SEMINAR OF ECOLOGY – 2015

WITH INTERNATIONAL PARTICIPATION

Proceedings

23-24 April 2015, Sofia, Bulgaria

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

Seminar of Ecology – 2015 with international participation Proceedings

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Този сборник съдържа доклади, изнесени на „Семинар по Екология - 2015“, с международно участие, проведен на 23-24 април 2015 г. в Институт по биоразнообразие и екосистемни изследвания – БАН, гр. София, България. Част от докладите са публикувани в пълен текст, а други като кратки съобщения. Семинарът е организиран от секция „Биология“ към СУБ, Институт по биоразнообразие и екосистемни изследвания – БАН, гр. София, България и с любезната финансова подкрепа на СУБ и на фирмите БУЛГАП ЕООД и Л.К.Б - България ЕООД. Публикуваните в Сборник „Семинар по Екология - 2015” материали са рецензирани и редактирани.

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ДО
УЧАСТНИЦИТЕ В НАУЧНИЯ
„СЕМИНАР ПО ЕКОЛОГИЯ - 2015"
С МЕЖДУНАРОДНО УЧАСТИЕ

УВАЖАЕМИ КОЛЕЖКИ И КОЛЕГИ!

С искрено удоволствие се отзовавам на поканата да поздравя участниците в станалия вече традиционен ежегоден Семинар по екология.

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.

Интердисциплинарният характер на тематиката повишава значението на научните форуми в областта на околната среда и устойчивото развитие.

На всички участници в семинара пожелавам успешна и ползотворна работа!

София, 23 април 2015 г.

акад. Дамян Дамянов,
председател на СУБ

БЪЛГАРСКА АКАДЕМИЯ НА НАУКИТЕ
ИНСТИТУТ ПО БИОРАЗНООБРАЗИЕ И ЕКОСИСТЕМНИ ИЗСЛЕДВАНИЯ
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23 април 2015 г, София

До проф. д-р Стефка Чанкова,
Председател на Организационния комитет
на Семинар по Екология 2015
с международно участие

Уважаеми организатори, уважаеми колеги и гости!

От името на Ръководството на ИБЕИ Ви приветствам с добре дошли на поредното издание Семинар по екология с международно участие, който се утвърди като традиционно място за среща на млади и утвърдени специалисти, провеждащи екологични изследвания в различни аспекти, както от ИБЕИ, така и от други научни институции в София, страната и чужбина.

През 2015 година програмата на семинара отново включва голям брой участници и разнообразна тематика: от една страна - ландшафтна екология, изследване икономическото значение на екосистемите и екосистемните услуги, от друга – влиянието на човешката дейност върху екосистемите и разнообразните механизми на адаптация на живите организми към това влияние. А всичко това ни дава гаранция, че и тази година на семинара ще бъдат представени нови научни постижения, ще възникнат нови творчески контакти, и ще се зародят идеи за нови съвместни разработки и проекти.

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

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

ДОКЛАДИ/REPORTS

Тематично направление:
БИОЛОГИЧНО РАЗНООБРАЗИЕ И КОНСЕРВАЦИОННА БИОЛОГИЯ

Topic:
BIOLOGICAL DIVERSITY AND CONSERVATION BIOLOGY

BIODIVERSITY OF VERTEBRATES IN ANTARCTICA

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Abstract: The conditions of existence of organisms and the vertebrate fauna, their position in the food chain and how they relate in the Antarctic marine ecosystem were presented. One of the features of this living environment is a combination of low temperatures, high light intensity during the summer and minimal in winter.

Antarctic animals - fish, penguins and other birds, seals and whales - mostly endemic species have evolved specific adaptations, unusual for other regions of the world. Coldwater Antarctic fish are active in a very narrow temperature range, often just below freezing. Birds are considered seabirds, some of them are nesting, others are migratory, nesting-migratory and birds occasional visitors. Most numerous are penguins - about 87% of the ornitofauna. Seals represent a large group of predatory animals getting through to amphibious lifestyle. In comparison with the terrestrial mammals, they have over 75% more blood. From a total of 100 species of whales inhabiting the ocean, about 20 species occur permanently or temporarily in Antarctica.

All the species found in the Southern Ocean and adjacent areas are under strict protection. The protection of the sensitive Antarctic marine ecosystem is extremely important, because the consequences of its violation could not be recovered, even for decades.

Keywords: Antarctica, Environment, Antarctic vertebrate animals

The living environment of Antarctica

The name of Antarctica often is used as a summary to indicate the ice mass covering the most southern area of the Earth. This circumpolar region mainly involves two different environments - continental and marine that form the Antarctic surface and the oceanic system surrounding them. Geographically there is no territory that determines the Antarctic region, as in the case of the Arctic. From a geopolitical point of view the 60° S parallel is considered its northern border.

The average distance between the Antarctic coast and the South Pole is about 2500 km, as the majority of the continent closes behind the southern polar circle (66° 33’ S). The Antarctic Ocean surrounding Antarctica separates it with a great distance from any land. The nearest continent is South America, which is about 1000 km away from it. The Antarctic’s geographical isolation is further strengthened by the climate characteristics of the Antarctic

Ocean, which is the coldest, deepest and the most stormy and is covered with a deep layer of sea ice which in the winter, makes approach to the continent almost impossible. Antarctica is the driest and windiest continent (winds reach record level, over 320 km/h), with the highest average altitude - 410 m. About 98% of its surface is covered with ice and only 0.2 - 0.3% is ice-free - some mountain peaks, ridges and oases along the coast.

The **Antarctic convergence (Polar Front)** is a curve continuously encircling Antarctica where cold, northward-flowing Antarctic waters meet the relatively warmer waters of the sub Antarctic. The Antarctic waters predominantly sink beneath the sub Antarctic waters, while associated zones of mixing and upwelling create a zone very high in marine productivity, especially for Antarctic krill. This line, like the Arctic tree line, is a natural boundary rather than an artificial one like a line of latitude. It not only separates two hydrological regions, but also separates areas of distinctive marine life associations and of different climates and creates a highly uniform in its spreading biological world.

The Polar Front sharply distinguishes the organism communities. The ocean waters south of it are rich in nutrients, so the Antarctic ecosystem is more productive than the sub-Antarctic. The Polar front strongly influences the plankton, fish and seabirds distribution. The species that exist south of the convergence have developed survival adaptations in this very specialized environment, forming a well-defined and separate marine ecosystem.

From the environmental point of view the biodiversity of the communities is more informative than the absolute number of the described species. Dominated by only a few species, which are usually in large numbers, is a sign of poor biodiversity, unlike the community where many species are presented with a similar incidence of appearance - an indicator of high biodiversity. The latter is an indicator of the complexity of the interactions in the food chain. High biodiversity may be retained for a long period of time under stable ambient conditions, i.e., without unforeseen extreme changes.

Factors determining the life in Antarctica

Antarctic animals exist in severe frosts and winds, because they have developed highly specialized adaptations. The most important is the homeothermy. Warm-blooded animals maintain a constant body temperature, regulated by the brain, regardless of the ambient temperature. This means that a large proportion of food consumed has to be used to maintain the body temperature. A further protection, which allows the heat preservation is a protection by fur, fat, feathers, shelters [1, 2]. Some species hibernate, others migrate. Internal physiological processes help to preserve, retain or radiate energy. According to the rule of Bergmann warm-blooded animals inhabiting cold areas are larger than those living in the tropics.

The insulation is also very important. It is mainly two types - subcutaneous fat layer or tissues impregnated with fat and dense body cover with fur or feathers. All Antarctic animals protect themselves from the cold basically by a huge layer of fat [3]. Color, posture and behavior are also of great importance. Some polar animals have the capacity to support two different temperatures - a high internal and the other, substantially lower for the extremities [4, 5]. The insulating protections are not perfect. Animals need a lot of energy and then spend it for swimming, flying, running, or for forming and restoring their own

tissues. They do not live only in these extreme areas, but their populations thrive, however only as long as there is abundant food [6].

Antarctic fauna

Antarctica is home of thousands of sea birds, penguins, seals, which cannot feed on land. They are subsisting in the Antarctic waters, which are the richest in plankton forms in the world and maintain the most animals in the relatively short Antarctic trophic chain. The populations of Antarctic animals are relatively large, what is the base of the Antarctic life, where the number of species is small and each is represented by a huge number.

Krill is the main planktonic crustacean distributed circumpolarly and only south of the Antarctic convergence. It is a key organism in the short food chain, with high biomass productivity therefore is the major food resource and most important component of the marine ecosystem. Krill forms a huge biomass and feeds the entire vertebrate Antarctic fauna - fish, numerous penguin populations, seabirds, seals and whales, which is only one conversion of solar energy into organic matter by microscopic algae.

The cold shelf waters of Antarctica are inhabited mainly by endemic fish species of the suborder Notothenioidei or etc. Antarctic cod [7]. About 100 species are representatives of the family Notothenidae. The most widespread representative is *Notothenia rossi* with a circumpolar distribution. Other species that are characteristic of more temperate waters are with limited distribution in different peripheral areas, which is an evidence that the Antarctic fish fauna evolved in isolation period of cold, probably during the Tertiary, about 70 000000 yr ago.

The genus *Nototheniiformes* covers 90% of all identified species in the marine waters of pack-ice. Very few species of these fish inhabit the area outside the Antarctic [7]. The body weight of certain fish varies between 18 and 70 kg.

The seawater temperature in the middle and at the bottom layers is around the freezing point, which depends on the salinity and pressure. Most of the fish species are kriopelegic and they inhabit this area annually in constant contact with the ice. This causes the development of an adaptation mechanism for spending energy for growth and the development of a specific protective agent - glycoprotein producing in their intercellular space thus reducing the blood freezing point. These fish have a fast metabolism and slow growth.

The Ice fish (*Champsocephalus gunnari*) is a representative of the family Chaenichthyidae. It inhabits sea water with a temperature between -2°C and 4°C and is unique in the animal world. The blood is colorless and diluted and does not contain hemoglobin and red blood cells. Their absence leads to heart enlargement of about 3 times. The Antarctic waters rich in oxygen make possible the diffuse gas exchange in the blood plasma both from the gills and skin.

As "Antarctic" are identified about 30 bird species, as 15 of them nest on the Antarctic continent. This number is quite small, but their populations' size is very high. The discrepancy between the species number and their population size confirms the general life rule in the Antarctic region. It also correlates with a similar discrepancy between favorable breeding sites and food resources. The food resources are practically inexhaustible, as the appropriate nesting places are not enough. This is the reason for a huge bird concentration of suitable nesting areas along the coast, as well as multiple uses. Birds like the underwater

inhabitants of the Antarctic waters are also endemic as a result of long term isolation.

Penguins (family Spheniscidae) are the most numerous, representing around 90% of all birds in Antarctica (their number is estimated at about 100 million individuals) and populate exclusively the southern hemisphere. Having lost the ability to fly they are fully adapted to the marine environment. They spend limited time on land until they finally complete the reproductive cycle and molting.

Penguins (6 genera and 18 species) are spread in Antarctica (17 spp.), only one species - the Galapagos penguin (*Spheniscus mendiculus*) occurs in the tropics. Antarctic penguins are: three species of the brush-tailed penguins (genus *Pygoscelis*) - Adelie penguin (*P. adeliae*), Chinstrap (*P. antarctica*) and Gentoo (*P. papua*), Emperor Penguin (*Aptenodytes forsteri*) and two species of the genus *Eudyptes* - Macaroni penguin (*Eudytes chrysolophus*) and the Rockhooper (*Eudyptes chrysocome*). They inhabit moderately cold circumpolar regions and only exceptionally reach higher latitudes.

The distribution of penguins is closely related to the possible nesting sites such as islands or mainly coastal areas located as close as possible to the feeding areas [8]. Rookeries were established in different locations - from thick sea ice for the emperor penguins, up to steep slopes to the Chinstrap.

The individual recognition between parents and the young is of enormous significance, as each bird found unmistakably partner or their young. The social behavior is also of great importance, especially in numerous colonies where territoriality and guarding are very highly developed [9, 10].

The Emperor penguin (*Aptenodytes forsteri*) is the largest of all penguins, with a height of 100 -120 cm, weight ranging between 20 and 40 kg. The variation is due to the fact that they remain for a long time without being fed during reproduction. The Emperor penguin is considered as a "real" Antarctic species. It inhabits the most remote and high latitudes and migrates into the sub-Antarctic areas.

Two peculiarities make it the most exceptional case in terms of adaptation to the environment [11]. It is the only creature in the world that does not build a nest and this forced it to keep on his feet lay an egg, protecting it in a skin fold, located at the bottom of the abdomen over a thick layer of fat. This explains why it lays only one egg. Its seasonal pattern does not match the other penguins. It spends the summer in the sea and begins its reproductive cycle in winter, when weather conditions are the most severe [12]. For penguins the level of territoriality depends on the occupancy of the colony. The greater density suggests minimum activity with minimal energy expenditure to spend the extremely low temperatures.

Some genera, including *Aptenodytes* and *Spheniscus* have social behavior under the water. During the breeding period they form groups that cooperate with other bird species, mammals, and even predatory fish while hunting food [13].

About 35 species of birds can be assigned as "Antarctic" but only about 15 of them nest on the continent. Their number is not large, but the population's size of many of them is too large, the mismatch in the number of species corresponds to the general life pattern in Antarctica. It may be of inconsistencies between nesting space and food resources. Access to food resources is huge, but suitable nesting locations are very limited. This leads to a

large concentration of birds into the accessible areas on the coasts, as well as a repeated use of the territories.

The Southern Giant Petrel (*Macronectes giganteus*) nests on open flat surfaces with Wilson's storm petrel (*Oceanites oceanicus*), which occupies the vacant places on the ground around and under them. Other Petrels (as *Hydrobates pelagicus*) occupy adjacent edges, rock niches or congregations.

Particularly important is the Snowy Sheathbill (*Chionis alba*), woodcocks atypical relative, but marine in habitus. It has salt glands and is the only bird in Antarctica bearing unwebbed toes. As a scavenger around the penguin colonies, it gets food from the sea, but second hand. The same is true also for the South Polar Skua (*Stercorarius maccormiki*), seagull's relative who hunted eggs and chicks of penguins [14].

The Antarctic Tern (*Sterna vittata*) is a true Antarctic coastal species that nests on many islands circumpolar, even on the continent. The Arctic Tern (*Sterna paradisea*) migrates further south when the winter comes in the northern hemisphere. This migration is most likely associated with the long daylight. During the Antarctic summer it accumulates fat feeding on krill in pack-ice.

Four endemic species of seals inhabit circumpolar the Antarctic, which is a proof of the existence in isolation for a long period of time in their evolutionary history. Antarctic seals belong to the family Phocidae, they are genetically distant from the northern ones, which is true for those inhabiting behind the Antarctic Front - southern seals and sea lions.

Six species of seals inhabit the Antarctica circumpolarly. They have been subjected to the same evolutionary divergence and are totally adapted to life at sea. Their ecological niches are so different that they completely avoid interspecific competition. Only one of them, the Crabeater seal (*Lobodon carcinophagus*) maintains the largest population - 8 000000 individuals. The Ross seal (*Ommatophoca rossii*) is the rarest and its population number is less than 130 000 individuals.

The southern Elephant seal (*Mirounga leonina*) is the largest representative of pinniped mammals. Females weigh an average of about 680 kg, while males weigh about 3600 kg. Males form large harems. They feed by diving at great depths - between 400 m and 1000 m, which is due to the physiological adaptation - enhanced capacity on oxygen saturation and reduced oxygen consumption. They use their extremely sensitive whiskers, which have a significant role in their food searching [15].

The Antarctic Fur seal (*Arctocephalus gazella*) is common in the Antarctic waters. 95% breed on the sub Antarctic Islands where males form harems. It feeds mainly on krill as single individuals consume about one ton per year.

The Leopard seals (*Hydrurga leptonyx*) are predominately found in the circumpolar region of the Antarctic pack ice. It is the only species of the genus *Hydrurga* and is typically a solitary predator. Its jaws are provided with sharp canines and molars, able to lock and allow the krill to be filtered [16] Teeth adaptation allows water filtration and retention of small crustaceans [17]. It inhabits ice floes and pack-ice.

The Crabeater seal (*Lobodon carcinophagus*) is a true seal with a circumpolar distribution around the coast of Antarctica. They dive to feed on krill at night and are known to dive for long periods of time up to 16 hours for feeding, traveling or migrating, and exploring. They

feed by swimming with open mouth, sieving the water out using their sophisticated teeth [17].

The Ross seal (*Ommatophoca rossii*) is a true seal in the Phocidae family, and can only be found on pack ice in Antarctica. Typically it appears to be solitary, but can occur in small groups, which are located far from other individuals. These seals communicate using a wide variety of vocalizations, consisting of twittering calls that can be made under water and on the ice, and can travel at long distances. Typical is that the number of teeth in the tooth row is reduced to curved, delicate and extremely sharp spikes. This gives reason to assume that it feeds mainly with squid and krill at great depth [13].The total population of Ross seals is thought to range between 20 000 and 227 000, although it is widely accepted that the number is actually around 130 000 individuals.

The Weddell seal (*Leptonychotes weddellii*) is the most southerly breeding mammal (77°S) and one of the best known Antarctic seal species. The Weddell seal is an accomplished diver, able to reach depths of over 600 m, ranging out to 5 km from a breathing hole in a single dive, and spending up to 82 minutes underwater before surfacing to breathe. This phenomenon is due to its high myoglobin concentration in the muscles, large amount of blood cells per unit volume of blood, in comparison with other mammals. It is able to reduce its heart rate and buffer its blood so that the pH avoids falling too low. The low pH of the blood is a signal to the brain that oxygen is required [19].

Its underwater vision is excellent, and a nictitating membrane protects the eyes from salt water and blowing snow. Holes in the ice, for breathing and hauling out, are created and maintained by cutting and sawing at the ice with the teeth. Its population is estimated at about 800 000 individuals.

Whales are found in all the oceans of the world, but Antarctica is the region where they are most numerous. Drawn to the cold, nutrient-rich Antarctic waters, ten species of whales spend their summers at the bottom of the world. They are the only mammals, other than manatees, that live their entire lives in the water, and the only mammals that have adapted to life in the open oceans. Toothed whales have narrow jaws lined with peg-like teeth which they use to catch fish, squid, and other marine mammals, swallowing them whole. They are smaller than the baleen whales and only have one blowhole. The Killer (*Orca*) whale (*Grampus orca*) and the Sperm whale (*Physeter microcephalus*) are the only toothed whales found in the Antarctic region. Baleen whales have comb-like structures instead of teeth that are used to filter krill and fish from the water. They are larger than toothed whales and have two blowholes. The Blue Whale (*Balaenoptera musculus*), the Fin Whale (*Balaenoptera physalus*), the Humpback Whale – (*Megaptera novaeangliae*), the Minke (*Balenoptera acutorostrata*), the Sei Whale (*Balaenoptera borealis*), and the Southern Right Whales (*Eubalaena australis*) are the baleen whales found in the Antarctic.

Antarctica is also the home to other small cetacean species about which there still isn't enough knowledge. These are endangered baleen species such as the Pygmy right whale (*Caperea marginata*) found in the Southern Ocean in the lower reaches of the Southern Hemisphere. Little is known about its population or social habits.

Most of the Antarctic animals are strongly attached to their territories, others have circumpolar distribution. Many of them migrate seasonally to other parts of the world. The Antarctic organisms due to their high specialization are extremely vulnerable even to minor

changes. They can be quickly re-adapted to environmental changes. The marine animals such as seals and penguins that breed on land are completely defenseless to the predators' alien land. These animals dependent directly on the sea and are specialized in feeding on plankton, so any change in the biological system occurs rapidly, and with far reaching consequences.

Conservation of the Antarctic Fauna

Compared to many other parts of the world the Antarctic is relatively unmodified by human activities. Protection of the native fauna and flora has been a concern for the Consultative Parties to the Antarctic Treaty from the beginning. In 1964 Agreed Measures for the Conservation of Antarctic Fauna and Flora were adopted, and many other measures dealing with related issues followed in the subsequent ATCMs.

Article 3.2 of the Environment Protocol provides that the activities to be undertaken in Antarctica shall be planned and conducted so as to avoid *"detrimental changes in the distribution, abundance or productivity of species or populations of species of fauna and flora" and "further jeopardy to endangered or threatened species or populations of such species"*. Annex II to the Protocol sets out specific measures to give effect to this. It provides several different mechanisms to protect Antarctic species, including:

• the prohibition of taking (removing) and of harmful interference, except in accordance with a permit;

• the prohibition of introducing non-native species, except in accordance with a permit; and

• the designation of Specially Protected Species.

The ATCM has also adopted specific measures to manage the human disturbance of Antarctic fauna and flora, including Recommendation XVIII-1: *Guidance for Visitors to the Antarctic*, adopted in 1994, and the *Guidelines for the Operation of Aircraft Near Concentrations of Birds in Antarctica*, adopted in 2004.

References:

1. Scholander P. F., Hock R., Walters V., Johnson F., Irving L. Biol. Bull., 99, 1950a, 237 – 258.

2. Scholander P. F., Hock R., Walters V., Johnson F., Irving L. Biol. Bull., 99, 1950b, 225 – 236.

3. Schmidt-Nielsen K. Fed. Proc., 29, 1970, 1524 – 1532.

4. Murrish D. E. Respir. Physiol., 19, 1973, 262-270.

5. Murrish D. E. Physiol. Zool., 55, 1982, 137-143.

6. Schmidt-Nielsen K. 1997. Animal Physiology. Adaptation and Environment (ed. 5). Cambridge University Press, Cambridge, UK. p. 617

7. Eastman J. T., Eakin R. R. Arch. Fish. Mar. Res., 48 (1), 2000, 11–20.

8. Ainley D. G., LeResche R. E., Sladen W. J. L. Breeding biology of the Adelie penguin. University of California Press, Berkey, 1983, p. 240.

9. Thompson D. H., Emlen J. T. Antarctic Journal of the United States., 3, 1968, 132.

10. Davis L. S. New Zealand Journal of Zoology., 9, 1982, 279–286.

11. Le Maho Y. American Scientist., 65, 1977, 680–693.

12. Williams T. D. The Penguins. Oxford University Press, Oxford, United Kingdom, 1995, p. 295.

13. Splettstoesser J., Gavrilo M., Field C., Field C., Harrison P. New Zealand Journal of Zoology, 27, 2000, 137-142.

14. Young E. Skua and Penguin: Predator and Prey. Cambridge University Press, Cambridge, United Kingdom, 1994, p. 472.

15. Klappenbach L. The 10 Largest Mammals. About. com., 2013, Retrieved 18 October – http://animals.about.com/od/mammals/tp/extreme-mammals.htm

16. Hill A. Hydrurga leptonyx. Animal Diversity Web., 2013, Accessed February 24 - http://animaldiversity.org/accounts/Hydrurga_leptonyx/

17. Burns J., Costa D., Fedak M., Hindell M., Bradshaw C., Gales N., McDonald B., Trumble S. and Crocker D Deep Sea Research Part II: Topical Studies in Oceanography, 51 (17-19), 2004, 2279-2303.

18. Bonner N. Seals and Sea Lions of the World. New York. Facts on File, Inc. 2004. p. 192.

19. Zapol W. M., Hill R. D., Qvist J., Falke K., Schneider R. C., Liggins G. C. & Hochachka P. W. Undersea Biomed, Res., 16 (5), 1989, 363–373.

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HABITAT DIVERSITY IN MALA PLANINA

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Abstract

Aim: The main aim of the current research is to identify and analyse the habitat types and the habitat diversity of Mala Planina.

Materials and Methods: Habitat types reflect the diverse array of organisms adapted to life in Mala Planina. Some typical mountainous habitats are found on this territory. Their investigation has theoretical and practical importance. Cameral and preparatory terrain research of the habitat types is done. Remote methods are used to accomplish the aim of the current work. The preliminary study is based on maps of the vegetation of Mala Planina, provided by Sofia Forestry and Svoge Forestry.

Results: The classical approach for classification helps for achieving better results in this study. All possible factors for differentiation of habitats are considered.

Conclusion: This research proves that the habitat diversity of Mala Planina is great. The area is not far away from the Bulgarian capital. The anthropogenic influence is indisputable and by revealing the preliminary habitat diversity authors aim to prove that conservation of habitats is necessary.

Key words: habitat types, diversity, Mala Planina, maps

Introduction

The issue of identifying, analyzing and protecting habitat diversity is a question of present interest. Mala Planina is situated near to the capital city of Sofia, most parts of it are easily accessible and the mountain's habitats are under anthropogenic pressure. Therefore the authors' aim is to contribute to the better understanding of the mountain's habitats which is advisable to lead to the protection of the habitat diversity. Habitats in Western Stara Planina are researched by Vassilev [1], Vassilev et. al. [2, 3].

An important document (Millenium Ecosystem Assessment) [4] was published in 2005. According to this assessment cultivated systems (areas where at least 30% of the landscape is in croplands, shifting cultivation, confined livestock production, or freshwater aquaculture) now cover one quarter of Earth's terrestial surface. Under the MA scenarios, a further 10–20% of grassland and forestland is projected to be converted by 2050 (primarily to agriculture). Therefore we should focus our efforts in preserving our planet's natural habitats.

The natural habitat is a place where floristic, faunistic species and abiotic factors combine. These components are in equilibrium. The habitats provide important ecosystem services (provisioning: timber, livestock, fresh water; regulating: pollination, climate regulation, pest regulation, erosion and water regulation; cultural: recreation, aesthetic).

Conservation of habitats is a main task for the European Union. By becoming a part of the European Union, the Bulgarian Ministry of Environment is now responsible for the application of the Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora [5].

As it is stated in the Interpetation Manual of the European Union about Habitats (2007) [6, 7], the Habitats Directive is a Community legislative instrument in the field of nature conservation that establishes a common framework for the conservation of wild animal and plant species and natural habitats of Community importance. It is the first normative document of united Europe which unifies the countries' efforts to preserve wild nature. It provides for the creation of a network of special areas of conservation - Natura 2000 which aim is to protect natural heritage and to encourage sustainable development of regions [8].

The Habitats Directive (together with the Birds Directive) forms the cornerstone of Europe's nature conservation policy. It protects over 1000 animals and plant species which are of European importance. Annex I lists today 231 European natural habitat types, including 71 priority ones (i.e. habitat types in danger of disappearance and whose natural range mainly falls within the territory of the European Union). Following the adoption of

the priority habitats manual, the experts also identified a set of 36 non priority habitat types causing interpretation problems.

A wide range of organizations on global or national scale work for the protection of habitats. WWF is among them. The organization has many programmes and one of them that concerns habitats in Bulgaria is the Danube-Carpathian Programme. It was established in 1998 to coordinate and lead WWF's conservation activities across the 19-country Danube-Carpathian ecoregion in cooperation with the WWF national organizations [9, 10]. The aim of the programme is to preserve, store and manage in a sustainable manner the natural values of the Danube-Carpathian ecoregions.

The territory of the current research is a part of the Western Balkan range. Mala Planina is a part of the biogeographic region of the Balkans [11]. Petrova and Vladimirov [12] contributed to the research of the floristic component of the habitats found in this range. They have identified 115 Balkan endemics and 50 Bulgarian endemics there. Velchev [13] contributed to the clarification of the current boundary of Mala Planina.

Matherials and Methods

The northern boundary of Mala Planina starts near the village of Golemo Malovo and goes to the east reaching the village of Tsraklevtsi. To the north is Chepan Mt. Then the boundary follows the river bed of Iskretska river. To the north is Ponor Mt. The eastern part of the northern boundary is described by Grigorov et. al [14].

The eastern border is marked by the Iskar river. To the east is Golema Planina. The southern boundary of Mala Planina starts from the town of Novi Iskar and goes to the west following the 600 m isohypse. That's the transition to the Sofia valley. The western borderline passes by the peak of Leshta (876.2 m) from the west and finishes its way near the village of Golemo Malovo (Fig. 1).

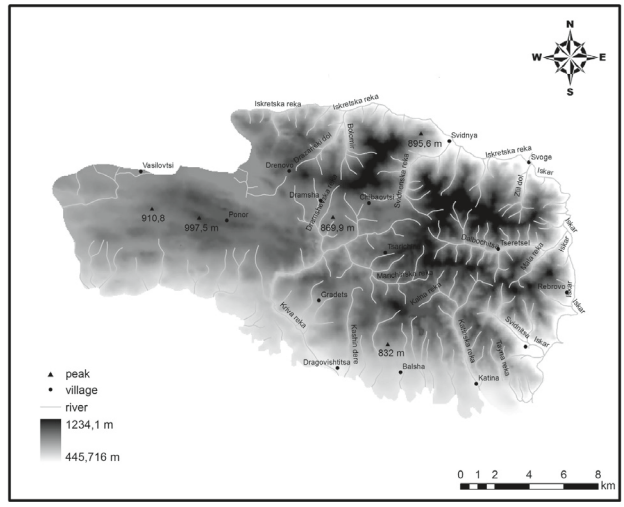

Fig. 1. Map of Mala Planina

The terrain research in Mala Planina is made in 2015. The route method is used for gathering information about the habitats. Specific habitats are chosen for collecting important data. The vegetation maps provided by Sofia and Svoge Forestries are processed in order to get more precise information. Habitats were verified during the terrain research and the conclusion was that the maps of the forestries are precise when it comes to dominant tree vegetation. These charts are used as a base for creating the map of the habitats in Mala Planina in this preliminary study.

Results

In order to reveal the habitat diversity in Mala Planina we have to consider the biodiversity of the mountains that border the area of the current research. Vassilev et. al [3] and Dimitrov and Petrova [15] study Ponor Mt. In the first article it is stated that 20 years of succession on a subset of grassland areas probably did not negatively affect the overall plant diversity of Ponor Mt. The same conclusion should be applied to Mala Planina.

Habitat diversity of Mala Planina and its importance is acknowledged by the fact that the western part of Mala Planina is included in NATURA's 2000 Dragoman protected area - BG0000322.

Fig. 2 illustrates the significant variety of habitats found in Mala Planina. They are of European and national importance.

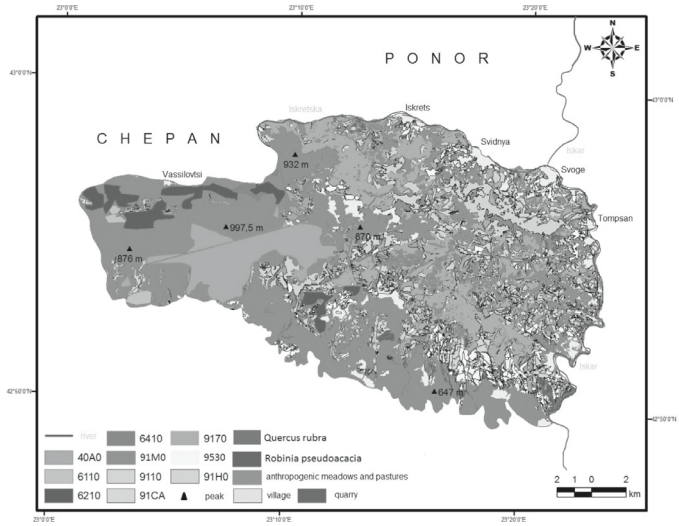

Fig. 2. Main habitats of Mala Planina

A brief description of each habitat along with its typical species can be found in the following lines [16]. There is a number from 1 to 5 after the name of each habitat. It characterizes the habitat according to the Conception for preservation of habitats by Belev et al. [17]. The explanation of the numbers is given below.

1. Well studied habitats (scientific reports, articles, etc.) and at least 50% of them are in protected areas (national parks, reserves, etc.).

2. Studied habitats (mainly old scientific reports) and less than 50% of them are in protected areas.

3. Poorly studied habitats but over 50% of the area they cover are in protected territories.

4. Poorly studied habitats and less than 50% of their area is a part of protected lands.

5. Practically unstudied habitats which are not represented in protected areas.

Typical species for the different habitats are listed bellow. There are invasive species like *Ailanthus altissima, Acer negundo, Robinia pseudoacacia* in some of the habitats including *Quercus* spp. and they represent a certain threat for the future stability of the habitats. The general condition of the habitats in Mala Planina is good. They show steady features and development. It has to be pointed out that habitats near the largest villages: Svoge and Novi Iskar and other big villages (Iskrets, Chibaovtsi, Kutina etc.) are more in danger because of the anthropogenic impact - trees are cut down for heating and building materials, grasslands are used for pastures.

5130 *Juniperus communis* formations on heaths or calcareous grassland - 5

These are mainly second-growth formations in calcareous terrains.

Species: *Juniperus communis, Rosa* spp*., Crataegus monogyna, Festuca* spp*., Carlina vulgaris, Euphorbia cyparissias, Plantago media, Dichanthium ischaemum, Chrysopogon gryllus, Bromus inermis, Salvia nemorosa, Eryngium campestre.*

6110 Rupicolous calcareous or Basophilic grasslands of the *Alysso-Sedion albi* - 5

These are open, pioneer communities on shallow soils in territories with carbonate rocks reaching 700-1000 m.

Species: *Alyssum alyssoides, Acinos arvensis, Arabis recta, Arenaria serpyllifolia, Cerastium* spp*., Erophila verna, Jovibarba heuffelii, Holosteum umbellatum, Medicago minima, Minuartia setacea, Poa bulbosa, Paronychia cephalotes, Saxifraga tridactylites, Scleranthus annuus, Sedum* spp*., Sempervivum* spp*., Teucrium montanum, Syntrichia ruralis, Grimmia pulvinata.*

6210 Semi-natural dry grasslands and scrubland facies on calcareous substrates (Festuco-Brometalia) - 4

These are xerothermic to meso-xerothermic grassland communities up to 1000 m.

Species: *Chrysopogon gryllus, Dichanthium ischaemum, Stipa capillata, S. pennata agg., Festuca valesiaca, Brachypodium pinnatum, Bromus inermis, B. erectus, Poa angustifolia, Anthyllis vulneraria, Coronilla varia, Carex caryophyllea, Carlina vulgaris, Centaurea scabiosa, Dianthus giganteus, D. moesiacus, Eryngium campestre, Koeleria macrantha, Filipendula vulgaris, Convolvulus cantabrica, Salvia nemorosa, Leontodon crispus, Medicago falcata, Anacamptis pyramidalis, Gymnadenia conopsea, Ophrys mammosa, O. cornuta, Orchis mascula, O. militaris, O. morio, O. purpurea, O. ustulata, O. tridentata, Origanum vulgare, Polygala vulgaris, P. major, Primula veris, Sanguisorba minor, Scabiosa columbaria, Veronica prostrata, V. teucrium, Helianthemum nummularium, Fumana procumbens, Adonis vernalis, Euphorbia nicaeensis, Silene otites, Thymus* spp.

6240 Sub-pannonic steppic grasslands - 4

These are open, xerothermic communities, developed on slopes with southern exposition on rendzinas.

Species: *Festuca valesiaca, F. rupicola, Stipa capillata, S. tirsa, Dichanthium ischaemum, Melica ciliata, Crupina vulgaris, Orlaya grandiflora, Carex humilis, Allium flavum, A. moschatum, Hesperis tristis, Iris pumila, Ranunculus illyricus, Teucrium chamaedrys, T. polium, Medicago minima, Rhodax canus, Poa bulbosa, Scorzonera mollis, S. hispanica, Salvia nutans, Sanguisorba minor, Potentilla recta* agg.*, Seseli rigidum, Sideritis montana, Alyssum alyssoides, Artemisia austriaca, Astragalus austriacus, A. onobrychis, Oxytropis pilosa, Herniaria incana, Gypsophila glomerata, Linum tenuifolium, L. tauricum, L. austriacum, Satureja montana, Achillea clypeolata, Vinca herbacea.*

6510 Lowland hay meadows - 4

These are mesophyll hay medows. They are developed on rich soils.

Species: *Poa sylvicola, Agrostis stolonifera, Festuca pratensis, F. arundinacea, Deschampsia caespitosa, Alopecurus pratensis, Holcus mollis, Cynosurus cristatus, Arrhenatherum elatius, Trifolium resupinatum, T. pratense, T. patens, Centaurea jacea, Cirsium canum, Moenchia mantica, Stellaria graminea, Knautia arvensis, Tragopogon pratensis, Daucus carota, Leucanthemum vulgare, Sanguisorba officinalis, Rhinanthus rumelicus, Carex distans, Lychnis flos-cuculi, Ranunculus acris, Gladiolus communis, Colchicum autumnale, Orchis laxiflora.*

8310 Caves not open to the public - 4

Plant species: mosses

Faunistic species: highly specialized endemic and relict species: *Carabidae, Lithobiidae, Isopoda, Amphipoda, Syncarida, Copepoda, Hydrobiidae.*

Bats: Rhinolophus sp.*, Myotis myotis, M. blythii, M. capaccinii, M. emarginatus, Miniopterus schreibersii.*

Other fauna: *Columba livia, Hirundo daurica, Strix aluco, Bubo bubo.*

9110 Luzulo-Fagetum beech forests- 3

These beech forests are developed on acidophilous, poor and eroded soils.

Fagus sylvatica, Abies alba, Picea abies, Luzula luzuloides, Lerchenfeldia flexuosa, Calamagrostis arundinacea, Vaccinium myrtillus, Pteridium aquilinum, Poa nemoralis, Oxalis acetosella, Dicranum scoparium, Polytrichum juniperinum, Leucobryum glaucum, Polytrichum formosum.

9130 Asperulo-Fagetum beech forests- 3

These are beech forests on neutral soils.

Species: *Fagus sylvatica, Abies alba, Picea abies, Anemone nemorosa, Lamiastrum (Lamium) galeobdolon, Galium odoratum, Melica uniflora, Aremonia agrimonoides, Cardamine bulbifera, C. pectinata, Mycelis muralis, Sanicula europaea, Viola reichenbachiana, Symphytum tuberosum, Allium ursinum, Mercurialis perennis, Corydalis* spp.*, Pulmonaria* spp.

9150 Medio-European limestone beech forests of the *Cephalanthero-Fagion* - 3

These are xero-thermophile forests.

Species: *Fagus sylvatica* ssp. *moesiaca, Tilia tomentosa, Carpinus betulus, Quercus frainetto, Q. cerris, Ostrya carpinifolia, Physospermum cornubiense, Lathyrus niger, L. laxiflorus, Galium pseudaristatum, Mycelis muralis, Euphorbia amygdaloides, Viola odorata, Hedera helix, Carex sylvatica, Brachypodium pinnatum, Epipactis* spp.*, Neottia nidus-avis, Cephalanthera* spp.*, Dactylorhiza cordigera, Ruscus* spp.*, Melica uniflora, Rubus hirtus, Helleborus odorus, Glechoma hederacea, G. hirsuta, Tamus communis.*

9170 *Galio-Carpinetum* oak-hornbeam forests - 3

These forests are formed over 500 m altitude. They are connected with mesophile beech woodlands.

Species: *Quercus petraea* agg. (including *Quercus dalechampii*)*, Carpinus betulus, Tilia cordata, T. platyphyllos, Acer platanoides, Sorbus torminalis, S. domestica, Acer campestre, Ligustrum vulgare, Cardamine bulbifera, Convallaria majalis, Festuca heterophylla, Mercurialis perennis, Corydalis* spp.*, Scilla bifolia, Poa nemoralis, Stellaria holostea*.

9180* *Tilio-Acerion* forests of slopes, screes and ravines - 3

These are second-growth, mixed forests.

Species:

Lunario-Acerenion: *Acer pseudoplatanus, A. hyrcanum, Fraxinus excelsior, Lunaria rediviva, Ulmus glabra, Allium ursinum, Mercurialis perennis, Actaea spicata.*

Tilio-Acerenion: *Tilia cordata, T. platyphyllos, Carpinus betulus, Corylus avellana, Quercus* spp.*, Anemone nemorosa, Corydalis* spp.*, Primula veris.*

91E0* Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior (Alno-Padion, Alnion incanae, Salicion albae)* - 2

These are riverside forests in lowlands and mountainous regions.

Species: *Alnus incana, A. glutinosa, Fraxinus excelsior, Salix fragilis, S. alba, Carex remota, C. sylvatica, Equisetum* spp.*, Filipendula ulmaria, Angelica sylvestris, Geranium sylvaticum, Geum rivale, Lycopus europaeus, Rumex sanguineus, Stellaria nemorum, Oxalis acetosella.*

91H0* Pannonian woods with *Quercus pubescens* - 4

These forests are much fragmented and occupyt small areas, mostly connected with shallow soils.

Species: *Quercus pubescens, Q. virgiliana, Q. frainetto, Q. cerris, Fraxinus ornus, Acer monspessulanum, Carpinus orientalis, Sorbus domestica, Cornus mas, Geranium sanguineum, Pyrus pyraster, Buglossoides purpurocaerulea, Campanula bononiensis, Filipendula vulgaris, Carex michelii, Euphorbia polychroma, Lactuca quercina, Limodorum abortivum, Acanthus balcanicus, Orchis purpurea, O. simia, Paeonia peregrina, Dictamnus albus, Scorzonera hispanica, Echinops sphaerocephalus, Laser trilobum, Helleborus odorus, Althaea cannabina, Chamaecytisus albus, Potentilla micrantha, Pulmonaria mollis, Tanacetum corymbosum, Viola suavis, V. hirta.*

91M0 Pannonian – Balkanic turkey oak-sessile oak forests - 2

These forests form the belt of the xerothermic oak forests reaching 600-700 m above sea level.

Species: *Quercus petraea* agg.*, Q. cerris, Q. frainetto, Acer tataricum, Ligustrum vulgare, Euonymus europaeus, Festuca heterophylla, Brachypodium sylvaticum, Poa nemoralis, Potentilla micrantha, Tanacetum corymbosum, Campanula persicifolia, Viscaria vulgaris, Lychnis coronaria, Silene viridiflora, Hieracium racemosum, H. sabaudum, Galium pseudaristatum, Lathyrus niger, Peucedanum alsaticum, Bupleurum praealatum, Helleborus odorus, Crocus flavus, Physospermum cornubiense.*

***91CA* Rhodopide and Balkan Range Scots pine forests - 2**

Species: *Pinus sylvestris, Vaccinium vitis-idaea, V. myrtillus, Juniperus communis, Chamaecytisus absinthioides, Genista rumelica, Digitalis viridiflora, Calamagrostis arundinacea, Luzula luzuloides, Pyrola chlorantha, Dicranum scoparium.*

9530 * (Sub-) Mediterranean pine forests with endemic black pines - 2

Species: *Pinus nigra* ssp. *pallasiana, P. sylvestris, Abies alba, Fagus sylvatica, Dorycnium herbaceum, Ostrya carpinifolia, Carex humilis, Cephalnthera longifolia, C. damasonium, Brachypodium pinnatum, Sesleria latifolia, Laser trilobum.*

Conclusion

Based on the collected information in this preliminary study the following conclusions could be made:

1. 16 habitat types are identified in the territory.
2. Mala Planina's geographical location near the capital city raises the attention to preserve its habitats.
3. This study can be used as a base for further detailed exploration of the habitats in Mala Planina.

The preliminary study is needed to become more familiar with area's habitat characteristics. They provide specific ecosystem services which are important for the population that inhabits Mala Planina. Despite being close to a large anthropogenic area there are some well preserved habitats (91H0*, 91M0), including 100-year old forests. There is lot more to be done in clarifying the area of the different habitats, their current condition, presence or absence of fragmentation and genuine threats.

The habitat diversity must be protected which can be helped by educating local residents about the importance of the habitats and by stronger implication of the laws for protection of the natural environment. The authors hope that the habitat diversity of Mala Planina can be kept for future generations by contributing to their better understanding.

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References:

1. Vassilev K. Grassland vegetation on limestone terrains western of Sofia. Dissertation, Sofia, 2012.

2. Vassilev K., Pedashenko H., Vassilev R. Floristic biodiversity and habitats of Kostinbrod Municipality. Ecological engineering and protection of the natural environment, № 2-3, 2008, 7-11.

3. Vassilev K., Pedashenko H., Nikolov S., Apostolova I., Dengler J. Effect of land abandonment on the vegetation of upland semi-natural grasslands in the Western Balkan Mts., Bulgaria, Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology: Official Journal of the Societa Botanica Italiana, 145:3, 2011m 654-665.

4. Millennium Ecosystem Assessment, 2005. Ecosystems and Human Well-being: Biodiversity Synthesis. World Resources Institute, Washington, DC.

5. http://ec.europa.eu/environment/nature/legislation/habitatsdirective/index_en.htm.

6. Intepretation manual of European Union Habitats (2007), European Comission DG Environment: Nature and biodiversity.

7. CORINE Biotopes manual, Habitats of the European Community. EUR 12587/3, Office for Official Publications of the European Communities, 1991.

8. http://ec.europa.eu/environment/nature/natura2000/index_en.htm.

9. http://www.bioregio-carpathians.eu/wwf-dcp.html.

10. WWF Danube-Carpathian Programme Bulgaria, Annual Report 2013.

11. Assenov A. Biogeography of Bulgaria, AN-DI, Sofia, 2006, p. 543.

12. Petrova A., Vladimirov V. Phytologia Balcanina 16 (2), 2010, 293 – 311.

13. Velchev A. Karst and karst forms in Sofia valley and the surrounding area (unpublished).

14. Grigorov B., Dimitrov P., Assenov A. Landscape biodiversity of Mala Planina mountain. Problems of Geography, Book 1 - 2, Sofia, 2015, 154-165.

15. Dimitrov M. A., Petrova D. D. Acta zool. bulg., 5, 2014, 9-20.

16. Kavrakova V., Dimova D., Dimitrov M., Tzonev R., Belev T. Fieldguide to habitats with European importance in Bulgaria, Sofia, 2005.

17. Belev T., Dimova D., Tzonev R. Conception for preservation of habitats of Application 1 of Directive 92/43/EEC of NATURA 2000 in Bulgaria, Sofia, 2005.

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INVASIVE PLANT SPECIES OF THE UNIVERSITY BOTANICAL GARDEN VARNA

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Abstract

Aim: The aim of this study is to identify the invasive alien species on the territory of the University Botanical Garden Varna, to describe the species composition, the degree of invasiveness and their impact on native vegetation.

Materials and Methods: An evaluation of the extent of invasive and potentially invasive plants was carried out on the territory of the University Botanical Garden Varna. The places with the densest populations of invasive plants were mapped. Extensive photographic data was collected.

Results: The studies found that the territory of UBG Varna has 28 invasive alien plant species, of which 5 species are included in Halting the Loss of Biodiversity by 2010: proposal for a first set of indicators to monitor progress in Europe – European Environment Agency Technical Report.

Conclusions: A Plan has been developed to limit the propagation and spread of invasive plant species.

Keywords: invasive alien plant species, University Botanical Garden

Introduction

The biological invasions by non-native or 'alien' species are one of the greatest threats to the ecological and economic well-being of the planet. Alien species can act as vectors for new diseases, alter ecosystem processes, change biodiversity, disrupt cultural landscapes, reduce the value of land and water for human activities and cause other socio-economic repercussions for man.

The invasive alien species are the second most important cause of biodiversity loss in the world, after the loss and destruction of the habitats. The fact that 6,658 species of terrestrial plants in Europe are identified as foreign is indicative of the scale of the process. Approximately 10-15% of these are considered to be invasive [1].

According to DAISIE (Delivering Alien Invasive Species Inventories for Europe) [1], in Bulgaria there are 736 species of terrestrial plants of foreign origin (17.93% of the vascular flora of Bulgaria). Of these, 60 species are described as being invasive, but their number is greater [2].

Most of the invasive plants in Bulgaria are introduced for a different purpose: decoration, erosion control, medical uses, food, wood and others. Only a few plants have infiltrated the country accidentally, for example by importing other types of plants. All alien species, once in the natural or semi-natural habitats and ecosystems, interact with the native species

and affect local biodiversity in the following ways: competition with the native species for water, nutrients and light; displacement of native species; hybridization with native species; changing of the habitats [3].

Besides biodiversity, invasive alien species threaten human health (some of them cause allergies) or cause serious economic damage (weeds or pests in agricultural and forestry crops). In Europe, most of the worst invasive species cause loss of biodiversity and lead to changes in the structure of the communities, while a small number cause direct damage to the species of conservation status [3].

The aim of this study is to identify the invasive alien species on the territory of the University Botanical Garden Varna, to describe the species composition, the degree of invasiveness and their impact on native vegetation.

Materials and Methods

The University Botanical Garden Varna is located in the resort "St. Konstantin and Elena", 8 km north of the town Varna. It occupies an area of 360 acres. The nearest distance from the sea is 200 m. The altitude is from 29 m to 85 m asl.

On the territory of the UBG Varna different habitats exist: forest and shrub, grass, rock and limestone terrain, wet riparian and aquatic biota which differ significantly in terms of the specific soil and microclimate conditions [4]. This creates conditions for the spread of invasive plants with different ecological requirements.

An evaluation of the extent of invasive and potentially invasive plants was carried out on the territory of the University Botanical Garden Varna. The places with the densest populations of invasive plants were mapped. Extensive photographic data was collected.

For the determination of plants the Handbook for Plants in Bulgaria was used [5]. Photos were taken with a Canon EOS 600D camera. The study was conducted during the vegetative season of 2014.

Results and Discussion

The studies found that the territory of the UBG Varna has 28 invasive alien plant species. This represents 2.57% of 1,090 species of vascular plants on the territory of the UBG Varna.

List of the invasive plants on the territory of Varna UBG:

Aceraceae: *Acer negundo* L.; **Amaranthaceae:** *Amaranthus albus* L., *Amaranthus hybridus* L., *Amaranthus retroflexus* L.; **Asteraceae:** *Bidens bipinnata* L., *Erigeron canadensis* L., *Symphyotrichum novi-belgii* (L.) G.L., *Galinosoga parviflora* Cav., *Xaithium strumarium* L., *Xaithium spinosum* L.; **Buddlejaceae:** *Buddleja davidii* Franchet.; **Cactaceae:** *Opuntia humifusa* (Raf.) Raf.; **Cuscutaceae:** *Cuscuta campestris* Yunck.; **Eleagnaceae:** *Elaeagnus angustifolia* L.; **Fabaceae:** *Amorpha fruticosa* L., *Gleditsia triacanthos* L., *Laburnum anagyroides* Medic., *Robinia pseudoacacia* L., *Spartium junceum* L.; **Moraceae:** *Broussonetia papyrifera* (L.) L'Her; **Onagraceae:** *Oenothera biennis* L.; **Oxalidaceae:** *Oxalis corniculata* L.; **Phytolaccaceae:** *Phytolacca americana* L.; **Poaceae:** *Sorghum halepense* (L.) Pers.; **Sapindaceae:** *Koelreuteria paniculata* Laxm.;

Simaroubaceae: *Ailanthus altissima* (Mill.) Swingle.; **Solanaceae:** *Lycium barbarum* L.; **Vitaceae:** *Parthenocissus quinquefolia* (L.) Planch. (Table 1).

Table 1. Taxonomic structure of invasive plants in Varna

№	Family	Genus	Species
1	Aceraceae	1	1
2	Amaranthaceae	1	3
3	Asteraceae	5	6
4	Buddlejaceae	1	1
5	Cactaceae	1	1
6	Cuscutaceae	1	1
7	Eleagnaceae	1	1
8	Fabaceae	5	5
9	Moraceae	1	1
10	Onagraceae	1	1
11	Oxalidaceae	1	1
12	Phytolaccaceae	1	1
13	Poaceae	1	1
14	Sapindaceae	1	1
15	Simaroubaceae	1	1
16	Solanaceae	1	1
17	Vitaceae	1	1

The most dangerous invasive alien species on the territory of the UBG Varna were *Ailanthus altissima, Robinia pseudoacacia* and *Sorghum halepense*. The most numerous invasive plant species belonged to the family Asteraceae - represented by 6 species, followed by family Fabaceae – 5 species. The remaining families were represented by only one species. These observations showed that they do not represent a threat to the changes in the natural vegetation of the area and were marked as potentially invasive. Those identified as potentially invasive species do not currently pose a threat to the spread and change of biodiversity, because due to agricultural activities taking place in the garden propagation and distribution are impossible.

Five species are included in Halting the Loss of Biodiversity by 2010: Proposal for a first set of indicators to monitor the progress in Europe – European Environment Agency Technical Report [6]: *Acer negundo, Ailanthus altissima, Amorpha fruticosa, Symphyotrichum novi-belgii, Robinia pseudoacacia* [6].

Five species are included in "The most problematic invasive alien species in Bulgaria": *Acer negundo, Ailanthus altissima, Amorpha fruticosa, Opuntia humifusa, Robinia pseudoacacia* [2].

The three most aggressive invasive plant species distributed on the territory of the UBG Varna are included in "List of the Worst Invasive Species in Europe", and among the ten most dangerous invasive species in Bulgaria [2] (Fig.1). Here is a brief description of each of the species:

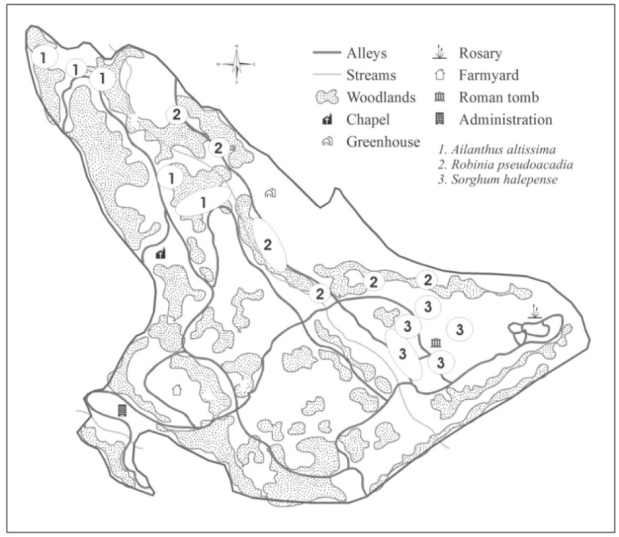

Fig. 1. Map of the most aggressive invasive species on the territory of UBG Varna

***Ailanthus altissima* (Mill.) Swingle (Simaroubaceae)**

Global distribution: The origin is China [7]. Imported in Europe in 1740 for ornamental purposes [2].

Distribution in Bulgaria: It was brought to the country in the period 1888-1900. The first evidence of its mass spontaneous spread in the country is from 1928 [2].

Morphology: Deciduous tree, up to 30 meters with a loose branched crown.

The bark is smooth, in old trees slightly cracked, grey-brown. The leaves are imparipinnate, 45-60 cm long. The flowers are greenish yellow, small and gathered in loose terminal panicles. It blooms from June to July, fruitful September - October [8].

Biology: Pollinated by insects. Propagated by seeds and vegetatively by root cuttings. Annually and abundantly fruitful. Fast-growing species.

Ecology: Prefers deep soils, but grows in very adverse soil conditions. Relatively warm temperatures, resistant to hot and cold climates. Does not suffer from mildew. Highly resistant to air pollution. Resistant to pests, glandule due to the leaves that issue strong odor. Stems and roots emit mucus that affects the surrounding plants [2].

On the territory of the UBG Varna *Ailanthus altissima* forms dense and clean communities by shifting autochthonous species such as: *Quercus* sp., *Acer campestre, Carpinus orientalis*. It captures the natural forest by spreading from the periphery to the inside (Fig. 2).

Fig. 2. ***Ailanthus altissima***

The sites with the greatest number of dominant form communities are comprised 100% only by the type. In the sites most endangered by *Ailanthus altissima* they are represented by seedlings to tree specimens of a height of 12-15 m.

***Robinia pseudoacacia* L. (Fabaceae)**

Global distribution: Originates from North America.

Distribution in Bulgaria: Middle XIX, after 1888. The first report of its wild and natural areas of penetration is from 1903 [2].

Morphology: Deciduous tree up to 25 m high. Dark-grey bark longitudinally cracked. Leaves consecutive imparipinnate. The truss 10-20 cm long, fibrous, located in the bosoms of the leaves. Blooms in May [9].

Biology: Pollinated by insects in September, October. Propagated by seeds and vegetatively from stem, root and vegetative shoots.

Ecology: One of the most tolerant to soil and climatic conditions tree species. Occupies heavily disturbed habitats.

On the territory of the UBG Varna *Robinia pseudoacacia* forms mixed communities with local vegetation. Seizes land that is not cultivated: gullies, natural forest and open lands (Fig. 3).

Fig. 3. ***Robinia pseudoacacia***

***Sorghum halepense* (L.) Pers. (Poaceae)**

Global distribution: Originates from the Mediterranean part of Europe and Asia. Becomes widely spread in Europe in the XIX century.

Distribution in Bulgaria: Established for the first time in 1885.

Morphology: Herbaceous perennial plant with short, thick, fleshy and creeping rhizome. Stem up to 150 cm high, smooth, glabrous, up to 1 cm thick. Leaves smooth sheath and white, wide, pointed with many parallel veins. Inflorescence conical, highly branched panicle [10].

Biology: Pollinated by wind, insects or self-pollinated. Blooms from June to September, fruiting from July to November. Reproduces very fast vegetatively by fragmentation of rhizomes and by seeds, which are produced in abundance [2].

Ecology: Adapted to the most varied habitats. It grows in grassy and sandy places, crops, roads, disturbed habitats, canals and wet places. It is considered one of the most dangerous weeds in the world [2].

On the territory of the UBG Varna *Sorghum halepense* occupies open arable and uncultivated areas. Dominant, forms dense populations (Fig. 4).

Fig. 4. *Sorghum halepense*

Conclusions:

A plan has been developed to limit the propagation and spread of invasive plant species. The possibilities for biological, chemical and mechanical control of invasive plant species were explored, as well as the possibility of replacing them with species of the native flora, which could displace or limit the spread of invasive plant species.

Information boards have been prepared in order to inform visitors of the garden of the damage they cause on biodiversity through invasive plants.

Referenses:

1. The DAISIE (Delivering Alien Invasive Species Inventories for Europe) website, http://www.europe-aliens.org/speciesTheWorst.do
2. Petrova A., Vladimirov V., Georgiev V., Invasive alien plants in Bulgaria, Institute of Biodiversity and Ecosystem Research, BAS, Sofia, 2012, p.319.
3. Zahariev D., Proceedings of the Seminar of Ecology – 2014 with International participation, dedicated to 70 years USB, FARAGO, Sofia, 2014, 148-157.
4. Boycheva P., Georgieva N., Tzonev R., Kosev K., Annuaire de l'Université de Sofia "St. Kliment Ohridski" Faculte de Biologie 100, 270-279.
5. Delipavlov D., Cheshmedzhiev I. (Eds.), Handbook for Plants in Bulgaria, Agrar. Univ. Acad. Press, Plovdiv, 2011, p. 591.
6. European Environment Agency (EEA), Halting the loss of biodiversity by 2010: proposal for a first set of indicators to monitor progress in Europe, Technical report No 11/2007, 107-108.
7. Jordanov D. (Ed. Princ.), Flora RP Bulgaricae, Acad. Press., Sofia, 7, 1979, p. 529.
8. Vakarelov I., Gencheva-Kostadinova S., Decorative dendrology., Matkom, Sofia, 2005, p.384.
9. Jordanov D. (Ed. Princ.), Flora RP Bulgaricae, Acad. Press., Sofia, 6, 1976, p. 596.
10. Jordanov D. (Ed. Princ.), Flora RP Bulgaricae, Acad. Press., Sofia, 1, 1963, p. 507.

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IN VITRO MICROPROPAGATION OF *DIANTHUS* SP. (CARYOPHYLLACEAE)

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Abstract

Aim: *Dianthus* species are perennial plants with high ornamental value, some of them are rare, and others are used in the traditional medicine. *In vitro* multiplication was tested to produce wild pinks.

Materials and Methods: Seeds gathered near Durankulak Lake (Bulgaria) were surface sterilized and germinated on half-strength MS medium, at 23±1°C and 16 h light daily. Shoots were sub-cultured every 8 weeks on media containing plant growth regulators (PGRs) BAP or Kin combined with NAA or IBA, in different concentrations. Multiplication efficiency was evaluated as the average number of shoots obtained

per explant. *In vitro* rooted plantlets were potted in soil mixture and *ex vitro* adapted in phytotron, then acclimated to greenhouse.

Results: Adventitious shoots were regenerated directly and via callus. Multiplication efficiency increased on media with less concentration of PGRs, reaching 10.2±2.8 shoots per explant on MS medium with 0.2 mg/l BAP and 0.1 mg/l NAA. *In vitro* rooting occurred on basal MS medium, and plants were successfully acclimated in greenhouse.

Conclusion: *In vitro* micropropagation of wild pinks could be an opportunity for their mass propagation.

Keywords: wild pink, *in vitro* multiplication, ornamental, greenhouse

Introduction

Dianthus species are perennial plants with high ornamental value and attractive fragrance. Seven of them are rare, and protected by the Biodiversity Act on the territory of Bulgaria: *D. carthusianorum, D. kladovanus, D. drenowskyanus, D. nardiformis, D. pallidiflorus, D. stribrnyi,* and *D. urumoffii*. Among the 106 vascular species from the Bulgarian flora which are included in the Red list of IUCN, there is one wild pink: *Dianthus nardiformis*. It can be found in the Nord Black sea coast floristic region, in Dobroudja, along with *D. palens*.

Some wild pinks like *D. chinensis* and *D. superbus* have been used in the traditional medicine in Asia and Europe. Ancient Greeks called wild pink plants as "the flower of God" because of their relaxing fragrance. In China wild pinks were appreciated for their anti-bacteria properties able to heal digestive and gastrointestinal problems, while in Africa and South America they were known as herbs for the treatment of rheumatism and arthritis. Infusion of wild pinks leaves and flowers are effective against skin rush, headache, and spasm. The healing effect is probably due to the saponins found out in these species.

In vitro rapid multiplication of *Dianthus* species could be applied to produce these nice small plants for the garden market. Successful micropropagation was reported of several endangered and vulnerable *Dianthus* species, among them *D. callizonus, D. giganteus, D. glacialis, D. pratensis, D. tenuifolius,* endemic for the flora of Romania, and *D. dobrogensis*, spread only in Bulgaria and Romania [1, 2]. In order to ensure long term *ex situ* conservation, cryopreservation was successfully applied to the endemic taxa *Dianthus giganteus banaticus* [3]. Authors noticed some specific features concerning the sterilization procedure and the multiplication rate of the studied species.

The focus of our research was put on the experimental determination of the most suitable conditions for *in vitro* rapid micropropagation of *Dianthus* sp. growing in the region of Durankulak. *In vitro* multiplication of wild pinks followed by *ex vitro* adaptation and acclimatization to open field could be an opportunity for the mass propagation of these nice small plants toward the garden market.

Materials and Methods

Seeds were gathered in august 2013 from *Dianthus* plants near the Lake of Durankulak, Bulgaria. They were surface sterilized by consecutive soaking in 70% ethanol for 30

seconds, commercial bleach (chlorine < 5%) for 10 minutes, and rinsed three times with distilled water for 10 minutes each.

Seeds were put for germination on half-strength MS medium [4] (medium MS/2), in Vitro vent containers with grids (Duchefa, NL), at constant temperature of 23±1°C and 16 hours artificial light daily, about 2000 lx. Temperature and light conditions were the same during the *in vitro* cultivation. Round plastic vessels with different height were used for sub-cultivated plants.

Shoots were sub-cultured every eight weeks on media supplemented with 100 mg myoinositol and plant growth regulators (PGRs). Combinations of two cytokinins: benzylaminopurine (BAP) or kinetin (Kin), with two auxins: α-naphthylacetyc acid (NAA) or isobutyric acid (IBA), in different concentrations, were tested for shoot multiplication enhancement (Table 1). Medium RI with 0.2 mg/l IBA as a sole PGR was tested for *in vitro* rooting. Media were supplemented with 30 g/l sucrose (except for the PGR-free media MS60 and MS60/2 which contained double sucrose amount and full or half amount of macrosalts, respectively), pH was adjusted to 5.75, then media were solidified with 5.5 g/l Plant agar (Duchefa, NL) and autoclaved at 121°C under 1 atm for 20 min.

Table 1. Composition of media used for shoot multiplication

Medium	BAP	Kin	NAA	IBA
BNi	1 mg/l	-	0.5 mg/l	-
KNi	-	1 mg/l	0.5 mg/l	-
B_2I_1	0.2 mg/l	-	-	0.1 mg/l
B_2N_1	0.2 mg/l	-	0.1 mg/l	-

Culture regeneration potential (RP) was evaluated as a percentage of the explants developing new shoots. Multiplication efficiency was evaluated by propagation coefficient (PC) representing the average number of shoots obtained per explant.

In vitro rooted plantlets were potted in soil mixture (soil, coconut fibre, and sand, in proportion 2:1:1) and *ex vitro* adapted in a climate chamber POL-EKO Aparatura, under strict control of the important parameters, for 4 weeks, simulating their natural daily dynamic: 10 h "day" under 2070 lx white light at 23°C, 8 h "night" in a dark, at 18°C, and two intermediate periods of 3 h each, under 1500 lx at 20°C. Special attention was put on the air humidity which is crucial for the success of the *ex vitro* adaptation: besides the daily dynamic with amplitude of 10%, higher in the night, it was progressively decreased, once a week, beginning from 80-90% and finally diminished to 55-65%. The second stage of adaptation was performed in a room phytotron with window, under less controlled conditions: temperature 22±4°C, natural light strengthened by LED light 16 hours daily, and air humidity between 35 and 65%. Plants were further acclimated to greenhouse conditions. In spring they were transferred to open field.

Results

Seeds germinated in only a week (Fig. 1). The sterilizing procedure ensured 75.4% survival seeds of 65 seeds gathered from the wild.

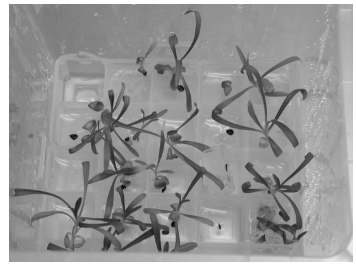

Fig. 1. Germination of *Dianthus* seeds *in vitro* in Vitro Vent containers with grids

After removal of seedling roots, stems were transferred to media BNi and KNi. Adventitious shoots rose from the stem base while callus formed under the explants instead of roots. Shoots were separated from the callus and put on fresh medium with the same composition while calli were transferred to PGR-free medium with half macrosalt strength and doubled sucrose concentration (MS60/2) (Fig. 2).

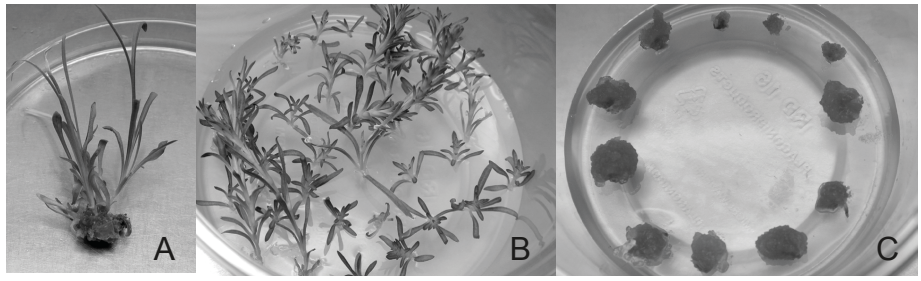

Fig. 2. First sub-cultivation of *Dianthus*: A) Adventitious shoots and callus formed on medium BNi; B) Shoots transferred to fresh BNi medium; C) Callus transferred to medium MS60/2

During the first sub-cultivations no significant differences were noticed concerning the direct shoot regeneration on the media containing different cytokinins although the regeneration potential of the medium containing BAP was better (Table 2). The 5-time decrease of the concentration of both, cytokinin and auxin, proved to be much more effective regarding the rate of the shoot multiplication. (Fig. 3). The regeneration potential of the shoots explants was 95.5% on medium B_2N_1 while it was only 66.7% on medium BNi. The maximal number of shoots obtained from one explant was 21 for medium B_2N_1 against 13 for medium BNi.

Table 2. Shoot multiplication efficiency on different media (RP – Regeneration Potential; PC – Propagation Coefficient)

Medium	BNi	KNi	RI	B_2N_1	B_2N_1
RP of the shoot explants [%]	66.7	55.0	13.8	100	95.5
PC of the First Sub-cultivation	1.9	2.3	-	-	-
PC of the Second Sub-cultivation	2.9	2.6	1.1	6.6	-
PC of the Third Sub-cultivation	-	-	-	6.7	10.2

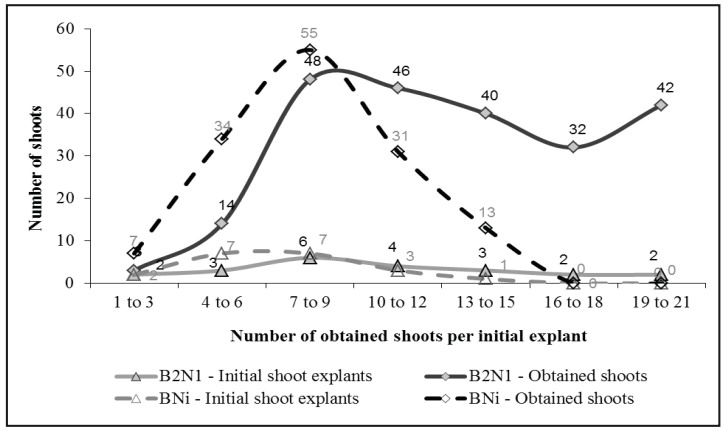

Fig. 3. Efficiency of shoot multiplication on media with different concentration of BAP and NAA

The comparison between the media B_2I_1 and B_2N_1 differing only in the auxin, showed that NAA was more suitable than IBA (Table 2). Shoots became long on both media and were additionally multiplied by vertical cutting during the sub-cultivation. Shoots grown on B_2N_1 were more numerous while those grown on B_2I_1 were longer (Fig. 4). The efficiency of the shoot multiplication was highest on medium B_2N_1 reaching an average of 10.2±2.8 shoots obtained per initial shoot explant. Shoots were strongly connected with each other which proved their direct regeneration; callus formed around shoots was easily removed (Fig. 5).

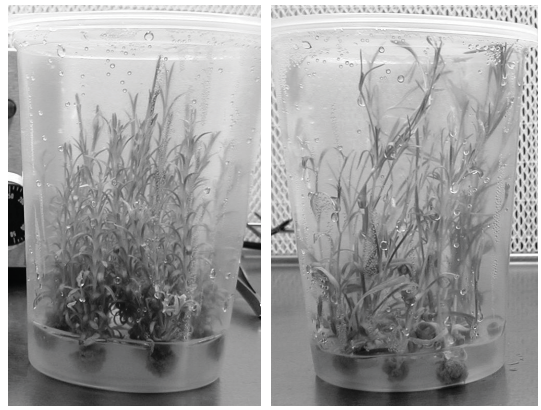

Fig. 4. *In vitro* plantlets grown on media B_2N_1 (left) and B_2I_1 (right)

Fig. 5. The lower part of the directly regenerated shoots, strongly connected with each other

Besides, 64.3% of the calli separated from the shoots regenerated new shoots, giving an average of 4.3 shoots per explant on MS medium, and 5.4 shoots per explant on medium MS60/2. However, the longer cultivation on medium with high sucrose concentration was unfavourable and led to morphological aberrations and hyperhydricity (Fig. 6). Normal shoots were separated from the callus and transferred to MS medium with 30 g/l sucrose for *in vitro* rooting.

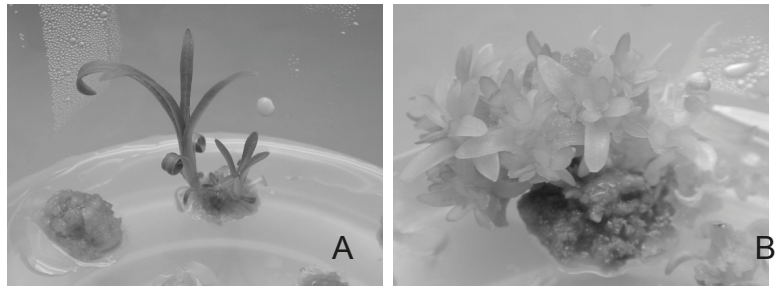

Fig. 6. Indirect shoot regeneration from callus, on medium MS60/2: A) Well developed shoots in the first month of cultivation; B) Hyperhydricity of numerous etiolated shoots cultivated several months

No spontaneous rooting was observed on media for shoot multiplication. The use of IBA as a sole PGR (medium RI) induced slight root formation in some shoots. The number of rooted shoots increased up to 57.1% on MS medium free of PGRs, for a period of 6 weeks, however roots were thin (Fig. 7-A). Rhizogenesis was further hampered by endophytic bacteria rising after the last sub-cultivation which caused loss of many shoots. The transfer of the upper part of the shoots on medium supplemented with active charcoal stimulated the rhizogenesis of the concerned plantlets. The survived plantlets were easily *ex vitro* adapted using a climate chamber, and then they were strengthened on the shelves of the room phytotron (Fig. 7-B). Plants were acclimated in the greenhouse for another 2 months (Fig. 7-C). Finally they were transferred to the experimental open-air field of the Institute of biodiversity and ecosystem research, in Sofia, where they are well developing. Several of the acclimated plants are currently blooming (Fig. 7-D).

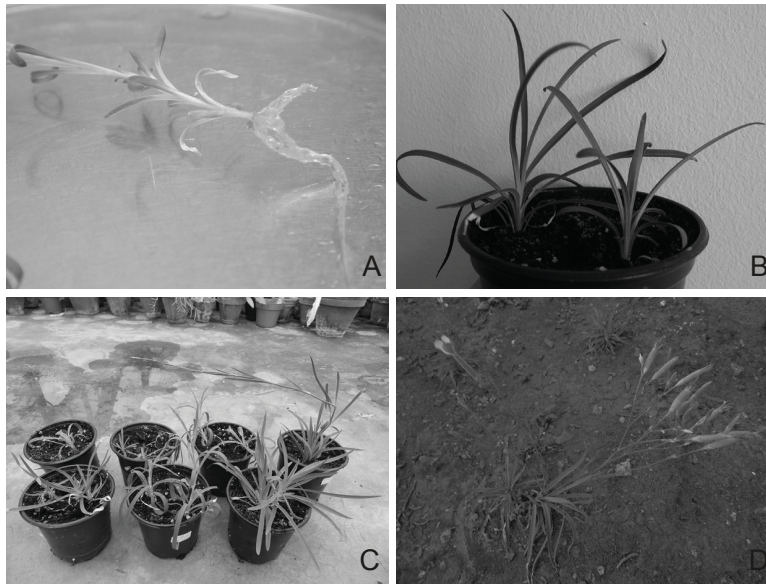

Fig. 7. Final steps of the method of *in vitro* micropropagation: A) *In vitro* rooted plantlet; B) *Ex vitro* adaptated plants in a room phytotron; C) Acclimated plants in a greenhouse; D) Acclimated flowering plant on the experimental field

Discussion

Seeds are most appropriate initial explants for *in vitro* micropropagation of rare plant species because of both: relatively easy disinfection from microbial contaminations and genetic diversity of the obtained cultures. Usually, the initiation of *in vitro* culture from plants living in the wild populations is difficult and needs special sterilization procedures. In our case the seed disinfection was successful as only ¼ of the seeds were eliminated due to microbial contamination. Some studies revealed very high contamination of the single stem nodes of *Dianthus nardiformis* causing between 51 and 75% lost explants [2]. Authors succeeded in seed disinfection reaching 100% effectiveness when using Domestos 2.4% for 10 minutes or two-step sterilization with hydrogen peroxide: first 2.5% for 16 h followed by 10% treatment for 15 minutes.

Between tested PGRs, BAP was found to be more appropriate cytokinin compared to Kin because of the better regeneration potential even if the propagation coefficient of the first two sub-cultivations was similar; NAA was chosen as more effective than IBA. The combination of BAP and NAA ensured good results especially after the decrease of their concentrations during the multiplication stage. This PGR combination was reported to be appropriate for different *Dianthus* species [3, 5]. Authors recommended a 10-fold decrease of the auxin in the multiplication stage compared to the initial medium, and PGR-free medium or further 10-fold decrease of both cytokinin and auxin for rhizogenesis [5]. Good rhizogenesis was reported as well on media containing 1 mg/l

NAA as a sole PGR or in combination with 0.1 mg/l BAP, for *Dianthus henteri*, endemic for the South Carpathians [6]. Our results on *in vitro* rooting of wild pink were limited because of the occasional manifestation of the endophytic microbial contaminants which impeded the *ex vitro* adaptation of the plantlets. This stage of the protocol could be further improved. Endophytic contaminants are frequent problem in *in vitro* cultures. There are different strategies to resolve this problem: identification of the bacteria and application of specific bacteriostatic or bactericidal substances e.g. antibiotics [7], or use of sugars free media for establishment of auxotrophic *in vitro* cultures which has also the advantage to be less costly [1].

The theoretical rate of the *in vitro* micropropagation evaluated about the best of the tested media is about 78 *in vitro* plantlets per seed, obtained for a period of eight months, including the time needed for *in vitro* rooting. The *ex vitro* adaptation and acclimation of the plants to the greenhouse took two additional months. They can stay in the greenhouse for a longer period in order to be transferred outside in spring. Plants strengthened and no more losses were noticed; however, after their transfer to the open field plot their stems elongated very fast and were binded to wood bars, otherwise they laid down. This step of the acclimatization could be further improved. The number of the plants regenerated by direct *in vitro* organogenesis was about three times higher than that of the plants obtained via indirect regeneration from callus. Morphologically, indirectly regenerated plantlets were normal and could not be distinguished from directly regenerated. However, to be sure about the absence of genetic aberrations due to somaclonal variation, additional tests should be applied.

In conclusion, after some improvement of the protocol of *in vitro* micropropagation, concerning both elimination of the endophytic bacteria *in vitro* and stem strengthening in the final stage of acclimatization, the method could be applied to multiply wild pinks originated from the region of Durankulak Lake. This would be a good opportunity for their mass propagation, aimed at their conservation and production for the gardening market. Further studies on the plants' metabolomics would be also of interest.

References:

1. Cristea V. Culturi *in vitro* fotoautotrofe la specii de *Dianthus* endemic şi periclitate din România. Todesco, Cluj-Napoca, 2010, p. 227.

2. Holobiuc I., Blindu R., Cristea V. Biotechn. Biotechn. Eq., 23:sup1, 2009, 221-224, DOI:10.1080/13102818.2009.10818405 2014

3. Jarda L., Cristea V., Halmagyi A., Plada M. Acta Hort., 918, 2011, 153-159.

4. Murashige T., Skoog F. Physiol. Plant., 15, 1962, 473-497.

5. Cristea V., Palada M., Jarda L., Butiuc-Keul A. Studia UBB Biologia, LVIII, 1, 2013, 57-69.

6. Cristea V., Deliu C., Oltean B., Butiuc-Keul A., Brummer A., Albu C., Radu G.L. Acta Hort., 843, 2009, 157-163.

7. Kulkarni A. A., Kelkar S. M., Watve M. G., Krishnamurthy K. V. Can. J. Microbiol., 53(1), 2007, 63-74.

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MORPHOMETRY OF EPIDERMIS OF LEAVES AND STEMS OF *RANUNCULUS ACRIS* (RANUNCULACEAE)

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Abstract

Aim: The aim of the study is to carry out ecological and anatomical investigation of different populations of *Ranunculus acris* in order to establish their variability.

M**aterials and Methods:** Four populations of *Ranunculus acris* from three floristic regions of Bulgaria were studied: the Rhodopes, the Danubian Plain and the Balkan Mountains.

Main results: The main characteristics of the leaf epidermis (upper and lower) and the stem were analyzed, including the size of the epidermal cells and stomata number. The epidermis analyzes of the anatomical characteristics of the leaves and stems of different populations of *R. acris* indicate that the length of the epidermal cells is the most variable parameter. The least variable is the number of the stomata.

Conclusions: The taxons of the population of *R. acris* from Chepelare have the largest size of epidermal cells and a greater number of stomata in comparison with the populations from Pavlikeni and Troyan. These differences are related to the environmental conditions and to the greater humidity, in particular.

Keywords: Ranunculaceae, *Ranunculus acris*, epidermis, anatomy, variability

Introduction

Ranunculaceae Juss. includes 59 genera and about 2500 species. The largest genus in the family is *Ranunculus* L. (Buttercup). Genus *Ranunculus* L. is one of the most abundant of the species and economically important genera of the family Ranunculaceae Juss. [1, 2]. The representatives of the genus occur in different environmental conditions and are characterized by high adaptability to the climate changes, which provides an opportunity for dissemination of large areas. In respect to their morphological characteristics, they have high variability in response to different ecological factors.

In Bulgaria, there are established 46 species of the genus [3]. The species are characterized

by high ecological plasticity [4]. They occur in different habitats – from dry areas to areas with humid climate, from low to high alpine areas, and show some morphological adaptations to the different environmental habitats [5]. Some taxa of the genus, which are result of extensive hybridization, have more specific requirements for the environmental conditions [6].

In Bulgaria, *R. acris* occurs in moist meadows and grassy places, along roads and ditches up to about 2000 m altitude [3]. The short life cycle, adaptability to a wide range of environmental factors, which provides distribution over large areas and diverse soil types, are typical for *R. acris* [7].

The aim of the study is to carry out ecological and anatomical investigation of different populations of *Ranunculus acris* in order to establish their variability.

Materials and Methods

Four populations of *Ranunculus acris* from three floristic regions of Bulgaria were studied: the Central Rhodopes (Asenovgrad and Chepelare), the Danubian Plain (Pavlikeni) and the Central Balkan Mountains (Troyan), with various environmental performances, which show the parameters of environmental variability. Ten individuals of each population were collected.

The geographical coordinates and the weather conditions of the settlements are:

Asenovgrad – 42.016667° N, 24.866667° E, 232 m altitude, 690 mm average annual rainfall, Rhodopes, Central;

Chepelare – 41.725833° N, 24.684444° E, 1232 m altitude, 800 mm average annual rainfall, Rhodopes, Central;

Pavlikeni – 43.242778° N, 25.321667° E 144 m altitude, 600 mm average annual rainfall, Danubian Plain;

Troyan – 42.883333° N, 24.716667° E, 400 m altitude, 620 mm average annual rainfall, Balkan Mountains, Central.

Anatomical and morphological indicators of the stem and leaves were analyzed – number of stomata, length, width and height of the upper and lower surface of the leaf and stem epidermis. Fresh material fixed in 75% ethanol was used. Semi glycerin preparations were prepared. The studies were conducted on a light microscope Amplival with eyepiece micrometer. The results were statistically analyzed on the basis of 20 measurements for each feature in each individual. The data obtained were processed by the method of descriptive statistics, with mean values (x) and standard deviation (stdv).

Results

Leaf epidermis

The main characteristics of the leaf epidermis (upper and lower) were analyzed, including the size of epidermal cells and stomata number of all four populations (Asenovgrad, Chepelare, Pavlikeni, Troyan).

The length of the upper epidermal cells is lower than the lower leaf epidermis. The smallest size of the upper (288.3±1.78 μm) and of the lower leaf epidermal cells (362.2±2.69

μm) was observed in the plants located in Pavlikeni. The highest length of the epidermal cells was observed in the plants from Chepelare (upper leaf epidermis 336.2±2.09 μm and lower leaf epidermis 421.2±2.63 μm). There are no major variations in the width of the upper epidermal cells – from 25.6±1.03 μm in Troyan to 29.6±1.06 μm in Chepelare. The width of the lower leaf epidermis varies from 23.6±1.06 μm in Troyan to 28.3±1.09 μm in Chepelare.

The height of the upper leaf epidermis varies from 34.6±1.03 μm (Troyan) to 40.7±1.09 μm (Chepelare). There are no big differences in the height of the main epidermal cells and of the lower epidermal cells. The data varied between 31.5±1.09 μm for plants from Troyan and 36.1±1.01 μm for plants from Chepelare. The data are presented in Table 1.

Table 1. Anatomical characteristics of the upper and lower leaf epidermis of four populations of *R. acris* L.

Taxon	*R. acris* L.			
Habitat	Asenovgrad	Pavlikeni	Troyan	Chepelare
Upper leaf epidermis, μm				
Length, μm	316.1±1.95	288.3±1.78	302.1±2.03	336.2±2.09
Width, μm	27.3±1.03	26.1±1.05	25.6±1.03	29.6±1.06
Height, μm	37.6±1.07	36.1±1.06	34.6±1.03	40.7±1.09
Stomata number/mm^2	28.4±1.08	24.3±1.11	21.2±1.16	33.7±1.15
Lower leaf epidermis, μm				
Length, μm	412.5±2.06	362.2±2.69	389.4±2.03	421.2±2.63
Width, μm	26.2±1.03	24.5±1.05	23.6±1.06	28.3±1.09
Height, μm	35.1±1.03	32.0±1.06	31.5±1.09	36.1±1.01
Stomata number/mm^2	38.6±1.01	34.5±1.01	31.8±1.22	41.3±1.05

The lower leaf epidermis contains more stomata compared to the upper leaf epidermis. The largest number of stomata is located in the lower leaf epidermis in the plants from Chepelare (41.3±1.05 number/mm^2) compared to those in the upper leaf epidermis (33.7±1.15 number/mm^2). There are no major variations in the stomata number in the upper leaf epidermis (21.2±1.16 number/mm^2 – 28.4±1.08 number/mm^2) and in the lower leaf epidermis (31.8±1.22 number/mm^2 – 38.6±1.01 number/mm^2) in the plants from Asenovgrad, Pavlikeni and Troyan (Table 1).

Stem epidermis

The size of the stem cells and stomata number of all four populations (Asenovgrad, Chepelare, Pavlikeni, Troyan) were investigated (Table 2).

The data in the table show that the length of the stems varied from 241.7±1.21 μm (Pavlikeni) to 322.9±1.01 μm (Chepelare). The largest width and height of the stem cells were observed in the plants from Chepelare. The values of the width of the stem cells in the plants from Cherelare were 32.3±1.23 μm. The width of the stem cells in the plants from

Asenovgrad, Pavlikeni and Troyan were 29.1±1.21 μm, 26.2±1.23 μm, and 24.6±2.01 μm respectively. The height of the stem cells varied from 31.5±1.05 μm (Troyan) to 41.6±1.23 μm (Chepelare).

The largest stomata amount was established in the plants from Chepelare 36.9±1.12 number/mm^2.

Table 2. Anatomical characteristics of the stems of four populations of *R. acris* L.

Taxon	Habitat	Length, μm	Width, μm	Height, μm	Stomata number/mm^2
R. acris L.	Asenovgrad	305.1±1.23	29.1±1.21	39.2±1.03	32.5±2.01
	Pavlikeni	241.7±1.21	26.2±1.23	32.1±1.09	30.4±1.40
	Troyan	270.2±1.61	24.6±2.01	31.5±1.05	27.9±1.25
	Chepelare	322.9±1.01	32.3±1.23	41.6±1.23	36.9±1.12

Discussion

A large part of the buttercups were found in a variety of soil and climatic conditions [8]. In general, *Ranunculus* L. species have a high capability of plasticity in new areas. They are adaptable to a wide range of climate change, which makes them able to spread over large areas. Many species have significant morphological variability [9]. In our previous studies, we have found a variability of the epidermal cells of *Ranunculus arvensis* L. from different habitats [10]. Exactly, these are the arguments on which we focused our studies to demonstrate the variability of the species *Ranunculus acris* L. depending on their habitat.

In the analysis of the main epidermal cells of the leaves and stems of *Ranunculus acris* L. from different populations, we have found significant differences in the width, height and length of the upper and lower leaf epidermal cells. The biggest length, width and height of the epidermal cells was found in the upper and in the lower leaf epidermis and greater number of stomata were observed in the plants from Chepelare compared to those in Asenovgrad, Pavlikeni and Troyan. Individuals from Chepelare were adapted to more humid environmental conditions. They are characterized by variability which defines them as mesophytic. They have larger epidermal cells and larger stomata. These plants are close in anatomical features to the plants in Asenovgrad, where the climate is more humid as compared to Pavlikeni. The plants from Pavlikeni are characterized by the smallest cell size of leaves and stems, and the lowest number of stomata, which is associated with the drier climate.

The number of stomata characterizes the ecological and geographical specialization of the species and shows certain variability specific for the individual taxa. However, the number of the stomata is the least variable, which is in compliance with other authors [11].

Conclusions

The individuals of the population of *R. acris* L. from Chepelare have the largest size of epidermal cells and a greater number of stomata in comparison with the populations from Pavlikeni and Troyan. These differences are related to the environmental conditions and to the greater humidity, in particular.

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References:

1. Tamura M. In: The families and genera of vascular plants (K. Kubitzki, J.G. Rohwer, V. Bittrich, Eds.) Springer, Berlin, 1993, 563-583.
2. Tamura M. In: Systematic Part. (P. Hiepko) Duncker & Humblot, Berlin, 1995, 223–519.
3. Delipavlov D., Cheshmedziev I. Guide to Plants in Bulgaria, Acad. Press, Plovdiv, 2003, p. 591.
4. Chen Q., Peng W., Qi S., Xu A. Planta Medica, 68, 2002, 550-553.
5. Paun O., Lehnebach C., Jahonsson J., Lockart P., Horandl E. Taxon, 54, 2005, 911-930.
6. Ovchinnikov P. N. In: Ranunculus (V. Komarov, Eds.), Institut Akademii Nauk USSR, Moscow, 1985, 271-388.
7. Hoffmann M., von Hagen K., Hörandl E., Röser M., Tkach N. International Journal of Plant Science 171, 2010, 90-106.
8. Tamura M., Sci. Rep. Osaka Univ. 12, 1963, 140-156.
9. Salisbury E. J. Bot. Land., 72, 1934, 185-196.
10. Dochev G. V., Zhalnov I., Docheva M. H. Turkish Journal of Agricultural and Natural Sciences, Special Issue: 2, 2014, 1464 – 1467.
11. Raschio N., Cuccato F., Dalla V. F., La Rocca N., Larcher W., Plant, cell and environment, 22, 1999, 205-212.

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A REVIEW OF THE MAIN CHARACTERISTICS OF *ELATOBIUM ABIETINUM* (WALKER) (HEMIPTERA, APHIDIDAE)

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Abstract

Elatobium abietinum (Hemiptera, Aphididae) is a pest infesting mainly *Picea* spp. This species was recently found for the first time in Bulgaria (in the spring of 2014) near Sofia on *Picea abies* and *Picea pungens*. The damage caused by the aphid leads to the full loss of needles of infested branches.

Aim: The aim of this paper is to present a review of the main characteristics of the newly detected aphid and its importance as a pest. The presented characteristics are based on a generalization of data from current studies carried out abroad.

Main characteristics: Description of host plants, damage, biology and ecological requirements of *Elatobium abietinum* are given.

Conclusion: *Elatobium abietinum* could survive in our country after mild winters and it could represent a serious problem for landscaping, nursery production, and forestry. Due to its harmful influence on *Picea* spp. and its potential for rapid spread, it is imperative that additional studies in Bulgaria are carried out on its biology and ecological characteristic. Such studies will contribute to a more appropriate control strategy of *Elatobium abietinum.*

Keywords: aphid, Aphididae, Bulgaria, *Elatobium abietinum*, *Picea*, pest

Introduction

Green spruce aphid *Elatobium abietinum* (Walker 1849) (Hemiptera, Aphididae) is a pest infesting the species from Pinaceae family [1, 2]. It originates from Central and Northern Europe and it is a major pest on spruce in many countries, especially in regions with maritime climate [3, 4, 5].

This species was recently found for the first time in Bulgaria (in the spring of 2014) near Sofia – in Bistritsa, Pancharevo, and Dragalevtsi on Norway spruce*, Picea abies* (L.) H. Karst. and Colorado spruce, *Picea pungens* Engelm. The damage caused by the aphid (Fig. 1) leads to the full loss of needles of infested branches [6]. This species diminishes the quality of ornamental plants and decreases the growth of affected trees, even though it seldom kills them [7]. The need for a high decorative effect requires such plants to be in good health and free from pests.

Fig. 1. Damage caused by *Elatobium abietinum* on *Picea pungens*

The aim of this paper is to present a review of data from current studies carried out abroad and to generalize the main characteristics of *E. abietinum* which will contribute to future studies and control of this pest species in Bulgaria.

Main characteristics

Host plants

Elatobium abietinum is a pest infesting mainly *Picea* spp. On rare occasions it may also infest *Abies* spp., *Pseudotsuga menziesii* (Mirb.), *Larix sibirica* Ledeb., *Pinus sibirica* Du Tour, *Pinus strobus* L. and *Pinus sylvestris* L. [1, 2, 3].

Damage

Elatobium abietinum feeds on the lower side of needles older than one year [4, 8] in the shaded part of the crown. An aphid outbreak results in significant losses of two and three year old needles during the following growing season [9]. Aphids can colonize the new growth and the upper crown of the infested plant if the pest numbers are very high.

Elatobium abietinum sucks phloem sap from the needles. The pest causes needle necrosis, chlorosis, dehydration, and premature drop of the needles [10]. Frequently the aphid feeding leads to severe defoliation and reduction in tree growth [4, 11]. Sometimes *Elatobium abietinum* may completely defoliate and kill trees [12]. Yellowing of the needles indicates a green spruce aphid infestation. The reason for this is the elicited chlorophyll loss which leads to discoloration of leaves and plant tissues and results in dropping of the needles of infested plants [13].

Trees stressed by drought and attacked by aphids are also more susceptible to bark beetle attack [14]. Heavy defoliation of plants, infested by *Elatobium abietinum*, diminishes defense mechanisms and significantly increases the host susceptibility to secondary pests [15, 16].

Biology and Ecology

Elatobium abietinum is a monoecious and holocyclic species with alatae males in some parts of continental Europe [17], but elsewhere it appears to be anholocyclic [2], especially in regions with maritime climate [4]. In the northwestern part of Europe onsets are usually detected in spring and early summer, but in Iceland outbreaks usually occur in fall or early winter [5]. Overwintering eggs hatch in April. They can resist severe winter frost [17, 18] and are able to withstand temperatures as low as -30°C [19].

The mean development time for one generation of green spruce aphid at 15°C is 15-24 days [12, 20, 21].

Anholocyclic aphids keep high population levels throughout the year by continuous viviparous reproduction [8, 22, 23]. The development threshold for this species is about 4°C. The aphid numbers start to increase exponentially, even in March, if the weather is favourable [24]. Aphid numbers build up rapidly to high densities soon after the bud-burst of the trees and when the nutritional quality of the phloem sap is at its highest (usually towards the end of April). They cause severe damage until their population peak in May [25]. The distribution of this pest is carried out by alatae, which are produced at high population levels during the spring peak in May and June [8, 22, 23]. The production of alatae for both, the holocyclic and the anholocyclic green spruce aphids is influenced by crowding, host quality and temperature. Another determinant of producing of alatae specimens in spring is the increasing day length with a critical limit of 14 hours, beyond which the proportion developing into alatae declines [26].

In response to a reduction of the nutritional quality of the phloem sap aphids decline and remain low until autumn [8, 22, 23]. The aphid numbers surviving the winter are generally low [24], because of the low winter temperatures, which cause high mortality rates in *Elatobium abietinum* populations [10, 11, 27]. Serious infestations usually occur after mild winters [25]. Long periods with relatively low, but not lethal, temperatures have a negative influence on *Elatobium abietinum* populations. The main reason for winter mortality is freezing temperatures. Adults and nymphs of green spruce aphid are able to survive temperatures as low as -15°C to -18°C when detached from the needles. In contrast, when aphids are feeding on the host plant most individuals die at temperatures of -10 to -12°C. The latter is explained with the ice nucleation in the phloem sap which spreads to the gut contents. The reason is the higher supercooling point of the sap than the aphids. When the sap freezes so do the aphids. At the same time unfed first-instar nymphs can withstand temperatures below -20°C [27, 28, 29, 30].

Mortality can also be caused by negative temperatures around -8°C, starvation by prolonged period with temperatures below the feeding threshold and temperatures lower than the reproductive threshold [27, 31].

There are differences in aphid density between open and shaded areas. This is due to a number of factors, acting either separately or together. These factors are plant defence mechanisms, plant nutritional quality (e.g. changes in nitrogen content, defensive terpene compounds, or concentrations of particular amino-acids), predator abundance and microclimate (e.g. sensitivity to UV). In the shade aphid population densities are significantly higher than in the open [32]. These observations are corroborated by Bladon

[33], who found that aphid population densities observed on P*icea sitchensis* (Bong.) Carr. seedlings grown under shade are three to four times higher than on seedlings grown in full light conditions. There are also suggestions that aphid populations are greater, or persist longer, in cooler habitats.

Conclusion

We conclude that the newly established *Elatobium abietinum* in Bulgaria, due to its main characteristics and the fact that *Picea* species are often used in landscaping and in forestry wood production in Bulgaria, is associated with a high potential risk of spread in our country. *Elatobium abietinum* could survive in our country after mild winters and it could represent a serious problem for landscaping, nursery production, and forestry. It could be a threat to both large- and small-sized spruce. Due to its harmful influence on *Picea* spp. and its potential for spread, it is imperative that additional studies in Bulgaria are carried out on its biological and ecological characteristic. Such studies will contribute to a more appropriate control strategy of *Elatobium abietinum*.

References:

1. Holman J. Host Plant Catalog of Aphids. Palearctic Region, Springer, 2009, p. 1216.
2. Blackman R. L., Eastop V. F., Aphids on the world's plants. Verson 2015. Online at http://www.aphidsonworldsplants.info/, 2015.
3. CABI, *Elatobium abietinum.* Verson 2015. Online at http://www.cabi.org/isc/datasheet/30897, 2015.
4. Straw N. A., Halldórsson G., Benedikz T. In: The Green Spruce Aphid in Western Europe; Ecology, Status, Impacts and Prospects for Management (K. R. Day, G. Halldórsson, S. Harding, N. A. Straw, Eds.) Edinburgh, Forestry Commission, Technical Paper, 24, 1998, 15-31.
5. Carter C. I., Halldórsson G. In: The Green Spruce Aphid in Western Europe; Ecology, Status, Impacts and Prospects for Management (K. R. Day, G. Halldórsson, S. Harding, N. A. Straw, Eds.) Edinburgh, Forestry Commission, Technical Paper, 24, 1998, 1–14.
6. Yovkova M., Pencheva A., Acta Zoologica Bulgarica (in press).
7. Halldórsson G., Benediktz Ó., Eggertsson Ó., Oddsdóttir E. S., Óskarsson H., Forest ecology and management, 181, 2003, 281-287.
8. Parry W. H., Oecologia, 23, 1976, 297-313.
9. Black K., Ray D. ECOFOR, Irish National Report, V1, 2009, p. 34.
10. Bevan D., Scottish Forestry, 20, 1966, 193–201.
11. Parry W. H., Forestry, 42, 1969, 69-82.
12. Hussey N. W., Scottish Forestry, 6 (4), 1952, 121-130.
13. Quiseberry S. S., Ni X. In: Aphids as Crop Pests (H. F. van Emden, R. Harrington, Eds.) CABI International, 2007, 331-353.
14. Ray D., Xenakis G., Semmler T., Black K. In: Forests, Carbon and Climate Change, Local and International Perspectives (E. Hendrick, K. G. Black, Eds.) COFORD, National

Council for Forest Research and Development, Dublin, Ireland, 2008, 27-33.
15. Raffa K., Krause S., Reich P., Ecology, 79, 1998, 2352-2364.
16. Lieutier F. In: Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis (F. Lieutier, K. R. Day, A. Battisti, J. C. Grégoire, H. F. Evans, Eds.) Kluwer Academics Publishers, Dordrecht, 2007, 135-181.
17. von Scheller H. D., Zeitschrift für Angewandte Entomologie, 51, 1963, 258-284.
18. Skov E., Wellendorf H., Forest Genetics, 7 (3), 2000, 233-246.
19. Carter C., Austarå O., Fauna Norvegica. Serie B, Norwegian Journal of Entomology, 41 (2), 1994, 53-58.
20. Fisher M., Forestry Commission Report on Forest Research, 1986, 71-72.
21. Cunliffe N., Quarterly Journal of Forestry, 18, 1924, 133-146.
22. Parry W. H., Oecologia, 15, 1974, 305-320.
23. Day K. R., Straw N. A., Harding S., In: The Green Spruce Aphid in Western Europe; Ecology, Status, Impacts and Prospects for Management (K. R. Day, G. Halldórsson, S. Harding, N. A. Straw, Eds.) Edinburgh, Forestry Commission, Technical Paper, 24, 1998, 97–105.
24. Crute S., Day K. In: Population Dynamics of Forest Insects (A. D. Watt, S. R. Leather, M. D. Hunter, N. A. C. Kidd, Eds.) Andover, UK, Intercept, 1990, 329-337.
25. Williams D. T., Straw N. A., Day K. R., Agricultural and Forest Entomology, 7, 2005, 95-105.
26. Fisher M., PhD Thesis, University of East Anglia, 1982.
27. Powell W., Parry W. H., Annales of Applied Biology, 82, 1976, 209-219.
28. Halldórsson G., Docherty M., Oddsdóttir E. S., Day K. R., Búvísindi icel. agr. sci., 14, 2001, 75-84.
29. Carter C. I., Forest Record, 84, 1972, p. 10.
30. Powell W., Annals of Applied Biology, 78 (1), 1974, 27-37.
31. Day K. R., Crute S. In: Population dynamics of forest insects (A. D. Watt, S. R. Leather, M. D. Hunter, N. A. C. Kidd, Eds), Intercept, Andover, UK, 1990, 25-33.
32. Bertin S., PhD Thesis, University of Edinburgh, School of GeoSciences, 2009.
33. Bladon F., PhD Thesis, University of Ulster, Coleraine, Northern Ireland, 2009.

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A STUDY OF AQUATIC VEGETATION IN TWO BULGARIAN DANUBE WETLANDS

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Abstract

Aim: The present study aims to investigate the macrophyte communities in two Danube swamps, situated on the river floodplain.

Materials and Methods: The species composition of aquatic plants was studied in Malak Preslavets Swamp and Garvan Swamp in 2014, following the transect methodology. Plant abundance was estimated according to a seven-point-scale established by Braun-Blanquet. Several environmental variables were measured: water temperature, oxygen saturation, electrical conductivity, pH and substrate type.

Results: 54 macrophytes were found, including seven rare species with limited distribution, a single species of liveworts and six invasive species. In Garvan Swamp the *Phragmites australis* community dominates, while in Malak Preslavets Swamp - the *Nymphae alba* community. The environmental conditions and parameters are similar across the 2 study areas. However, there are differences between some of the parameters, such as the oxygen saturation and the electrical conductivity. The data analysis reveals 41% similarity in the species composition between the two wetlands.

Conclusions: The overall species diversity is relatively high. We found around 34% of the total number of macrophytes in Bulgaria. The biodiversity is higher in Malak Preslavets Swamp, which is due to the better ecological conditions in this water body.

Keywords: freshwater aquatic vascular plants, Danube wetlands, biodiversity, environmental monitoring

Introduction

The Danube wetlands play a very important role in maintaining and enriching the biodiversity in the Danube River Basin. Macrophyte vegetation also contributes greatly to the biodiversity in the Danube region.

The present study aims to investigate the species diversity of macrophyte communities, their syntaxonomic position, as well as several physico-chemical parameters in two Danube swamps situated on the river floodplain - Malak Preslavets Swamp and Garvan Swamp. Data on the aquatic vegetation of these two swamps is very sparse. The only publications are mainly single studies of aquatic vegetation along the Danube River floodplain from the 1980s, such as [1, 2] and one recent publication [3], which present information about other Danube wetlands. They provide only fragmentary information and apply a different methodology for the vegetation surveys; moreover, only [1] and [2] contain data particularly on the aquatic vegetation of the two studied swamps. The present study aims to contribute to the data on the current state of aquatic macrophyte biodiversity in the Danube wetlands.

Materials and Methods

The species composition of aquatic plants was studied in Malak Preslavets Swamp and Garvan Swamp (Fig. 1) in 2014. The study sites are stagnant water bodies situated on the Danube River floodplain. They are located next to Malak Preslavets and Garvan villages respectively. Both swamps are part of the Natura 2000 network [4].

After building a protective embankment in the 1950s that separates Garvan Swamp from its natural source of fresh water, it began to shrink. Usually water is retained in the swamp only until the beginning of summer. The majority of the protected site is a large reed and swamp area with water mirrors which vary between 120 and 160 ha. The depth of the water also varies - from 1.50-1.60 m in high-water years to 0.70 - 0.80 m in low-water years, depending mainly on the water level of the Danube. Very often there is water in the swamp from March to June, then it dries up completely in July and August, and is filled again during the remaining months of the year.

Malak Preslavets Swamp was transformed into a reservoir and isolated from the Danube River by a dam in the 1960s. Currently its fresh water supply is from surface water, and possibly from groundwater springs. Its surface is higher than the average level of the Danube River. Therefore, the direction of the water flow is one-way - from the swamp to the Danube River. Its depth reaches four-five meters, and its area is 38.5 to 51.48 ha.

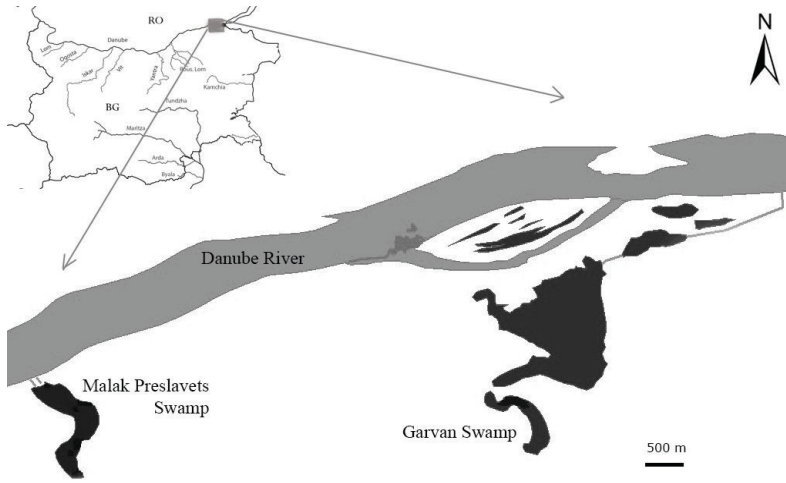

Fig. 1. Map of the study area

Following the transect methodology, transects long enough to encompass the macrophyte vegetation present at each site were established.

The fieldwork was carried out during the June-October period, which is characterized by maximum aquatic vegetation development.

For the purposes of the syntaxonomic work on site, the 7-degree scale of Braun-Blanquet was used [5]. The syntaxa were determined according to [6].

In order to fully characterize the macrophyte habitats, several environmental variables were also measured: water temperature, oxygen saturation, electrical conductivity, pH and substrate type. Measurements were made with WTW multi1960i set.Vascular plant species were determined on site according to the field guide of vascular plants of Bulgaria [7]. The plant names and authors were verified after Flora Europaea [8]. The invasive species are determined according to [9].

Results and Discussion

During the study 54 macrophyte species were found, belonging to 48 genera and 27 families (Table 1).

Table 1. Distribution of species in the study area. Superscripts refer to: [1] - invasive species; [2] -not macrophyte

Taxa/Site	Garvan Swamp	Malak Preslavets Swamp
Amorpha fruticosa [1,2]	+	+
Artemisia annua [2]		+
Ballota nigra [2]		+
Berula erecta		+
Bidens frondosa [1]		+
Bidens tripartita		+
Butomus umbellatus		+
Calystegia sepium [2]	+	
Carex sp.	+	
Ceratophyllum demersum		+
Ceratophyllum submersum	+	
Cichorium intybus [2]		+
Clematis integrifolia [2]		+
Convolvulus arvensis [2]	+	
Dactylis glomerata [2]		+
Echinochloa crus-galli		+
Elodea nuttallii [1]		+
Erigeron annuus [1, 2]		+
Erodium cicutarium [2]	+	
Glyceria maxima		+
Glyceria fluitans		+
Glycyrrhiza echinata		+
Hydrocharis morsus-ranae	+	+

Iris pseudacorus		+
Juncus effusus	+	
Lemna gibba		+
Lemna minor	+	+
Lemna trisulca	+	+
Lolium perenne [2]		+
Lycopus europaeus		+
Lycopus exaltatus		+
Lysimachia nummularia	+	
Lysimachia vulgaris		+
Lythrum salicaria	+	+
Lythrum virgatum		+
Mentha aquatica		+
Myosoton aquaticum	+	
Najas marina		+
Nymphaea alba	+	+
Oenanthe aquatica	+	+
Paspalum distichum [1]		+
Persicaria amphibia	+	
Persicaria hydropiper	+	
Persicaria lapathifolia	+	
Persicaria minor	+	
Persicaria mitis	+	
Phragmites australis	+	+
Plantago altissima [2]		+
Plantago major [2]		+
Potamogeton lucens		+
Ranunculus repens		+
Ricciocarpus natans	+	
Rorippa amphibia	+	
Rubus caesius [2]		+
Rumex hydrolapathum	+	+
Salvinia natans	+	
Schoenoplectus lacustris	+	+
Scutellaria galericulata		+
Senecio paludosus		+
Sicyos angulatus [1,2]		+

Solanum dulcamara [2]	+	+
Sparganium erectum	+	+
Spirodela polyrrhiza		+
Stachys palustris	+	+
Tanacetum vulgare [2]		+
Trifolium pratense [2]		+
Typha angustifolia	+	+
Typha latifolia	+	+
Typha laxmannii		+
Urtica dioica [2]		+
Utricularia vulgaris	+	
Veronica longifolia [2]		+
Xanthium italicum [1]	+	
Wolffia arrhiza		+

Seven of the observed plant species are rare and have limited distribution: *Nymphaea alba* L., *Typha laxmannii* Lepech., *Ceratophyllum submersum* L., *Wolffia arrhiza* (L.) Horkel ex Wimm., *Lemna gibba* L., *Salvinia natans* (L.) All. and *Potamogeton lucens* L. [10]. The white water lily *Nymphaea alba* has an 'endangered status' both in the *Red list of the Bulgarian vascular plants* [11] and in the *Red Data Book of the Republic of Bulgaria* [12]. It is also included in the *Biological Diversity Act* [13]. The species *Salvinia natans* All. has a 'vulnerable' status in the *Red list of the Bulgarian vascular plants*. It is included in the *Biological Diversity Act* and in the *Bern Convention* [14]. *Wolffia arrhiza* and *Lemna gibba* are also included in the *Red list of the Bulgarian vascular plants* with 'vulnerable' and 'nearly threatened' status respectively.

A single species of liverworts was found in the course of our fieldwork: *Ricciocarpus natans* L. in Garvan Swamp.

The invasive species play a crucial role in the macrophyte communities' formation in the two studied wetlands. Six invasive species, which participate in the community development, were found on the territory of the two swamps: *Xanthium italicum* Moretti, *Elodea nuttalli* (Planch.) H. St. John, *Bidens frondosa* L., *Paspalum distichum* L., *Erigeron annuus* (L.) Pers. and *Sicyos angulatus* L.

The aggressive and adaptable *Amorpha fruticosa* L. was found along the banks at all sites and therefore plays a crucial role in the vegetation assemblages even though it is not a macrophyte.

The Poaceae and Lamiaceae families were the most diverse families with seven and six species respectively, followed by Polygonaceae and Lemnaceae with 5 species each. The family Asteraceae and Typhaceae were represented by 3 species each. The rest of the families were represented by two or a single species.

The macrophyte diversity in the study area is relatively high. We found around 34% of the total number of macrophytes in Bulgaria (163, according to the 'Atlas of aquatic and

wetland plants in Bulgaria' [10]). The rich biodiversity could be the result of the stagnant type of the studied water bodies, as well as the well-developed communities there.

The data analysis reveals 41% similarity in the species composition between the two wetlands.

The two studied wetlands are part of the Natura 2000 network and are fundamental elements of the habitat '3150 Natural eutrophic swamps with *Magnopotamion* or *Hydrocharition* - type vegetation' [4].

In Garvan Swamp the *Phragmites australis* community dominates, while in Malak Preslavets Swamp - the *Nymphae alba* community.

The macrophyte communities determined in the two swamps belong to twelve associations, seven alliances, four orders and four classes, from a syntaxonomical point of view.

The syntaxa which were established belong to the following subtypes:

1. In Malak Preslavets wetland:

Class Lemnetea O. Bolos et Masclans 1955

Order ***Lemnetalia minoris*** Tuxen ex O. Bolos et Masclans 1955

Alliance ***Lemnion minoris*** Tuxen ex O. Bolos et Masclans 1955

Ass. ***Lemnetum minoris*** Th. Muller. et Gors 1960 – the macrophyte communities in this association are established on the periphery of the open water. Fragments could be established between the stems of the reed beds and bullrushes. The species *Lemna trisulca, Spirodela polyrhiza* and individual specimens of *Ceratophyllum demersum* could be observed beside the dominant species.

Ass. ***Lemno-Spirodeletum*** W. Koch 1954 – the distribution of this association is similar to the previously described one. It is very often that communities of this association form complexes with communities of ass. *Lemnetum minoris*.

Alliance ***Lemnion trisulcae*** Hartog et Segal ex Tuxen et Schwabe-Braun in Tuxen 1974

Ass. ***Lemnetum trisulcae*** Knapp et Stoffers 1962 – around the periphery of the open water areas. The dominant species is typically accompanied by species with similar biology and lifestyle such as *Spirodela polyrhiza* and *Lemna minor*.

Class Potametea pectinati Klika in Klika & Novák 1941

Order ***Potametalia*** W. Koch 1926

Alliance ***Nymphaeion albae*** Oberd. 1957

Ass. ***Nymphaeetum albae*** Vollmar 1947 – the macrophyte communities of this association are almost solely composed of the dominant species *Nymphaea alba*. Individual specimens of *Ceratophyllum demersum, Lemna minor* and *Spirodela polyrhiza* are often observed in the water under the leaves of *Nymphaea alba*. The biggest white water lily population in Bulgaria is found in this wetland.

Alliance ***Potamion polygonifolii*** Hartog & Segal 1964

Ass. ***Potametum lucentis*** Hueck 1931 – the communities of this association cover relatively small parts which are located around the inflow section. They are usually monodominant. The species *Lemna minor*, *L. trisulca* and *Spirodela polyrhiza* are rarely found beside the dominant species *Potamogeton lucens*.

Class Phragmiti australis-Magnocaricetea elatae Klika in Klika & V.Novák 1941

Order ***Phragmitetalia australis*** W. Koch 1926

Alliance ***Phragmition communis*** W. Koch 1926

Ass. ***Typhetum angustifoliae*** Pignatti 1953 – the communities are pure and form complexes with the association phytocoenoses from this alliance.

Ass. ***Typhetum latifoliae*** G. Lang 1973 – the communities are almost pure, composed only by *Typha latifolia*. They cover bigger areas than the previously described association.

Ass. ***Phragmitetum communis*** Soo 1927 – the communities are almost pure, comprised only of reed. Sometimes the species *Typha angustifolia, T. latifolia, Sparganium erectum* and *Schoenoplectus lacustris* participate in the community formation.

Ass. ***Sparganietum erecti*** Roll 1938 – these communities prefer shallows along the periphery of the open water areas. Sometimes they form almost pure assemblages of different sizes. It is common for several species with the same ecology to participate actively in their communities, such as *Typha angustifolia, T. latifolia* and *Schoenoplectus lacustris*.

Class Molinio-Arrhenatheretea Tüxen 1937

Order ***Molinietalia*** W. Koch 1926

Alliance ***Veronico longifoliae-Lysimachion vulgaris*** (Passarge 1977) Bál.-Tul. 1981

Ass. ***Glycyrrhizo echinatae- Bolboschoenetum maritimae*** [3]

These hydrophytic highgrass communities have a secondary origin. They cover relatively small areas along the periphery of the inflow section. The dominant species in these phytocoenoses is *Glycyrrhiza echinata.* The species *Artemisia annua, Iris pseudacorus, Lysimachia vulgaris, Lythrum salicaria, L. virgatum, Veronica longifolia, Rubus caesius, Senecio paludosus, Stachys palustris, Tanacetum vulgare, Urtica dioica* very actively participate in the grassland communities formation. The invasive species *Amorpha fruticosa, Bidens frondosus, Erigeron annuus* and *Sicyos angulatus* also play a crucial role in the macrophyte communities formation [3, 15].

2. In Garvan wetland

Class Lemnetea O. Bolos et Masclans 1955

Order ***Lemnetalia minoris*** Tuxen ex O. Bolos et Masclans 1955

Alliance ***Lemnetalia minoris*** Tuxen ex O. Bolos et Masclans 1955

Ass. ***Lemnetalia minoris*** Th. Muller. et Gors 1960 – the macrophyte communities are spread along the periphery of the open water areas. The species *Lemna trisulca, Spirodela polyrhiza* and individual specimens of *Ceratophyllum submersum* are also part of the community formation.

Alliance ***Lemnion trisulcae*** Hartog et Segal ex Tuxen et Schwabe-Braun in Tuxen 1974

Ass. ***Lemnetum trisulcae*** Knapp et Stoffers 1962 –around the periphery of the open water areas. The dominant species is almost always accompanied by species with similar biology and lifestyle, such as *Spirodela polyrhiza* and *Lemna minor.*

Class Potametea pectinati Klika in Klika & Novák 1941

Order ***Potametalia*** W. Koch 1926

Alliance ***Nymphaeion albae*** Oberd. 1957

Ass. ***Nymphaeetum albae*** Vollmar 1947 – the communities are dominated only by *Nymphaea alba* and cover small areas.

Class Phragmiti australis-Magnocaricetea elatae Klika in Klika & V.Novák 1941

Order ***Phragmitetalia australis*** W. Koch 1926

Alliance ***Oenanthion aquaticae*** Hejny ex Neuhausl 1959

Ass. ***Rorippo amphibiae–Oenanthetum aquaticae*** (Soo 1928) Lohmeyer 1950 – these communities are developed mostly along the shallow parts of the open water. Small groups of *Lythrum salicaria, Sparganium erectum, Typha latifolia* and *Lemna minor* usually also participate in the community formation.

Alliance ***Phragmition communis*** W. Koch 1926

Ass. ***Typhetum angustifoliae*** Pignatti 1953 – these communities are dominated mostly by *Typha angustifolia*.

Ass. ***Typhetum latifoliae*** G. Lang 1973 – these communities consist entirely of *Typha latifolia* and form complexes with the association phytocoenoses from this alliance. They are situated along the periphery of the open water.

Ass. ***Phragmitetum communis*** Soo 1927 – the communities are unmixed, composed only of reed patches. Sometimes *Typha angustifolia, T. latifolia, Sparganium erectum* and *Schoenoplectus lacustris* are also present in the assemblage structure.

Ass. ***Sparganietum erecti*** Roll 1938 – these communities prefer shallow places around the periphery of the open water. Sometimes they form small, nearly pure patches. Species with similar ecology, such as *Typha angustifolia, T. latifolia* and *Schoenoplectus lacustris* participate in the community formation.

Ass. ***Eleocharietum palustris*** Ubrizsy 1948 – this syntaxon is scarcely presented in this wetland's vegetation. The communities cover small surface in the open-space areas between the reedbeds. From a floristic point of view they have a poor species composition. The community is formed mainly of hygrophytes: *Rorippa sylvestris*, species from *Carex* sp. and *Oenanthe aquatica* (if the water level is high).

Comparison with the data from [1] shows that in Garvan Swamp the number of species is nearly the same in both studies, but we didn't find some of the species, which were recorded in [1], such as *Marsilea quadrifolia, Ranunculus aquatilis* and *Potamogeton pusillus*.

In Malak Preslavets Swamp a big difference in species composition is registered: we found 39 species, and in [1] they were 46. Some of the species we didn't find are: *Azolla filiculoides, Myriophyllum verticillatum, Marsilea quadrifolia, Potamogeton crispus, Potamogeton fluitans, Ceratophyllum submersum*. This could be due to misdetermination of some of the species, but some of them probably have really disappeared, and there is degradation in the species biodiversity.

[2], only found one association in Malak Preslavets Swamp - the *Salvinio-Spirodolletum* Slavnic 1965. Not only this association, but also the species *Salvinia natans* was not registered in Malak Preslavets Swamp during our study. The association in Garvan Swamp registered during the study of [2] was *Nymphoidetum peltatae* (Allorge 1922) Th. Muller et Gors 1960. We found neither the species *Nymphoides peltata* nor the association in this swamp either.

This disappearance of some of the species since the study of [2] could be a result of the change of the hydrological conditions in both swamps in the 50s and 60s, and also from the human impact on them as a whole.

The environmental conditions and parameters are similar across the two study areas. However, there are differences between some of the parameters, such as the oxygen saturation (43.5% in Garvan Swamp; 112% in Malak Preslavets Swamp) and the electrical conductivity (880 µS/cm in Garvan Swamp; 680 µS/cm in Malak Preslavets Swamp) (Table 2).

Table 2. Values of the physico-chemical parameters in the studied wetlands

	Malak Preslavets Swamp	Garvan Swamp
pH	8.20	7.46
water temperature (°C)	26	28.1
O_2 (mg/L)	9.7	3.16
O_2 (%)	112	43.5
C (µS/cm)	680	880
Substrate	mud	mud

Conclusions

The overall species diversity is relatively high. We found a total of 54 macrophyte species – 28 species in Garvan Swamp and 39 in Malak Preslavets Swamp.

The biodiversity is higher in Malak Preslavets Swamp, which is due to the better ecological conditions in this water body.

The macrophyte communities in the study area belong to the habitat '3150 Natural eutrophic swamps with *Magnopotamion* or *Hydrocharition* - type vegetation', which is a habitat with European significance.

The measured parameters show that the major reason for the bigger biodiversity in Malak Preslavets Swamp is the high amount of dissolved oxygen in its water. This is a result of the bigger flow of the river which is flowing into the water body of Malak Preslavets Swamp.

The comparison with data from [1] and [2] shows that there is a degradation in the species biodiversity since the study of [2] which could be a result of the change of the hydrological conditions in both swamps in the 50s and 60s, and also from the human impact on them as a whole.

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Reference:

1. Kochev H., Yordanov D. Vegetation of water basins in Bulgaria. Ecology, protection and economic importance, Academic Press, Sofia, 1981, p. 183

2. Kochev H., Husak S., Otahelova H. 1987. In: Int. Symp. 'The role of wetlands

in preserving the genetic material' Srebarna, 8-12 October 1984. Collected works (S. Nedialkov, H. Kochev, T. Michev, A. Damyanova, V. Velev, Eds), BAS, Sofia, 1987, 81-97

3. Tzonev R. In: Proc. Fourth Balkan Bot. Cong., Sofia 2006. (D. Ivanova, Ed.), Publishing House Bulg. Acad. Sci., Sofia. 2009a, 321-331.

4. http://natura2000.moew.government.bg/Home/ProtectedSite?code=BG0000517&siteType=HabitatDirective

5. Braun-Blanquet J. Plant Sociology: The Study of Plant Communities. Authorized English translation of 'Pflanzensoziologie' by G. D. Fuller & H. S. Conard. McGraw-Hill Book Company, New York, 1932, p. 439.

6. Tzonev R. T., Dimitrov M. A, Roussakova V. H. Phytol. Balcan., 15 (2), 2009, 209 – 233.

7. Kozhuharov S. (Ed). Field Guide to the Vascular Plants in Bulgaria. Nauka & Izkustvo, Sofia, 1992, p. 788 (in Bulgarian).

8. http://rbg-web2.rbge.org.uk/FE/fe.html

9. Petrova A., Vladimirov V., Georgiev V. Invasive alien plant species in Bulgaria. IBER, BAS, Sofia, 2012, p.320 (in Bulgarian).

10.Tsoneva S., Georgiev V., Valchev V., Ganeva A. Atlas of aquatic and wetland plants in Bulgaria. IBER, Sofia, 2012, p. 200 (in Bulgarian).

11. Petrova A., Vladimirov V. (Eds). Phytol. Balcan., 15 (1), 2009, 63-94.

12. Peev D. (Ed). Red Data Book of the Republic of Bulgaria,Vol. 1. Plants and Fungi. Digital edition. http://e-ecodb.bas.bg/rdb/en/vol1/.

13. Biological Diversity Act. 2002. Decree no. 283 accepted by the 39th National Assembly on 02 August 2002. – Durzhaven Vestnik, no.77/09.08.2002. Pp. 9-42 (in Bulgarian).

14. Bern Convention (Convention on the Conservation of European Wildlife and Natural Habitats). Appendix I.1979

15. Tzonev R. Biotechnol. & Biotechnol. EQ., 23:sup1, 2009, 354-359. http://www.tandfonline.com/doi/pdf/10.1080/13102818.2009.10818438

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ECOLOGICAL STATUS OF THE RIVERS IN "CENTRALEN BALKAN" NATIONAL PARK (BULGARIA) ACCORDING TO BOTTOM MACROINVERTEBRATES

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Abstract

Aim: To present preliminary data about the ecological conditions of some rivers from "Centralen Balkan" National Park (Bulgaria).

Materials and Methods: The composition and structure of invertebrates were used to determine the ecological status of 16 selected rivers according to the Water legislation in Bulgaria (Regulation N-4/2012). The "hand net" (EN ISO 10870:2012) was used for collecting the fauna. Ten subsamples from each sampling site were taken according to "multihabitat sampling" (EN 16150:2012). The Biotic index and the index TTN (total taxa number) were used to evaluate the ecological status.

Results: Macrozoobentic invertebrates from the more sensitive "A" and "B" taxa groups (according to tne Biotic index) predominated. The studied rivers showed "good" and "high" ecological status according to the standardized indices.

Conclusion: The studied rivers maintained conditions close to the referent for those river types, ensuring the existence of undisturbed macroinvertebrate communities with high biodiversity.

Keywords: mountain rivers, macroinvertebrates, Biotic index, Total taxa number, ecological status

Introduction

The objects of the present study were the mountain rivers of "Centralen Balkan" National Park. Taking in account data from routine hydrobiologic monitoring performed by the Ministry of Environment and Water, although there were registered single cases of decline, there are no lasting trends for the decrease of the ecological condition. The favorable situation of the river stretches (above towns, villages and industrial plants) as well as their inaccessibility exclude serious anthropogenic influence. This is a precondition for the rivers to show "good" and "high" ecological condition.

Materials and Methods

The study was carried out during October and November 2014. A single sampling site was selected for each river. The sites were chosen to be relevant for a complex assessment of the water bodies as a part of the river ecosystems and to be representative for the catchment area. They were selected to be informative for the different river types. According to the altitude, width of the river, the slope of the river valley, the character of the bottom sediments

and geographic position we established several river types (according to Cheshmedjiev et al. 2010) [1] and Directive 60/2000/EC [2]. In ecoregion 7 (Eastern Balkans), we found mountain river (R3) and semi-mountain river (R5) types. In ecoregion 12 (Pontic Province) we found mountain river (R2) and semi-mountain river (R4) types. Typology of different rivers, as well as geographic coordinates and altitude of the sampling sites are shown in Table 1.

The sampling was carried out with hand held, 30 x 30 cm with mesh size 500 μm in accordance with European standard EN ISO 10870:2012 [3] and Regulation N-4/2012 for characterization of the surface waters [4]. The approach was according to the "multi-habitat sampling" method, standard EN ISO 16150:2012 [5] in its version adapted for the Bulgarian conditions [6] with the gathering of ten subsamples from different microhabitats of the river bed.

Table 1. Date of sampling, river type geographic coordinates and altitude of the studied rivers in "Centralen Balkan" National Park

Sampling site	River type	Date	Latitude (N)	Longitude (E)	Altitude [m]
Zavodna	R2	07-11-14	N42°47.956'	E24°22.678'	821
Stara Ribarica	R2	07-11-14	N42°48.050'	E24°25.157'	759
Zhidov dol	R2	08-11-14	N42°45.840'	E24°44.913'	792
Cherni Osam	R2	08-11-14	N42°45.974'	E24°44.372'	759
Lyava Vidima	R2	09-11-14	N42°45.254'	E24°54.487'	912
Praskalska	R2	09-11-14	N42°45.373'	E24°56.582'	916
Bagreneshtitsa	R2	10-11-14	N42°47.056'	25°03.636'	830
Tazha	R3	18-10-14	N42°44.294'	25°00.806'	1390
Tundzha	R5	21-11-14	N42°39.966'	24°58.553'	917
Malkata	R3	21-11-14	N42°39.079'	24°54.523'	1040
Gabrovnitsa	R3	22-11-14	N42°43,100'	25°10.405'	714
Byala	R5	19-10-14	N42°39.490'	24°57.706'	586
Korudere	R3	23-11-14	N42°45.610'	24°34.652'	872
Vartopa	R3	23-11-14	N42°43.799'	24°21.762'	1306
Damladere	R3	23-11-14	N42°45.173'	24°32.457'	752
Mominska	R2	24-11-14	N42°48.605'	24°13.543'	926

The collecting and following processing and conservation of the samples was carried out accordance with standard ISO 5667-3:1995 [7]. The samples were analyzed microscopically in laboratory "Hidrobiological monitoring" in the Faculty of Biology, Sofia University. In the laboratory the samples were washed, filtered and sorted using stereomicroscope (3-10 fold magnification). The samples were sorted in "the basic hydrobiologic groups" to the lowest possible taxonomic level for the calculation of the Biotic index, the taxonomic determination of the macrozoobentos was made according to Uzunov et al. [8].

The taxonomic composition was used for assessment of the ecological condition in accordance to the manual for monitoring of "macrozoobenthos" [9] and Regulation N-4/2012. For the determination of the ecological condition in the rivers the Biotic index (BI) and the Total taxa number (TTN) were used.

Results

Using BI and TTN indices the studied rivers showed "good" (11 to 15 taxa, BI between 3.5 and 4) and "high" (16 and more taxa, BI between 4.5 and 5) ecological condition (Fig. 1 and Fig. 2).

The fauna typical for unaltered ecosystems predominated in the studied rivers, which is common for the mountain rivers. Macroinvertebrates such as the species from the family Heptageniidae (order Ephemeroptera), which according to the BI are classified as "sensitive forms", were frequent in the samples – *Epeorus* sp.*, Ecdyonurus* sp.*, Rhithrogena* sp. A commonly found, clean water species *Perla marginata* (Panzer, 1799) from order Plecoptera presented in all 16 rivers. A species of stonefly larva *Taeniopterix* sp., was discovered in 6 of the rivers. These insect larvae are typical for stable ecosystems and usually are found in the autumn-winter period. In three sampling sites *Austropotamobius torrentium* (Schrank, 1803) was found – a NATURA 2000 species included in the Annexes of Habitat Directive [10]. Since the group of the dragonflies (order Odonata) prefers macrophyte patches and fine substratum they were very rare in the samples. An exception was Damladere River where in a relatively large zone of fine sand the species *Aeshna cf. cyanea* (Muller, 1764) was found. In Malkata River a caddisfly larva from the family Hydroptilidae (order Trichoptera) was found, which is the only site where this group was established.

The studied rivers have shown high taxonomic diversity (established by the TTN), which is typical for balanced biocenosis.

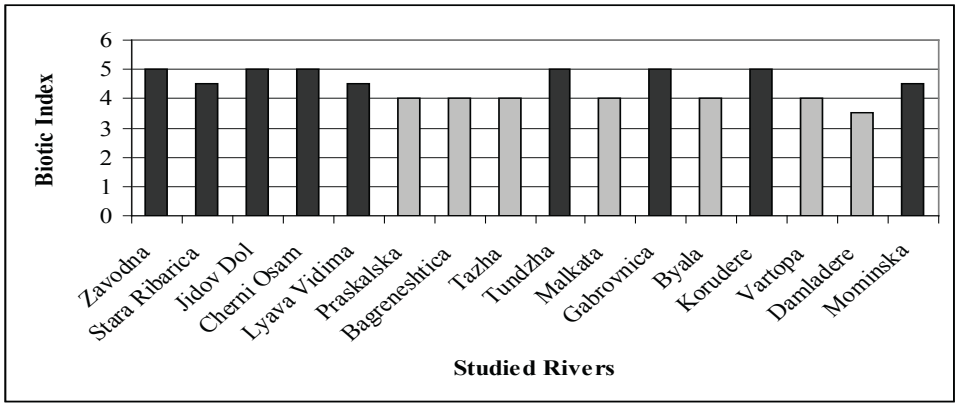

Fig. 1. Values of BI for each of the studied rivers in "Centralen Balkan" National Park. The rivers with high ecological status are shown in black and the rivers with good ecological condition are shown in light gray.

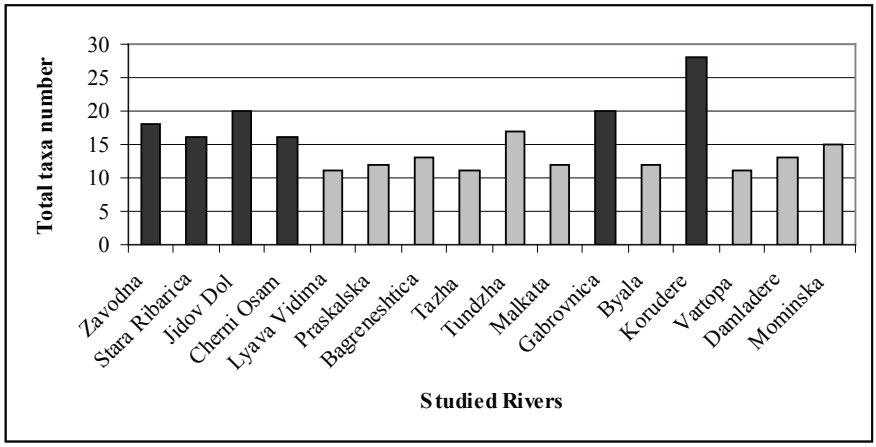

Fig. 2. Values of TTN for each of the studied rivers in "Centralen Balkan" National Park. The rivers with high ecological status are shown in black and the rivers with good ecological condition are shown in light gray.

Conclusion

The results of the evaluation of the ecological status of the rivers of "Centralen Balkan" National Park satisfy the criteria of the Water Framework Directive (Directive 2000/60/EC) and the national legislation (Regulation 4/2012) and no additional measures for improvement of the conditions are needed. Such ecological conditions match the xenosaprobity and oligosaprobity situation. Taking in account the taxonomic diversity (high values of TTN) the water macroinvertebrate communities support stable populations, the species composition is close to the undisturbed for the mountain river ecosystems – predominant are the insect water larve (Ephemeroptera, Plecoptera, Trichoptera, Diptera) as well as organisms with an entirely water life cycle – class Oligochaeta, subphylum Turbellaria, crustaceans (order Amphipoda, order Decapoda) etc. The river conditions were close to the referent for those river types, ensuring the existence of undisturbed macroinvertebrate communities with high biodiversity.

References:

1. Cheshmedjiev S., Karagiozova Tz., Michailov M., Valev V. "Revision of River & Lake Typology in Bulgaria within Ecoregion 12 (Pontic Province) and Ecoregion 7 (Eastern Balkans) According to the Water Framework Directive", Ecologia Balcanica., 2, 2010, 75-96.

2. DIRECTIVE 2000/60/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 23 October 2000 establishing a framework for Community action in the field of water policy, Official Journal of the European Communities 22.12.2000.

3. EN ISO 10870:2012, Water quality - Guidelines for the selection of sampling methods and devices for benthic macroinvertebrates in fresh waters.

4. Regulation № N-4 from 14.09.2012 for characterization of the surface waters, published by the Ministry of Environment and Water, renewed in State Gazette issue 22 on 05.03.2013 in effect on 05.03.2013; changed in issue 79 on 23.09.2014 in effect on 23.09.2014.

5. EN ISO 16150:2012 Water quality. Guidance on pro-rata Multi-Habitat sampling of benthic macro-invertebrates from wadeable rivers.

6. Cheshmedjiev S., Soufi R., Vidinova Y., Tyufekchieva V., Yaneva I., Uzunov Y., Varadinova E. Water Research and Management., 1 (3), 2011, 55-58.

7. ISO 5667-3:1995 Water quality - Sampling - Part 3: Preservation and handling of water samples.

8. Uzunov Y., Yaneva I., Parvanov D., Varidinova E., Kenderov L., Presolska Y., Tyufekchieva V., Panova-Karajova A., Soufi R., Petrova A. Sort definer of bottom invertebrate fauna (marcozoobenthos) from the Bulgarian rivers and lakes (Uzunov Y., Naumova S., Eds), Ministry of Environment and Water, contract № 6/27.03.2009 by operational Program ”Environment” 2010, p. 134.

9. Cheshmedjiev S. & Varadinova E. Biological analysis and ecological assessment for the types of surface water in Bulgaria (Uzunov Y. Eds), University Press “Paisii Hilendarski”, 2013, 147-163.

10. COUNCIL DIRECTIVE 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora http://europa.eu.int/comm/environment/nature/habdir.htm

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DEVELOPMENT OF MACROZOOBENTHOS COMMUNITY IN THE EPHEMERAL ALDOMIROVSKO MARSH (NORTHWEST BULGARIA)

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Abstract

Aim: The aim of the study was to investigate the macrozoobenthos community in the ephemeral Aldomirovsko Marsh with respect to its specific hydrological regime and the environmental factors.

Materials and Methods: The samples were taken at 4 sampling sites in July in 2013 and one of them was sampled again 5 times in 2014 from May to August. The main physical and chemical parameters of the water body were measured along with the nutrients concentrations. Macrozoobenthos samples were collected according the standard multihabitat approach. The statistical analysis of the data was performed in package PAST Version 3.0.

Results: In 2013 no statistically significant correlations were found between the abundance of the macrozoobenthos and the environmental factors, such as: depth, water temperature and transparency. The total abundance of macrozoobenthos significantly correlated with the water conductivity and the concentration of nitrogen forms. On the other hand, in 2014 the most significant factors for the dynamics of the abundance were the changes of the depth and water temperature.

Conclusions: The results suggest that the temporary character of the marsh, i.e. its hydrological regime and life conditions that it forms was of greater relative importance for the abundance of macrozoobenthos community.

The environmental conditions at different sampling sites of one ephemeral marsh were very similar and did not have any significance for the abundance of macrozoobenthos community.

Keywords: abundance, macrozoobenthos, ephemeral marsh

Introduction

Temporary water bodies are usually filled with fresh water and have dry phase with different continuity. Periodically drying out are those which have cyclicity in the periods of drying and those periods are comparatively predictive. Most often this is a result of the seasonal changes in the moderate latitudes and Mediterranean regions.

Aldomirovsko Marsh is situated at the most west part of the Sofia hollow, at the south side of the mountain ridge Tri ushi. Its surface area is almost 130 ha. The real part which collects and retains water is more than 10 times smaller than whole surface area of the marsh. It has karst character which suggests easy seepage and drainage of the water. Despite of that it existed as a permanent water body until 1984 when the access of the only one runnel, which fed the marsh with water, was interrupted. Since then the marsh has too changeable regime and respectively mutable character according to the hydrological regime through the years.

According to Silver et al. [1] temporary wetlands present a more unpredictable and variable habitat for macroinvertebrate and species found there must be able to survive or escape the drying wetland using different strategies as relocating to a permanent wetland, possessing a terrestrial adult form, or having a desiccation-resistant stage that can survive in the substrate until the wetland fills with water again.

Thus, the macrozoobenthos in temporary water objects is too different, but usually abundant. The structure of the macrozoobenthos community in such objects depends on many physical, chemical and biotical factors, but especially on the geography of the water object and the duration of the period during which it retains water (hydroperiod).

The aim of the study was to observe the development of the macrozoobenthos community in relation to various habitats and to the hydrological regime of the body of water. The first part of the study, in the year 2013, was to find if different habitats have substantial importance for the diversity and abundance of the macrozoobenthos community. The second part of the study, in the year 2014, was to observe the temporal development of the

macrozoobenthos community and its species richness in relation to the hydrological regime and changes in the physical and chemical parameters of the marsh. The size of the marsh was expressed through the water volume, depth, alkalinity, conductivity, concentration of the dissolved oxygen and water temperature.

Materials and Methods

The samples were collected during July in the year 2013 from different habitats in 4 different sampling points (13.1, 13.4, 13.5, 13.6) characterized with a different degree of plant cover. The first sampling point (13.1) is located in a zone fully covered with vegetation. The next one (13.4) is located on the border between water and reed and the last two (13.5, 13.6) are situated in the open water (Fig. 1).

During 2013 the hydroperiod was very short, less than 6 weeks and in the beginning of August the marsh became completely dry (Fig. 2).

During 2014 5 samples were collected from one sampling point, located in the open water (14.1, 14.2, 14.3, 14.4, 14.5) (Fig. 1), coincident with sampling point 13.6. The samples were collected from the end of May until the middle of August.

Fig.1. Location of the sampling points

Fig. 2. The dry Aldomirovsko Marsh

The main physical and chemical parameters of the water (dissolved oxygen, oxygen saturation, temperature of the water, alkalinity, conductivity and depth) were measured in situ along with the measurement in the laboratory of the nutrients in the water (NH_4, NO_2, NO_3, Total Nitrogen, PO_4, Total Phosphorus). The analytical methods used were as follows: water temperature (^OC) - BDS 17.1.4.01 (Method Detection Limit-0.1); alkalinity (pH) – BDS ISO 10523: 2012 (MDL-0.00); conductivity ($\mu S.cm^{-1}$) - BDS EN 27888:2000 (MDL-0.0); dissolved oxygen/ oxygen saturation ($mg.l^{-1}$/ %) – BDS/EN/ ISO 5814:2012 (MDL-0.00); ammonia –NH_4 ($mg.l^{-1}$) - ISO 7150/1 (MDL-0.01); nitrite – NO_2 ($mg.l^{-1}$) - EN 26777 (MDL-0.002); nitrate – NO_3($mg.l^{-1}$) - ISO 7890-1 (MDL- 0.2); P- orthophosphate – PO_4/ Total Phosphorus (TotP, $mg.l^{-1}$) - EN ISO 6878 (MDL-0.01). Water samples were collected and preserved according to BDS 16777.

The macrozoobenthos samples were collected according to an adapted version [2] of the multi-habitat sampling methodology of AQEM/STAR [3]. Benthic samples were taken in correspondence to the European standards EN ISO 10870:2012 using a hand-net (frame 30x30 cm, mesh size 500 μm) and were preserved according to BDS EN ISO 5667-3: 2012. Then the macroinvertebrates were separated and conserved in 70% ethanol. The benthic samples were separated by the taxonomic group and then species were determined by qualified professionals (see "Acknowledgements").

Statistical analyses were performed using Statistical software package PAST Version 3.0 [4]. Pearson's linear coefficient was used to estimate the correlations among the environmental variables and macroinvertebrate abundance and species richness.

Results and Discussion

In the samples, collected in 2013 47 taxons of macroinvertebrates were determined and in those samples from 2014 – 39 taxons. The abundance in the 2013 samples were much more higher – average 2226.5 ind./ m², while in the 2014 samples it was 216.4 ind./ m² or almost 10 times lower. In the samples from both years the dominated main taxonomic group was family Chironomidae with 5605 ind./ m² for 2013 from total collected 8906 ind./ m² and 519 ind./ m² for 2014 from total collected 1082 ind./ m².

The statistical analysis of the data from the year 2013 showed that there is no statistical significant ($p<0.05$) correlation between abundance of the benthic community and environmental parameters (depth, alkalinity, temperature of the water, dissolved oxygen, oxygen saturation, total nitrogen, nitrites, total phosphorus and orthophosphates).

Table 1. Pearson`s correlation coefficients among the abundance, species richness and some of the measured environmental parameters

	Abundance 2013	Species diversity 2013	Abundance 2014	Species diversity 2014
NH_4	-0.958*	-0.024	-0.336	0.069
NO_3	0.958*	0.024	-0.303	0.275
Conductivity	0.981*	0.300	-0.493	-0.665
Dissolved O_2	-0.730	0.407	0.885*	0.487
Temperature of water	-0.886	-0.019	0.562	0.904*
Depth	-0.783	-0.062	-0.623	-0.923*
Volume	n.a.	n.a.	-0.658	-0.901*

* values are significant ($p<0.05$)

The analysis of the samples from 2013 showed strong correlations between the abundance of the macrozoobenthos community and the conductivity and nitrogen forms NH_4 and NO_3 (negative with ammonia and positive with nitrates and conductivity) (Table 1, Fig. 3).

On the other hand, the analysis showed that during 2014 a significant factor for the abundance was the dissolved oxygen and for the species diversity – the temperature of the water having strong positive correlations. Also the depth of the marsh and its water volume had strong negative correlations with the species diversity (Table 1, Fig. 4). Fig. 3 and Fig. 4 also showed that the macrozoobenthos in 2013 was influenced by the oxidized nitrogen forms (nitrates and nitrites) and the higher oxygen saturation in the marsh.

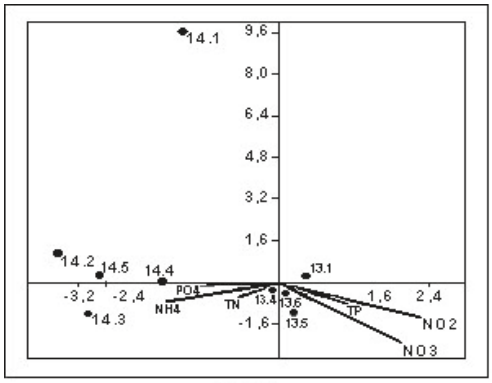

Fig. 3. Correspondence analysis (PCA) of nutrients and samples of two years (p=0.9901)

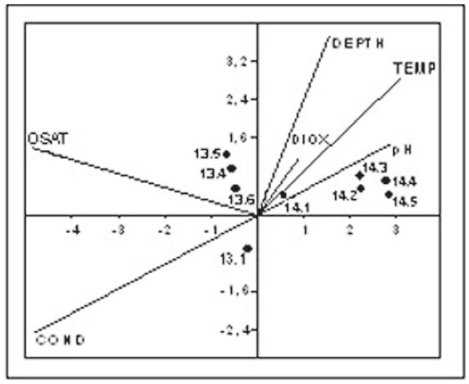

Fig. 4. Correspondence analysis (PCA) of physical and chemical parameters and samples of two years (p=0.0396)

In 2014 the abundance of the macrozoobentos decreased with the increasing of the water volume except in July when a high increasing was observed and the water volume was lower (Fig. 5). The similarity analysis of the abundance (Fig. 6.) reaffirmed this as samples with high water volume in 2014 formed well expressed clusters except 14.4 which was characterized with a low water volume. Samples from 2013 13.5 and 13.6 formed one cluster probably because both sampling points were located where depth was bigger. At sampling point 13.4 the depth was smaller than the previous two and at sampling point 13.1 it was only 15 cm (Fig. 5.)

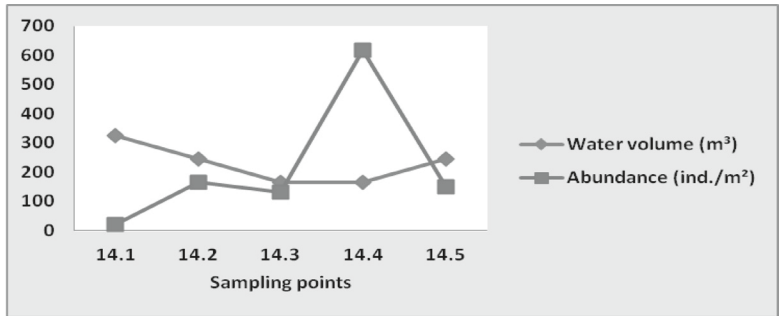

Fig. 5. Comparison of abundance and water volume of the Aldomirovsko marsh

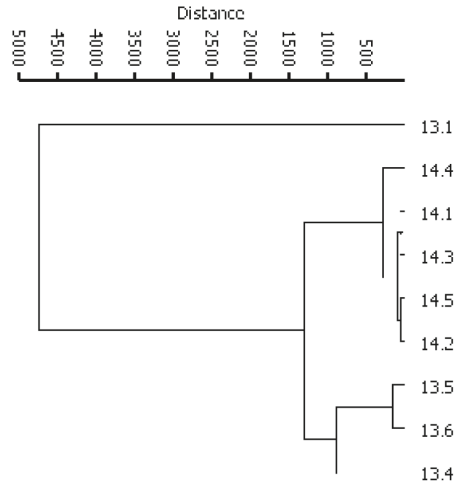

Fig. 6. Cluster analysis of abundance of macrozoobenthos between samples of two years, stress 0.9702

Conclusion

The results lead to the conclusion that the hydroperiod, e.g. duration of water in Aldomirovsko ephemeral marsh a was more significant factor for the development of the macrozoobenthos community than the environmental parameters or the different habitats. Physical, chemical parameters and nutrients also played a role for the development but more or less secondary. The environmental parameters were depended on the water volume, which was a result of the extremely variable water regime. Thus, the hydroperiod, expressed through the water volume was the leading factor in the forming and development of the macrozoobenthos community.

The taxon diversity was relatively small, but the abundance of a limited number of species was high and they rapidly occupied the available habitats. This suggested a change in their life strategies due to the extreme environmental conditions in such water bodies [5].

Acknowledgements: We thank the qualified professionals that helped in determination of species: Yanka Vidinova – Ephemeroptera; Stefan Stoichev – Chironomidae; Desislava Stoianova – Heteroptera; Galia Georgieva – Oligochaeta; Emilia Varadinova – Trichoptera.

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References:

1. Silver C. A., Vamosi S. M., Bayley S. E., Acta Oecologica, 39, 2012, 1-10.

2. Cheshmedjiev S., Soufi R., Vidinova Y., Tyufekchieva V., Yaneva I., Varadinova E., Water Research and Management, 1 (3), 2011, 55-58.

3. Barbour M. T., Gerritsen J., Snyder B. D., Stribling J. B., Rapid Bioassessment Protokols for Use in Streams and Wadeable Rivers: Periphyton, Benthic Macroinvertebrates and Fish. Second Edition. EPA/841-B-99-002. U.S. EPA, Office of Water, Washington, D.C, 1999, p. 197.

4. Hammer Ø., PAST, Paleontological Statistics Version 3.0, Refernce Manual. Natural History Museum, University of Oslo, 2013, p. 221.

5. Wellborn G. A., Skelly D. K., Werner E. E., Annu. Rev. Ecol. Syst., 27, 1996, 337-363.

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TRENDS OF MESOZOOPLANKTON COMMUNITY DYNAMICS IN THE BULGARIAN PART OF THE BLACK SEA

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Abstract

Goal: The aim of the present study was to analyze the long-term quantitative fluctuations and structural modifications of the mesozooplankton community in the Bulgarian part of the Black Sea.

Materials and Methods: A long-term spring data set (1967-2009) of the mesozooplankton abundance and biomass along transect c. Galata was analysed.

Results: The analysis shows some trends of recovery of the mesozooplankton community after the period of eutrophication (1980s and 1990s): occurrence of sensitive species; development of rarely observed copepods *Paracalanus parvus* (Claus, 1863) and decline of the opportunistic one *Acartia clausi* Giesbrecht, 1889; a reduced impact of the Jellyfishes; abrupt increase (regime shift) in spring values of the biodiversity indexes.

Conclusions: The positive trends are still not clear due to the observed large inter-annual deviations of the parameters studied and significant dynamics in the quantitative values of *Noctiluca* population.

Key words: zooplankton, Western Black Sea, eutrophication, fluctuations, regime shift

Introduction

The Black Sea is a semi-enclosed basin whose isolation from the World Ocean makes the environment very sensitive to external impacts. The low productive ecosystem characterized by a natural variability and lack of anthropogenic influence until the mid 1970s (referent period) underwent significant transformations, environmental degradation and instability due to bottom-up (significant river nutrients enrichment) and top-down effects (invasion and outburst of ctenophora species *Mnemiopsis leidyi* (Agassiz, 1865), high biomass of *Aurelia aurita* (Linnaeus, 1758), overfishing of pelagic fishes, trawling for benthic organisms) in 1980s and 1990s [1, 2]. Currently there are some signs of ecosystem recovery which cannot be considered as an improvement or restoration of the ecosystem [3, 4, 5, 6, 7, 8, 9].

The zooplankton community shows significant long-term dynamics in the qualitative and quantitative parameters due to the impact of anthropogenic and climatic factors as well as changes in the internal ecosystem balance [6, 10, 11].

Goal

The aim of the present study was to analyze the long-term quantitative fluctuations and structural modifications of the mesozooplankton community in the Bulgarian part of the Black Sea.

Materials and Methods

The zooplankton data set combined 37 spring samples derived from the database of the Institute of Oceanology - BAS, Varna (Bulgaria), collected in the period 1967 - 2009 on a station located 3 miles off c. Galata (43º.10'N; 28º.00'E) (Fig. 1). The large amount of existing data and the variety of pressures determined the selected area as adequate for analysis. The size group of the mesozooplankton (20 - 200 μm) and in particularly its trophic compound without non-trophic dinoflagellata species *Noctiluca scintillans* (Macartney) Kofoid & Swezy, 1921) was investigated. Standard methods of sampling and analysis were used. Samples were collected by using vertical plankton Juday nets (0.1 m^2 mouth opening area, 150 μm mesh size) towed from 2 meters above the bottom to the surface (20 - 0 m) and fixed in buffered 4% formalin solution. Species abundance per cubic meter was calculated according to Dimov's method [12] and presented by anomalies of the values. Biomass ($mg.m^{-3}$) was estimated by using individual standard weight [13]. The biodiversity was assessed using the indexes of Shannon-Weaver (H'), Pielou (J) and Margalef (d) by abundance (N) and biomass (B).

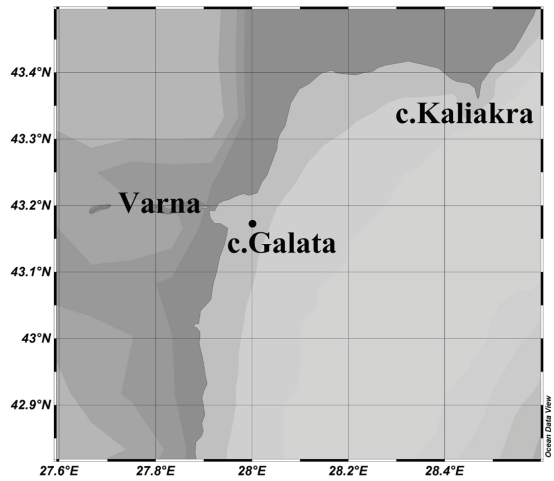

Fig. 1. Map of sampling area

Results

Pristine period

During the period 1967 - 1972 the spring mesozooplankton abundance and biomass, at a station 3 miles off c. Galata, were with values above the average for the studied period and narrow ranges of variation. The community was in its positive phase in terms of the quantitative parameters (Fig. 2). Its structure was dominated by benthic larvae (meroplankton) (Fig. 3) and eurytherm copepoda species such as *A. clausi* and *P. parvus* (Fig.3, Fig. 4). The average density of the heterotrophic dinoflagellate *N. scintillans* was insignificant (Fig. 3). The trophic and climatic conditions in the coastal area induced

development of both eurytherm organisms and thermophilic cladocerans (Fig. 3, Fig. 4).

The values of the biodiversity indexes were high and relatively stable (Fig. 5).

Period of intensive eutrophication

In the late 1970s - early 1980s in front of c. Galata the zooplankton average quantitative values were measured to be 5-fold lower in comparison with the previous period (Fig. 2). While the abundance of the essential mesozooplankton groups such as meroplankton, copepoda and cladocera decreased among 3 to 17-folds, *Noctiluca* population density increased 3-folds (Fig. 3).

Despite the effects of the ongoing eutrophication in the period 1981 - 1990, low spring mesozooplankton standing stock was registered due to the strong predatory pressure of the jellyplankton species *A. aurita* and *M. leidyi* in the late 1980s. In that decade the mesozooplankton community abundance and biomass showed significant variability. The copepods were most abundant with quantitative values similar to the previous periods (Fig. 3). *Noctiluca* retained the increasing trend (3-folds) while the average values of the meroplankton and cladocerans increased insignificantly (Fig. 3). In 1981 was registered the maximum density for the whole studied period due to the prevalence of *A. clausi* with 97.3% of the total abundance (Fig. 4).

In the period of intensive eutrophication some sensitive mesozooplankton species as *Centropages ponticus* Karavaev, 1894, *Anomalocera patersoni* Templeton, 1837, *Pontella mediterranea* (Claus, 1863), *Penilia avirostris* Dana, 1849, *Evadne spinifera* Müller, 1867, *Evadne nordmanni* Lovén, 1836, *Pseudoevadne tergestina* (Claus, 1877) almost disappeared and others just like *P. parvus* were rarely observed. In the studied aquatory after 1997 the cladocera species *Oithona nana* Giesbrecht, 1893 had not been detected in spring samples. On the contrary, the opportunistic copepoda species *A. clausi* showed significant quantitative development.

The changes in the ecosystem structure and functioning resulted in an abrupt decrease (regime shift) in spring values of the biodiversity indexes (Fig. 5).

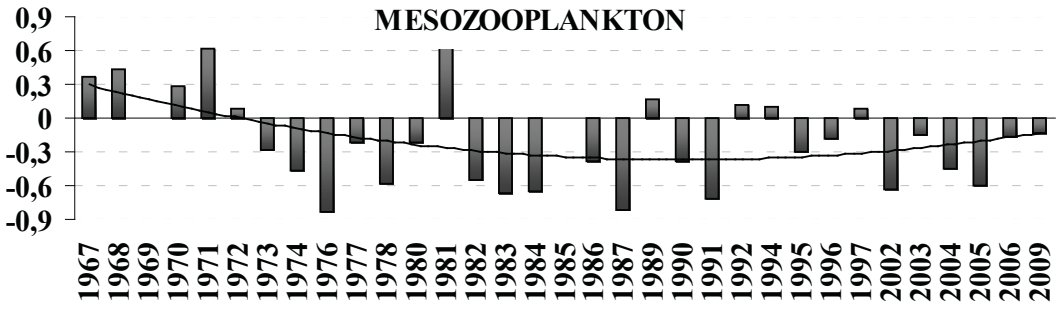

Fig. 2. Anomalies of the total spring mesozooplankton abundance [ind.m^{-3}] at a station 3 miles off c. Galata transect in the period 1967 - 2009 (Used data of Konsulov for the period 1967-1994 and Kamburska: 1995-2005)

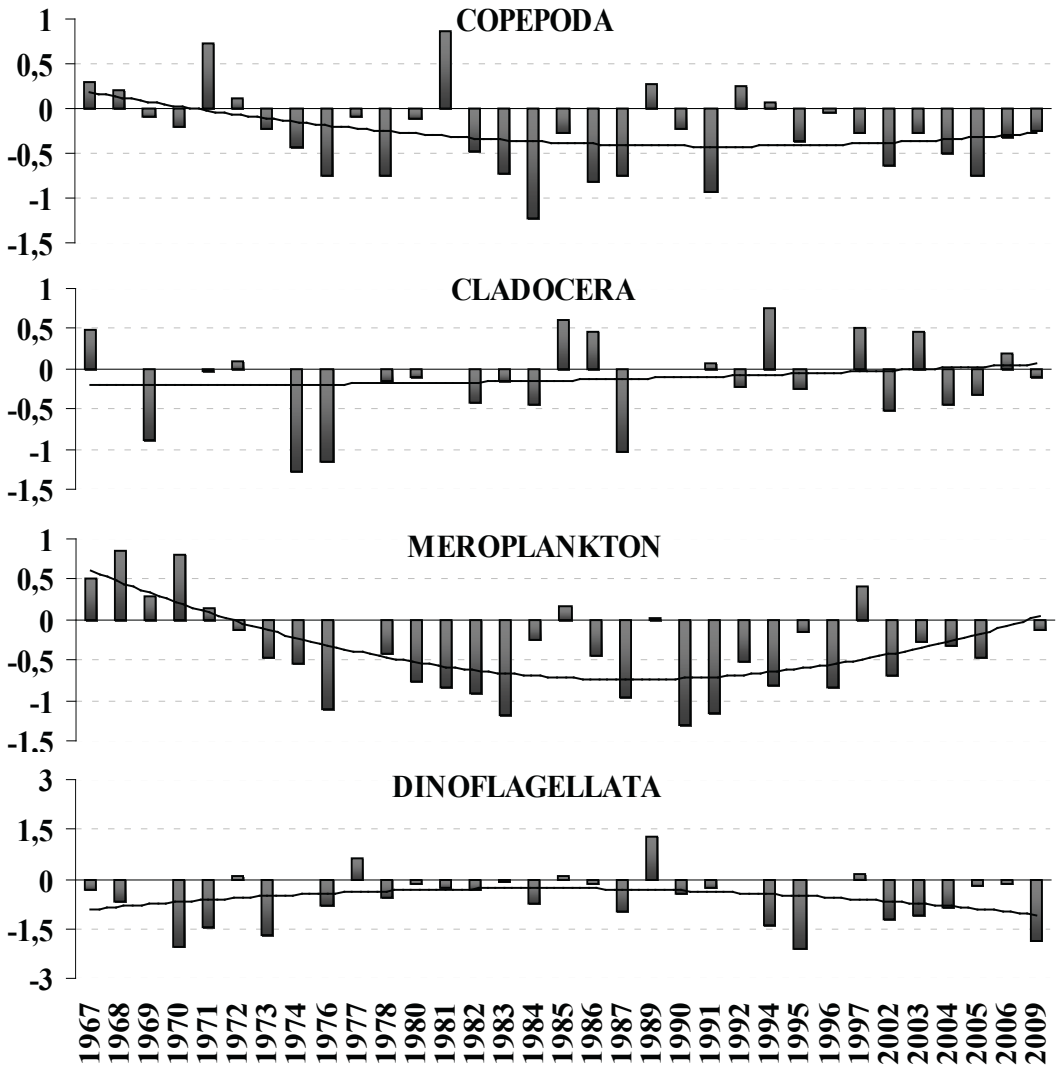

Fig. 3. Anomalies of the abundance [ind.m^{-3}] of the main mesozooplankton groups: Copepoda, Cladocera, Meroplankton and Dinoflagellata at a station 3 miles off c. Galata transect in the period 1967 - 2009 (Used data of Konsulov for the period 1967-1994 and Kamburska: 1995-2005)

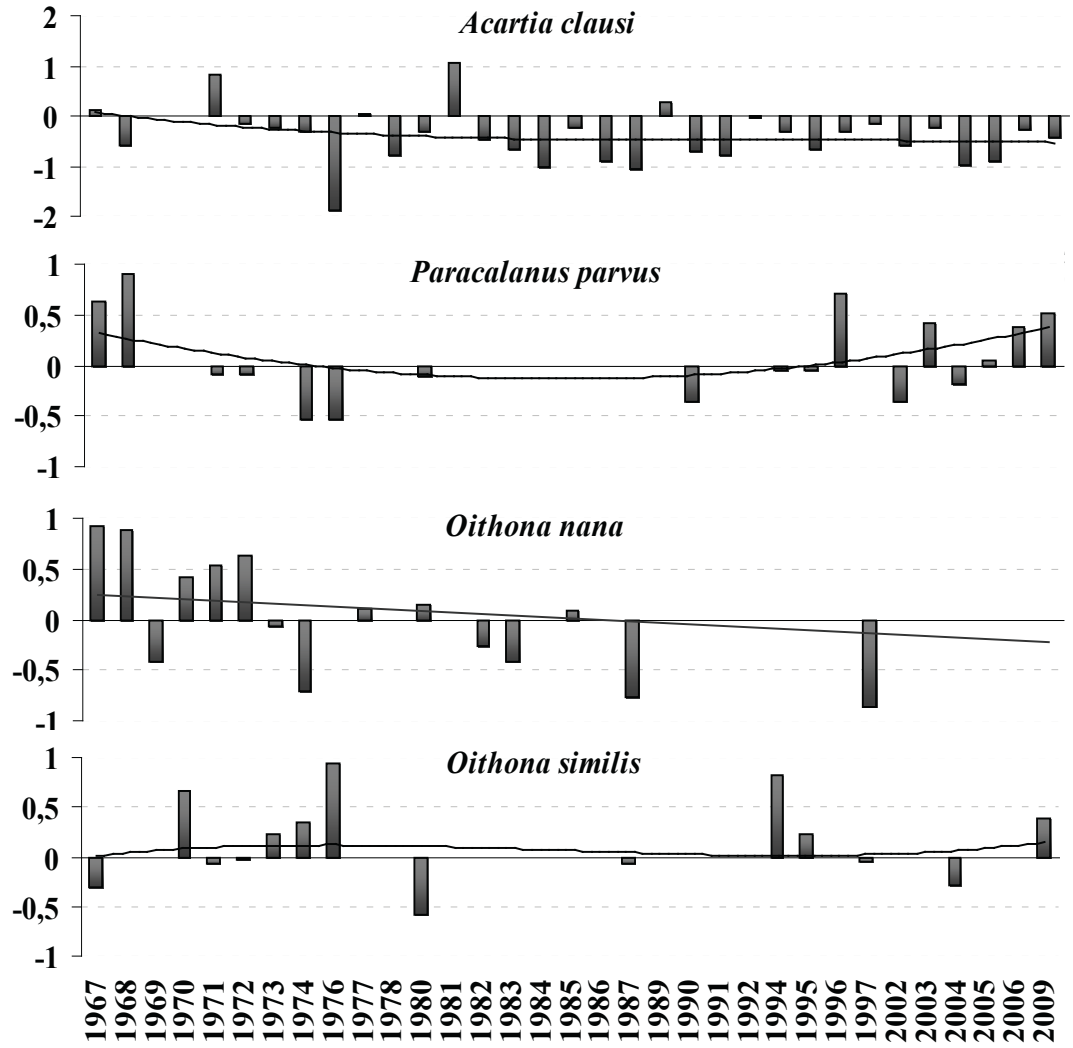

Fig. 4. Anomalies of the abundance [ind.m^{-3}] of the dominant copepods: *Acartia clausi, Paracalanus parvus, Oithona nana* and *Oithona similis* at a station 3 miles off c. Galata transect in the period 1967 - 2009 (Used data of Konsulov for the period 1967-1994 and Kamburska: 1995-2005)

Post- eutrophication period

During this period (1990s till now) in front of c. Galata the mesozooplankton quantitative values showed an increasing trend but they were lower in comparison with the average ones for the entire investigated period (Fig. 2). The abundance of the meroplankton and the cladocera complexes increased 2-folds while *Noctiluca* population density decreased 7-folds (Fig. 3). The average quantitative values of the copepoda complex were comparable

with the previous but with some specifics in the ratio of the essential species. The average abundance of the opportunist *A. clausi* decreased 3-folds while for the sensitive species *P. parvus* an increase of 12-folds was registered (Fig. 4).

An abrupt increase (regime shift) was observed in the spring values of biodiversity indexes of Shannon-Weaver, Pielou and Margalef characterized with significant inter-annual deviations (Fig. 5).

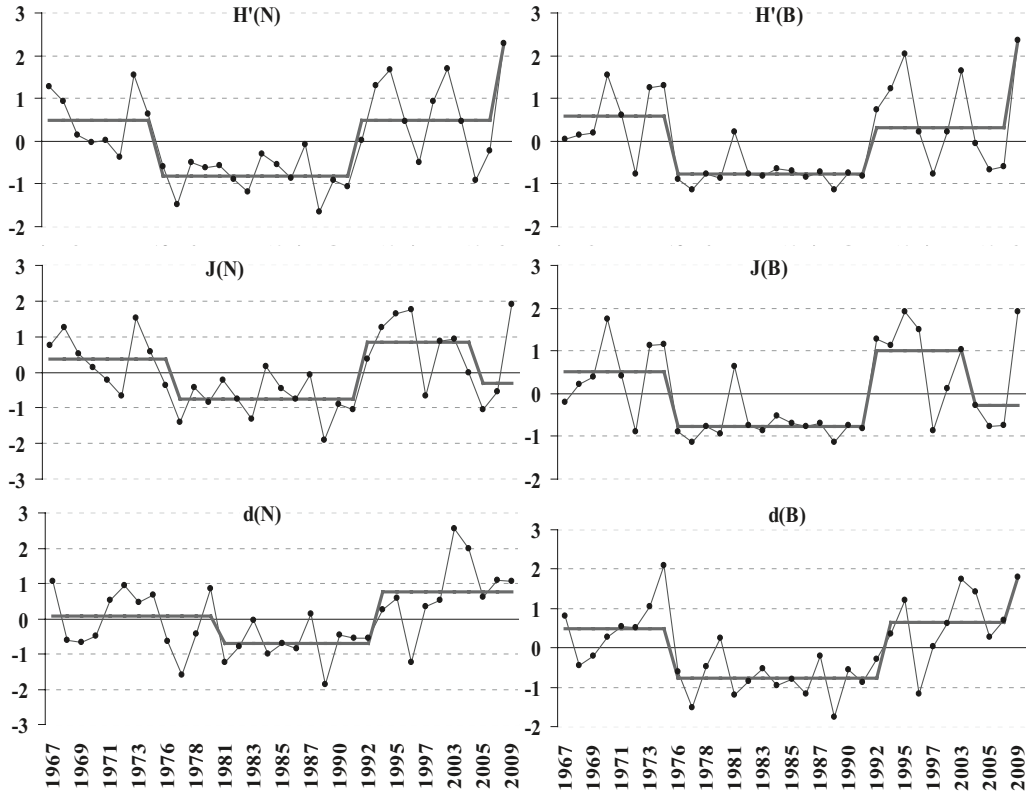

Fig. 5. Dynamics of the spring values of the indexes of Shannon-Weaver (H'), Pielou (J) and Margalef (d) by abundance (N) and biomass (B) at a station 3 miles off c. Galata transect in the period 1967 - 2009 (Used data of Konsulov for the period 1967-1994 and Kamburska: 1995-2005)

Discussion

The data set analysis clearly distinguishes the main periods of the Black Sea ecosystem evolution.

The high average quantitative values with narrow ranges of variation and relatively even distribution of mesozooplankton species in the community are a result of the relatively stable ecosystem conditions. The trophic capacity allows complete nutrient utilization

and regeneration [6, 10] and the zooplankton parameters depend on the optimal climatic factors [14].

The ecosystem functioning in the period of intensive eutrophication was strongly affected by a complex of factors which led to structural and functional modification, ecosystem instability and degradation. The alteration of the spring mesozooplankton parameters along c. Galata shows a strong decline in numerical values and especially in its sensitive part - meroplankton, cladocerans and a lot of copepoda species. Another community respond was mass development of the opportunistic species *N. scintillans* and *A. clausi*. The lost of species richness and evenness resulted in an abrupt decrease (regime shift) in spring values of the biodiversity indexes.

The analysis shows some trends of recovery of the mesozooplankton community during the recent period: an increase of the quantitative values, occurrence of some sensitive species; increase of *P. parvus* (constant in the recent period) and a decline of *A. clausi* (Fig. 4); a reduced impact of *N. scintillans, A. aurita* and *M. leidyi*; an abrupt increase (regime shift) in the spring values of biodiversity indexes.

Conclusions

The positive trends of the mesozooplankton community recovery are still not clear due to the observed large inter-annual deviations of the parameters studied and the significant dynamics in the quantitative values of the *Noctiluca* population.

References:

1. Mee L. D. Ambio 21: 1992, 278–286.

2. Yunev O. A, Vedernikov V. I., Basturk O., Yilmaz A., Kideys A. E., Moncheva S., Konovalov S. K. Mar. Ecol. Prog. Series, 230, 2002, 11-28.

3. Velikova V., Moncheva S., Petrova D. Water Science and Technology, 39 (8), 1999, 27-36.

4. Stefanova K., Todorova V., Kamburska L., Dencheva K. In: Bulgarian biodiversity platform. Contemporary status of biodiversity in Bulgaria – problems and perspectives, Sofia, 2005, 447-468 (In Bulgarian).

5. Oguz T. Black Sea Oceanography, 18 (2), 2005a, 122-133.

6. Oguz T. Black Sea Oceanography, 18 (2), 2005b, 112-121.

7. Shtereva G., Krastev A., Hristova O., Dzhurova B. In: Proceedings of IO, 5, 2005, 103 - 111.

8. Moncheva S., Shtereva G., Stefanova K., Slabakova N., Krastev A., Hristova O., Djurova B., Slabakova V., Mavrodieva R. In: Proceedings of tenth international conference of marine sciences and technologies “Black Sea’2010”, 2010, 288 -296.

9. Oguz T., Velikova V. Marine Ecology Progress Series, 405, 2010, 231–242.

10. Prodanov K., Moncheva S., Konsulov A., Kamburska L., Konsulova Ts., Dencheva K. Recent ecosystem trends along the Bulgarian Black Sea coast. Studies of Institute of Oceanology. Bulgarian Academy of Sciences, Varna, 3, 2001, 110-127.

11. Shiganova T., Musaeva E., Araskievich E., Kamburska L., Stefanova K., Michneva V., Polishchuk L., Timofte F., Ustun F., Oguz T., Khalvashi M. In: BSC, 2008. State of the Environment of the Black Sea (2001 - 2006/7), (T. Oguz, Ed.). Publications of the Commission on the Protection of the Black Sea Against Pollution (BSC) 2008 - 3, Istanbul, Turkey, 2008, p. 448.

12. Dimov I. Improved Quantitative Zooplankton Sampling Method. Reports Bulgarian Academy of Sciences, 12, 5, 1959, 427 429.

13. Petipa T. S. On average weight of main forms of zooplankton. The studies of Sevastopol Biological Station, 5, 1959, p. 13.

14. Konsulov A. In: Proceedings of RIOF, XII:64, 1974, (In Bulgarian).

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ASSESSMENT OF TURBOT (*SCOPHTHALMUS MAXIMUS* L.) STOCK STATUS ALONG THE BULGARIAN BLACK SEA COAST ACCORDING TO THE INDICATORS OF EU MARINE STRATEGY FRAMEWORK DIRECTIVE

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Abstract

Aim: The current study is aimed to perform an assessment of the turbot stock by a set of indicators under the three criteria of D3 using data from the regional analytical stock assessments and the research surveys along the Bulgarian Black Sea coast.

Materials and Methods: Six indicators were calculated and applied to assess the turbot stock status in the Bulgarian Black Sea area based on the data collected within 12 research surveys during 2006–2012.

Results: The indicators demonstrate a high level of fishing pressure - fishing mortality and catch/biomass ratios are above the reference levels. Reproductive capacity is likely to be at very low levels and the stock spawning biomass and biomass indices are below the precautionary limits.

Conclusions: The Indicator-based assessment according to the criteria under Descriptor 3 demonstrate that the turbot stock along the Bulgarian coast has not achieved good environmental status due to the high fishing pressure, reduced reproductive capacity and negative alterations in the size structure.

Keywords: turbot, Bulgarian Black Sea area, Marine Strategy Framework Directive, Descriptor 3, indicators

Introduction

The Common Fishery Policy (CFP) is the main official instrument for managing of the fish stocks in the Community waters, ensuring consistency between the Member States in the process of sustainable use of marine biological resources. Achieving a healthy marine environment depends on the range of desirable aspects of the ecosystem, identified in the Marine Strategy Framework Directive (MSFD) and formulated in eleven descriptors of good environmental status (GES), which have to be achieved by 2020 [1]. Descriptor 3 (D3) addresses the status of the commercial fish stocks, which should be exploited sustainably consistent with the high long-term yields, to have full reproductive capacity ensuring the necessary recruitment and the age and size structure, consisting of older fish. The GES is monitored through a list of indicators and reference levels that are suggested at the national level. The indicators of D3 are grouped within three criteria - level of pressure of the fishing activity, the reproductive capacity of the stock and the population age and size distribution [2].

The commercially exploited fish stocks at the Black Sea regional level are assessed annually by the Scientific, Technical and Economic Committee for Fisheries (STECF) through the Black Sea working group (EWG Black Sea assessments) since 2008 [3, 4, 5]. The scientific stock assessments usually require extensive knowledge about the age and size structure, growth, maturity and other life-history parameters of the stocks over the whole distributional range of the species. These types of data are collected under the National Data Collection program of Bulgaria, where the turbot (*Scophthalmus maximus*) is one of the monitored species.

Turbot is a long lived demersal fish distributed over the Black Sea shelf area up to 120 m depth [6]. Species become a preferred target for the Bulgarian commercial fisheries due to the high market demand and prices. The studies on turbot distribution, stock dynamics and population structure along the Bulgarian Black Sea coast were initiated during the 1950s [7, 8] and the recent studies cover the period 2006-2012 [9, 10, 11].

The current study is aimed to perform an assessment of the turbot stock in the Bulgarian Black Sea area by calculation of the indicator metrics according to the Descriptor 3 of the EU Marine Strategy Framework Directive by aggregating data available from the analytical stock assessments [3, 4, 5] and the scientific research surveys along the Bulgarian coast during the period 2006–2012 [9, 10, 11].

Materials and Methods

Turbot is included in the National Data Collection Programme of Bulgaria for collection and management of fisheries data since 2008 and was selected according to the requirements of MSFD to ensure consistency between the Data Collection Framework (DCF) and the MSFD.

Time series of six indicators were calculated for the turbot stock using the data collected during 12 research surveys in the Bulgarian Black Sea shelf area (Fig. 1). For the present study, data from 2006 to 2012 were used to calculate the annual indicator metrics. During the studied period, the number of survey hauls per year ranged from 47 to 80. Spawning

stock biomasses (SSB) and fishing mortality (F) estimates were extracted from the summary tables in the single-species regional stock assessment report of STECF [5].

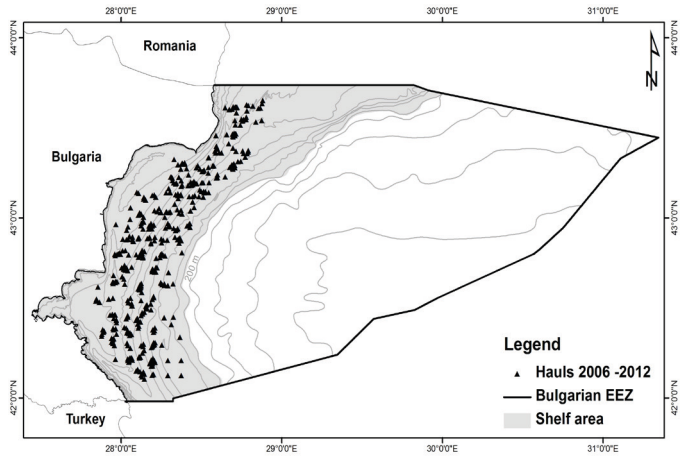

Fig. 1. Investigated area and distribution of survey hauls in the Bulgarian Black Sea during the period 2006–2012.

The following indicators were chosen and estimated to cover the three criteria of Descriptor 3:

1. *Criterion 3.1: Level of pressure of the fishing activity.*

A fishing mortality (F) is selected as the primary indicator. The annual estimates of the F and the reference level - F at a maximum sustainable yield (F_{MSY}) with value of 0.26 were extracted from the analytical stock assessment based on the historical turbot catches during 1950–2011 and refer to the whole Black Sea area [5]. The analytical assessments for the Bulgarian Black Sea area are not available and the ratio between the catch and biomass index was applied as a secondary indicator. The annual values of the indicator were calculated as a proportion between the biomass of commercial landings and the average biomass estimated from the annual research surveys along the Bulgarian Black Sea coast. The average of the ratios within the period 2007–2009 was applied as reference level with the value of 0.033 at national level, because the turbot stock abundance and biomass during the pointed period were the highest observed [9]. The GES under *Criterion 3.1* will be achieved when the estimated values of the indicators are below the reference level.

2. *Criterion 3.2: Reproductive capacity of the stock.*

The Spawning Stock Biomass (SSB) is selected as the primary indicator and the annual estimates of SSB and the precautionary reference level (SSB_{pa}) with a value of 4080 t were extracted from the analytical stock assessments, referring to the whole Black Sea area [5]. The lack of a separate analytical assessments for the Bulgarian Black Sea area requires the application of the biomass indices as a secondary indicator. The annual values of secondary indicator were estimated from the research surveys along the Bulgarian coast during the period 2006 – 2012. The reference value was set at 1700 t [12] and calculated as the average

over the period 2007 – 2009, when the estimates of the relative biomass of turbot stock in front of the Bulgarian coast were the highest [9]. The GES under *Criterion 3.2* will be achieved, if the estimated values of each of the indicators are above the reference level.

3. *Criterion 3.3: Population age and size distribution.*

The proportion of the fish larger than the mean size of the first sexual maturation and the 95% percentile of the fish length distribution observed in the research vessel surveys were applied as primary and secondary indicators. The annual values of the indicators were calculated using the data derived from the research surveys [9]. The reference values at the national level were set at 74% for the proportion of the larger fish and 62 cm for the 95% percentile of the observed length distribution [12], estimated as an average of the annual values over the period 2006–2011 due to the lack of historical data. The GES under *Criterion 3.3* will be achieved, if the estimated values of each of the indicators are above the reference level.

The final assessment of the state of the turbot stock will be done according to the "one-out-all-out" principle (OOAO).

Results

The Turbot stock in the Bulgarian Black Sea area was assessed by six indicators under the three criteria of the Descriptor 3. The level of the pressure of fishing activity (*Criterion 3.1*) was assessed as high and above the reference values. The primary indicator - fishing mortality - at regional level is more than three times higher than the F_{MSY} level (Fig. 2) and the stock of turbot in the Black Sea has been exploited unsustainably and at a risk of collapse. In the Bulgarian area till 2009, the catch/biomass ratio was bellow or around the reference level and then continuously started to increase after 2009 (Fig. 2). During the last three years, the estimated values are significantly over the reference level of 0.033, suggesting the overexploitation and ineffective management of the turbot stock. According to the results obtained from the two indicators, the GES regarding the turbot stock is not achieved and the pressure of fishing activity is high both at the regional and at the national levels.

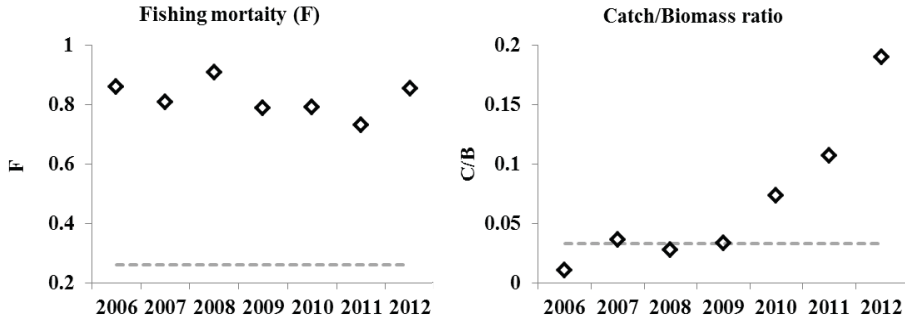

Fig. 2. Assessment of turbot stock according to the indicators under the *Criterion 3.1* (Level of pressure of the fishing activity) during the period 2006-2012. The fishing mortality and the Catch/biomass ratios by years were shown against the reference levels (grey dotted line).

The reproductive capacity of the turbot stock (*Criterion 3.2*) was assessed by two indicators – the SSB and the biomass indices, obtained from the research surveys. The overall spawning biomass of turbot in the Black Sea is likely to be at very low levels and consistently below the SSB_{pa} during the period 2006–2012 (Fig. 3). The SSB at the regional level diminished steadily during the years and reached its low in 2012. However, it is necessary to adopt precautionary measures for managing of the stock at the Black Sea level. At national level, the biomass indices were above the reference value until 2008, when started to decrease below the safe limits (Fig. 3). Hence, the GES is not achieved in terms of the two indicators.

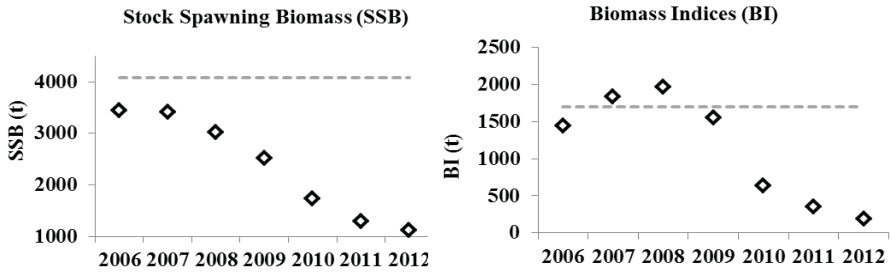

Fig. 3. Assessment of turbot stock according to the indicators under the *Criterion 3.2* (Reproductive capacity of the stock) during the period 2006-2012. The stock spawning biomass (SSB) and the biomass indices by years were shown against the reference levels (grey dotted line).

Two indicators under *Criterion 3.3* (Population age and size distribution) were estimated, referring to the Bulgarian Black Sea area: the proportion of fish larger than the mean size of the first sexual maturation and 95% percentile of the fish length distribution observed in the research vessel surveys – Fig. 4. The results demonstrated, that the negative trend was observed in the abundance of the larger fish during the last two years, although the 95% percentile is still above the reference level (Fig. 4), but the GES is not achieved.

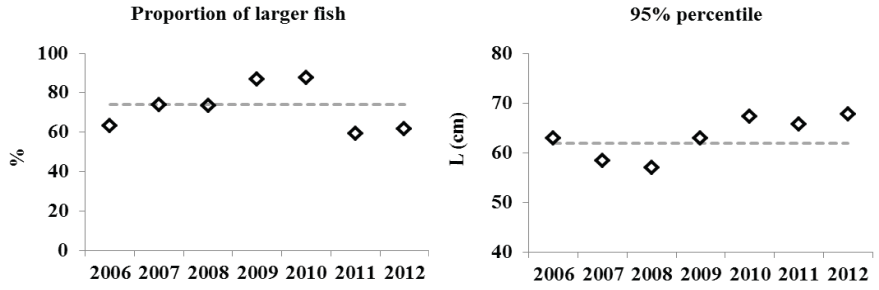

Fig. 4. Assessment of turbot the stock according to the indicators under the *Criterion 3.3* (Population age and size distribution) during the period 2006-2012. The proportion of the larger fish and the 95% percentile of the population length distribution by years were shown against the reference levels (grey dotted line).

Discussion

The fish stocks in the Black Sea have been exploited for human consumption and trade from centuries. As a high valued species, turbot is harvested by all the six riparian countries and the majority of the landings until 1992 were taken by the former USSR and Turkey, and afterward – by Turkey and Ukraine [5]. Hence, the share of Bulgaria and Romania in the total turbot landings in the Black Sea is negligible since 2000 (about 8%). Moreover, the existence of the illegal, unreported and unregulated (IUU) fishing on turbot as a common practice since 1990s [13] is considered as a major issue leading to the unknown harvest rates and the failed species management. The more recent expert assessments of IUU fishing on turbot showed that the unreported quantities exceeded approximately five times the officially reported catches [5]. Along with the turbot oriented fisheries, the species is also by- caught during the fishing by pelagic and beam trawls, long lines and purse seiners with unknown rates. In general, the fishing pressure on the turbot population in the Black Sea has been altered over the decades depending on the development of fishing gears and the technologies and also on the market demands. The main fishing gears used in the Black Sea region are the bottom set gillnets and the bottom trawls. The negative impact of the bottom trawl gears on the benthic communities causes their prohibition in the legislation of all coastal countries since the 80s except in Turkey [14] and recently the gillnets are recognized as the main legal fishing gear [5].

The Bulgarian turbot landings ranged between 0.9-460.2 t over the period 1925-2012 [5] – Fig. 5. The catches started to increase during the 1950s when the fishing by bottom trawls was introduced and maintained high quantities till the 1980s. After 1980, a strong decreasing trend of turbot catches was observed and the mean multi-annual yield reduced from 244.88 t for the period 1950–1980 to the 12.99 t for the period 1980–1981. In 1984, the bottom trawling was forbidden in the Bulgarian Black Sea area and till now the turbot fishery has been accomplished by gillnets only. Due to low the turbot stock size, a four years fishery ban was introduced during the period 1990–1994, which allowed the recovery of the turbot population to some extent. Over the period 1995–2012, the average turbot landings reached the average value of 51.47 t (Fig. 5).

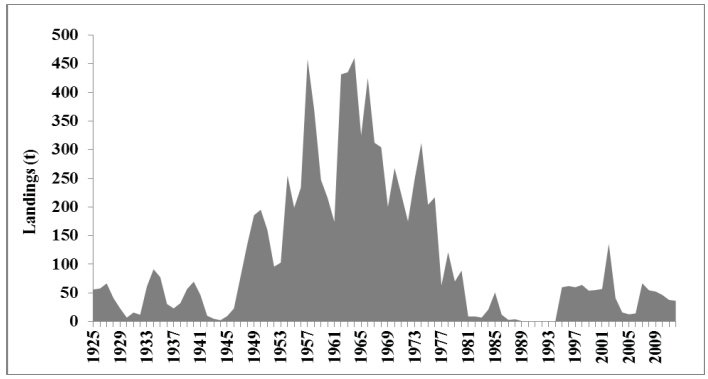

Fig. 5. Turbot landings (in tonnes) of Bulgaria during the period 1925-2012.

From a biological point of view, turbot is a good indicator species for the state of the Black Sea environment. As a key predator and component of the both pelagic and benthic food webs, turbot is vulnerable to the various anthropogenic and environmental factors. The reproduction and the survival rates of the species strongly depend on the status of the environment because throughout its life-span, the turbot occurs in both pelagic and benthic habitats along the shelf, where the anthropogenic impacts such as fisheries and pollution are intense. The survival of the recruitment (1 and 2-years old fish) is related to the state of the coastal benthic communities and the quality of the nursery and feeding grounds. The physical disturbance of the seabed due to the coastal development and the usage of destructive fishing gears lead to the reduction or loss of the nursery and the spawning grounds, which combined with the selective fisheries on the spawning population, negatively affects the sex ratio and the egg production and significantly decrease the recruitment and the spawning stock size.

The recent regional stock assessments showed that the turbot population in the Black Sea has been over-exploited during the last decades and recently is at the verge of collapse [4, 5]. Accordingly, this study found that the fishing mortality at the regional level is above the level of F_{MSY} and the good environmental status failed to be achieved (Fig. 2). The catch/biomass ratio indicator demonstrated that after 2009, the exploitation level of the turbot population along the Bulgarian coast is greatly above the reverence level (Fig. 2) and the estimated biomass indices from the research surveys continuously decreased after 2008 – Fig. 3. The survey data indicated a decrease in the abundance of the larger fish at the national level (Fig. 4) and undesirable effects on the age and size structure of the population [15]. The negative impact of the fisheries was observed over the reproductive capacity (SSB) of the turbot population in the Black Sea (Fig. 3) and currently the SSB is three times lower than the precautionary level (SSB_{pa}). The Achievement of the GES regarding the turbot population at the national and at the regional level requires reduction in a range of anthropogenic pressures, but the most important among them is the fisheries.

The recovery of the turbot population in the Black Sea is difficult to be predicted and managed. The governance of the turbot fisheries in the Black Sea is challenging due to the intermingling of the resource conservation goals with the economic and the social issues. The existing legal frameworks addressed the aspects needed for the effective management of the resource, but the geopolitical complexity of the region impedes their implementation by all the coastal states. With the accession of Bulgaria and Romania in the European Union (EU) in 2007, the Common Fishery Policy (CFP) has been extended into the Black Sea. Turbot fisheries in the EU waters (Bulgaria and Romania) have been managed through the annual establishment of fishing opportunities, set of technical measures regarding the fishing gears and the temporal fisheries bans. During the last four years, the turbot quota in the EU Black Sea waters has been fixed at 86.4 t and allocated as 50% to Bulgaria and 50% to Romania – Table 1.

Table 1. Annual turbot quotas of Bulgaria during 2008-2014.

Year	Quota (Bulgaria)
2008	45 t
2009	50 t
2010	48 t
2011	43.2 t
2012	43.2 t
2013	43.2 t
2014	43.2 t

The current situation with the management of turbot stock in the Black Sea is characterized by the restrictions for the turbot fishery, based on the scientific advice, but obligatory only for the European countries (Bulgaria and Romania). The rest of the countries manage the turbot stock according to their internal national restriction and rules. Therefore, no substantial improvement and stock recovery could be expected while all these measures have not been implemented on the basin scale. The assessment of the turbot stock according to the indicators under the D3 of the MSFD along the Bulgarian coast in 2006–2012 demonstrate that the species population is far away from achieving of good environmental status under all three criteria of the D3 and the negative alterations been observed in the stock spawning biomass and the size structure due to the high impact of the fishing pressure. For the recovery of turbot population, additional reduction of the fishing pressure and more conservation activities are necessary to be implemented at the national and the regional level.

References:

1. EU-COM. 2008. Directive 2008/56/EC establishing a framework for community action in the field of marine environmental policy (Marine Strategy Framework Directive).

2. EU-COM. 2010. Commission decision of 1 September 2010 on criteria and methodological standards on good environmental status of marine waters. (2010/477/EU).

3. Pilling G., Abella A., Di Natale A., Martin P., Guillen J., Cardinale M., Accadia P., Anastopoulou I., Colloca F., Daskalov G., Dimech M., Fiorentino F., Karlou-Riga C., Katsanevakis S., Lleonart J., Maximov V., Murenu M., Panayotova M., Petrakis G., Quetglas A., Radu G., Raykov V., Santojanni A., Sartor P., Shlyakhov V., Spedicato M. T., Tsitsika E., Vasiliades L., Zengin M., Cheilari A., Rätz H. J. JRC Scientific and Technical Reports, 2008, p. 525.

4. Daskalov G., Cardinale M., Charef A., Duzgunes E., Genç Y., Gümüş A., Maximov V., Mikhaylyuk A., Nikolaev S., Osio G. C., Panayotova M., Radu G., Raykov V., Shlyakhov V., Yankova M. and Zengin M., STECF members: Casey J., Abella J. A., Andersen J.,

Bailey N., Bertignac M., Cardinale M., Curtis H., Daskalov G., Delaney A., Döring R., Garcia Rodriguez M., Gascuel D., Graham N., Gustavsson T., Jennings S., Kenny A., Kirkegaard E., Kraak S., Kuikka S., Malvarosa L., Martin P., Motova, A., Murua H., Nowakowski P., Prellezo R., Sala A., Somarakis S., Stransky C., Theret F., Ulrich C., Vanhee W. and Van Oostenbrugge H. JRC Scientific and Policy Reports, 2012, p. 279.

5. Scientific, Technical and Economic Committee for Fisheries (STECF) – 2013. STECF 13-20, 2013, JRC Scientific and Policy Reports, p. 430.

6. Stoyanov S., Georgiev Z., Ivanov L., Hristov D., Kolarov P., Alexandrova K., Karapetkova M.. Fishes in the Black Sea (Bulgarian coast). State press, Varna, 1963, p. 246.

7. Martino K., Karapetkova M.. Sci. An. Res. Inst. Fish. and Fish Indust., 1, 1957, 45-51.

8. Ivanov L., Beverton R.J.H. GFCM Studies and Reviews, 60, 1985, p.135.

9. Panayotova M., Todorova V. Acta Zool. Bulg., Suppl. 2, 2008, 317-324.

10. Panayotova M., Raykov V., Maximov V., Radu Gh., Anton E. Compt. Rend. Acad. Bulg. Sci., 65 (1), 2012, 63-66.

11. Panayotova M., Raykov V., Todorova V. Acta Zool. Bulg., 64 (1), 2012, 85-91.

12. Panayotova M. In: National report – Formulation of good marine environmental status, vol. II, MOEW, 85 – 100.

13. GFCM. GFCM Secretariat, 2013, p. 58.

14. Zengin M. In: Turkish Fisheries in the Black Sea, 40, Turkish Marine Research Foundation, Istanbul, p. 450.

15. Panayotova M., Raykov V. In: National report – Initial assessment of marine environmental status according to Art. 8 of MSFD, vol. I, MOEW, 392 – 410.

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Тематично направление:
БИОЛОГИЧНО РАЗНООБРАЗИЕ И КОНСЕРВАЦИОННА БИОЛОГИЯ

Topic:
BIOLOGICAL DIVERSITY AND CONSERVATION BIOLOGY

CERCOSPOROID FUNGI (*ASCOMYCOTA, MYCOSPHAERELLACEAE*) ON AGRICULTURAL CROPS IN BULGARIA

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Summary: The systematic position of the cercosporoid fungi included in the following text and their current names are presented after [1] and MycoBank. All taxa (from the genera *Cercospora, Passalora, Pseudocercospora, Ramularia*) of cercosporoid fungi, included in the present work, are listed alphabetically, following the most recently adopted system of the Phylum *Ascomycota*.

Aim: The aim of the article is to make an attempt to present the systematic position of a part of the cercosporoid fungal pathogens on some agricultural plants using their current names and also to stress at their modern systematic position in the Kingdom Fungi. They cause diseases, known in Bulgaria as 'cercosporoza' and 'ramularioza', usually appearing as spots on leaves or stems. These spots may have various forms, sizes, coloration and localization in the plant organs.

Materials and Methods: The systematic position of the cercosporoid fungi included in the following work and their current names are presented after [1] and according to the requirements of the latest ICN [2], the databases consisting of fungal names of various taxa such as MycoBank (http://www.mycobank.org/Basic search on names). The present list of the diseases and their causal agents are arranged alphabetically, according to the Latin names of the fungal pathogens.

Results and Discussion: The most common and harmful diseases of plants are known to be caused by fungi. The control of those plant pathogens is of priority for the phytopathologists. The taxonomists are used to study the fungi from specific point of view, emphasizing at their systematic arrangement in the Kingdom Fungi. They aimed at the exact and correct naming, conforming the rules of the International Code for Nomenclature for algae, fungi and plants – ICN [2]. In the phytopathological literature, the fungi presented herein are treated as belonging to the division *Deuteromycota*, class *Hyphomycetes*, families *Moniliaceae* and *Dematiaceae*. All of the 113 taxa, included in volume 7 of the monographic work 'Fungi of Bulgaria' [3] are studied as Anamorphic fungi – a formal taxonomic group. After 2011 and 2012 years the known cercosporoid fungi are included in the system of the division *Ascomycota*.

Systematic position of a part of the cercosporoid fungi on agricultural crops in Bulgaria:
Phylum **Ascomycota**
Subphylum **Pezizomycotina**
Class **Dothideomycetes**
Subclass **Dothideomycetidae**
Order **Capnodiales**
Family ***Mycoshaerellaceae***
Cercospora Fresen.
Cercospora apii Fresen.
Causal agent of yellow leaf spots on celery (*Apium graveolens* L.)
Cercospora beticola Sacc.
Causal agent of 'cercosporoza' on sugar-beet (*Beta vulgaris* L.)
Cercospora carotae (Pass.) Kazn. & Siemaszko
Causal agent of leaf blight on carrot (*Daucus carota* L.)
Cercospora physalidis Ellis (sub. ***Cercospora capsici*** Heald & F.A. Wolf).
Causal agent of 'cercosporoza' on pepper (*Capsicum annuum* L.)
Cercospora physalidis Ellis (sub. Cercospora nicotianae Ellis & Everh.)
Causal agent of 'leaf spots (frog eyes) on tobacco (*Nicotiana tabacum* L.)
Cercospora zonata G. Winter (sub ***Cercospora fabae*** Fautrey).
Causal agent of 'cercosporoza' on broad beans (*Vicia faba* L.)
Passalora Fresen.
Passalora bupleuri (Pass.) U. Braun (sub. ***Cercospora coriandri*** Rjach.).
Causal agent of blight (cercosporoza) on coriander (*Coriandrum sativum* L.)
Passalora fraxini (DC.) Arx (sub. ***Cercospora fraxini*** Sacc.).
Causal agent of brown leaf spots on ash (*Fraxinus*)
Passalora malkoffii (Bubák) U. Braun (sub. ***Cercospora malkoffii*** Bubák).
Causal agent of blight (cercosporoza) on anise (*Pimpinella anisum* L.)
Pseudocercospora Speg.
Pseudocercospora cruenta (Sacc.) Deighton (sub. ***Cercospora cruenta*** Sacc.).
Causal agent of 'cercosporoza' on soybean (*Glycine max* (L.) Mill.)
Pseudocercospora griseola (Sacc.) Crous & U. Braun [sub. ***Phaeoisariopsis griseola*** (Sacc.) Ferraris].
Causal agent of angular leaf spots on bean (*Phaseolus vulgaris* L.)
Ramularia Unger
Ramularia armoraciae Fuckel
Causal agent of leaf spots on horseradish (*Armoracia rusticana* G. Gaertn., B. Mey & Scherb.)
Ramularia betae Rostr.
Causal agent of 'ramularioza' on sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *saccharifera* Alef.)
Ramularia beticola Fautrey & Lambotte
Causal agent of leaf spots (ramularioza) on sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *saccharifera* Alef.)

Ramularia grevilleana (Oudem.) Jørst (sub. ***Ramularia tulasnei*** Sacc.)
Causal agent of white leaf spots on strawberry (*Fragaria vesca* L.)
Ramularia heraclei (Oudem.) Sacc. (sub. ***Ramularia pastinacae*** Bubák).
Causal agent of leaf spots on parsnip (*Pastinaca sativa* L.)
Ramularia onobrychidis Allech.
Causal agent of brown leaf spots on sainfoin (*Onobrychis pulchella* Schrenk)
Ramularia rubella (Bonord.) Nannf. (sub. ***Ovularia obliqua*** (Cooke) Oudem.).
Causal agent of leaf spots on dock (*Rumex obtusifolius* L.)
Ramularia tiliae Lobik
Causal agent of grey-brown spots on linden (*Tilia*)

Conclusion: From the taxonomic point of view phytopathological literature and volume 7 of the monographic work 'Fungi of Bulgaria', are not currently interpreted in accordance with ICN and therefore their up-to-date adaptation is fairly needed.

References:

1. Hyde K. D., McKenzie E. H. C. & KoKo T. W. Mycosphere, 2 (1), 2011, 1-88.
2. McNeill J., Barrie F. R., Buck W. R., Demoulin V., Greuter W., Hawksworth D. L., Herendeen P. S., Knapp S., Marhold K., Prado J., Prud'homme van Reine W. F., Smith G. F., Wiersema J. H., Turland N. J. (Eds.). International Code of Nomenclature for algae, fungi and plants (Melbourne Code) adopted by the Eighteenth International Botanical Congress Melbourne, Australia, July 2011. Regnum Vegetabile, 154. Königstein: Koeltz Scientific Books, 2012, xxx, p. 208. [excl. Appendices II–VIII]
3. Bakalova G. G. & Borisova T. I. Cercosporoid hyphomycetous fungi. In: Fungi of Bulgaria. Volume 7. (C. M. Denchev, Ed.). Institute of Botany, Bulgarian Academy of Sciences, Sofia, 2010, p. 259 (In Bulgarian).

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BACTERIOPLANKTON ABUNDANCE AND DIVERSITY IN THE OKOTO LAKE, SEVEN RILA LAKES

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Aim of the study: The aim of the study was to determine the temporal diversity, and the key environmental factors influencing the bacterioplankton of the Okoto Lake.

Materials and Methods: The Okoto Lake is situated in the territory of the Rila National park, Rila Mountain and it is the deepest glacial lake (37.5 m) on the Balkan Peninsula. The lake is located at 2 440 m altitude and its surface area is 6.8 ha, water volume is 860 000 m^3 and it has a watershed area of 36 ha. Water samples for microbial (bacterial number and diversity) and physic-chemical analyses (temperature (ºC), pH, TDS (mg l^{-1}), dissolved oxygen (mg l^{-1}), NO_3-N (µg l^{-1}), NH_4-N (µg l^{-1}), PO_4-P (µg l^{-1}), electric conductivity (µS)) were collected from the surface layer of the lake (0.5 m depth) in July and September 2006 and in July 2007. The bacterial number was counted under epifluorescence microscopy. Metagenomic DNA was extracted by direct lysis and purified through AXG-100 cartridges according to the method of Selenska-Pobell et al. [1]. PCR amplification was carried out using specific and universal bacterial primers: $16S_{8\text{-}27}$F (5'-AGAGTTTGATYMTGGCTCAG-3') and $16S_{1492\text{-}1513}$R (5'-TACGGYTACCTTGTTACGACTT-3') (*E. coli* numbering). The 16S rRNA gene library construction approach was used to assess the bacterial diversity and communities' composition. Three clone libraries were constructed and 288 clones were analysed using RFLP (rare-cutting *Msp*I (Fermentas/Thermo Fisher Scientific)) determining 62 16S rDNA profiles, in total. The dominant 16S rDNA clones were Sanger sequenced. Cluster and correlation analyses were performed for determining the similarity among the samples and the relationships between the abiotic and biotic parameters using software product NCSS 97 (http://www.ncss.com/).

Results: The abiotic parameters did not vary widely between the sampling occasions except for July 2006, when the temperature was 2.4 ºC, compared to the other sampling periods when it varied from 12 ºC to 15.7 ºC. NO_3-N was higher in July 2007 (356.0 µg l^{-1}) than in July 2006 (up to 96.0 µg l^{-1}), which is probably due to the earlier vegetation season and higher activity of the biota in 2007. The bacterial abundance increased during the sampling periods and varied from $0.63x10^5$cfu ml^{-1} in July 2006 to $35.11x10^5$cfu ml^{-1} in July 2007 [2].

In general, the bacterioplankton composition varied in time as the diversity increased from July to September 2006, in contrast to July 2007, when a decreasing trend was recorded. The dominant 16S rDNA profiles comprised 45 – 74% of the respective clone libraries, and they were represented by *Betaproteobacteria, Bacteroidetes* and *Actinobacteria*. *Betaproteobacteria* was presented in a higher percentage during July 2006

and 2007 (76% and 55%, respectively), whereas *Bacteroidetes* dominated in September 2006 (44%). *Actinobacteria* comprised from 19% in September 2006 to 22% in July 2007 from the 16S rDNA sequences and were not found in July 2006.

There was not found a certain trend in the bacterioplankton diversity composition between the sampling occasions. Cluster analysis showed high similarity of abiotic environments between July and September 2007, in a contrast to the bacterial properties, where the bacterioplankton from September 2007 differed dramatically in number and diversity from the bacterioplankton from July 2007.

Relationships, although insignificant, between bacterial number and dissolved oxygen (-0.76), phosphates (-0.76) and bacterial diversity (-0.71), and between bacterial diversity and ammonium (0.75) were recorded.

Conclusion: The lake environment was relatively stable during the sampling period, except in July 2006, when the water temperature was very low. The bacterial abundance fluctuated from 0.63 x 10^5 cfu ml^{-1} to 35.11 x 10^5 cfu ml^{-1}. The dominant phyla of the bacterioplankton communities were *Betaproteobacteria, Bacteroidetes* and *Actinobacteria*.

References:

1. Selenska-Pobell S., Kampf G., Flemming K., Radeva G., Satchanska G. Antonie van Leeuwenhoek, 79, 2001, 149-161.

2. Boteva S. PhD thesis, Sofia University, Faculty of Biology, 2011, p.191.

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DIVERSITY OF SOFT-BOTTOM MACROZOOBENTHIC COMMUNITIES IN THE SHALLOW COASTAL ZONE OF BURGAS BAY (SOUTH-WESTERN BLACK SEA)

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Introduction and aims: During the 1970-1980s, the biodiversity of the Black Sea was reduced dramatically due to the impact of anthropogenic eutrophication, with a disappearance of many sensitive taxa, and a shift of numerical dominance from molluscs to

polychaetes [1, 2]. In more recent years, the state of the Black Sea ecosystems has begun to improve. However, the macrobenthic communities, and particularly those in the coastal zone, are still recovering [3, 4].

The present study aims to characterize the current state of macroinvertebrate biodiversity in soft-bottom habitats from the shallow areas (up to 10 m depth) of the Burgas Bay (south-western Black Sea).

Materials and Methods: Sampling was carried out in June 2013, at 6 monitoring stations in the shallow coastal zone (depth = 10 m), which are situated in a stable eutrophication gradient and were used for water quality measurements in previous studies (2009-2011). The stations are located at an increasing distance from the city of Burgas, as follows: station 1 – Kraimorie, 2 – Chukalya, 3 – Akin, 4 – Sozopol, 5 – Agalina, 6 – Sv. Paraskeva. Triplicate samples were collected at each station with a Van Veen grab (sampling surface 0.05 m^2). The samples were fixed in a 10% formaldehyde solution, and sieved through a 0.5 mm-mesh sieve. The macronivertebrates were sorted, identified to the lowest possible taxonomic level, and counted. For the Turbellaria, Phoronida, Nemertea, Oligochaeta and Hirudinea, further taxonomic identification was not attempted. These groups, together with the Holothuroidea and Chordata but excepting the oligochaetes, were pooled in the group Varia. Oligochaetes were counted as a distinct group in the analyses because of their high numerical abundance at certain stations. For each station, macrozoobenthic community parameters (abundance, number of taxa, taxonomic composition) and diversity indices (Shannon-Wiener diversity H', Pielou evenness J') were calculated. Statistical analyses were carried out using the PAST software package.

At each station, the samples for the analysis of granulometric composition of the sediments and for total organic matter content were also collected. The granulometry was determined as the proportions of 3 fractions (gravel, sand and mud) after wet sieving on a set of 2 mm and 0.063 mm-mesh sieves, complemented by a visual characterization, and the total organic matter was determined as % of the weight loss on ignition at 520°C.

Results: A total of 7498 macroinvertebrate individuals were collected, representing 108 taxa (all samples pooled). The average abundance per station tended to decrease towards the outer Burgas Bay, while the average number of taxa slightly increased – from an average of 9829 individuals/m^2 and 28 taxa for stations 1-3 to 6863 individuals/m^2 and 29 taxa at stations 4-6. The polychaetes were the most abundant group, contributing to 42.4% of the total abundance (all stations pooled), followed by the molluscs (21.3%), oligochaetes (19.9%) and crustaceans (13.2%). The polychaetes and oligochaetes were most abundant at stations located in the inner Burgas Bay, together accounting for on average of 83.75% of the abundance at those stations. By contrast, the proportion of the other groups increased at the outer Burgas Bay stations, where the molluscs, crustaceans and the group Varia represented on average 75.95% of the abundance. The polychaetes were also represented by the highest number of taxa (49), followed by the molluscs (27) and the crustaceans (24). The Shannon-Wiener diversity H' was relatively low, varying from 1.13 (station 4) to 4 (station 5). The Pielou evenness J' was relatively high at most stations, with the exception of stations 3 and 4 where its values were the lowest (0.47 and 0.27 respectively), revealing a high dominance of one or a few taxa in those communities.

Three main groups of stations were separated by cluster analysis and confirmed by analysis of the similarities (ANOSIM, $R = 0.9453$, $p < 0.05$): stations 1 and 2, stations 4 and 6, and a more indistinct group of stations 3 and 5. The overall average dissimilarity determined by the similarity percentage (SIMPER) procedure was 85.93%, with the following taxa contributing the most to it: *Chamelea gallina* (Linnaeus, 1758) (18.14%), oligochaetes (15.83%), *Protodorvillea kefersteini* (McIntosh, 1869) (12.12%), *Heteromastus filiformis* (Claparède, 1864) (6.2%), *Microdeutopus versiculatus* (Bate, 1856) (5.87%), *Melinna palmata* (Grube, 1870) (5.45%), *Prionospio cirrifera* (Wirén, 1883) (2.84%), *Melita palmata* (Montagu, 1804) (2.04%), *Polygordius neapolitanus* (Fraipont, 1887) (1.87%). The bivalve *C. gallina* dominated the communities at stations 4 and 6, characterized by fine sand. At station 3, where the sand was medium-grained, the communities were dominated by the polychaete *P. kefersteini* and *P. neapolitanus*, with other characteristic soft-bottom species such as *Ophelia limacina* (Rathke, 1843) and *Branchiostoma lanceolatum* (Pallas, 1774), also very abundant. However, the unusually high abundance of oligochaetes at that station is what distinguishes it the most from the others, and accounts for their high contribution to the overall dissimilarity. At stations 1 and 2, where the sediment contained high amounts of mud and organic matter, tolerant polychaete species *H. filiformis, Melinna palmata, P. cirrifera,* as well as oligochaetes, were very abundant. Station 5 was characterized by shelly coarse-grained sand, and the dominant taxa there were crustaceans *M. versiculatus* and *Melita palmata; P. kefersteini, P. neapolitanus* and *B. lanceolatum* were also present.

Conclusions: The average number of taxa tended to increase towards the outer Burgas Bay, where with the increasing distance from the city of Burgas – the main source of anthropogenic influence in the area - the stations are subjected to lower amounts of pressure from urbanization, agriculture, tourism and other activities in the coastal zone. There was also a decrease in the abundance of polychaetes and oligochaetes, with a parallel increase in the abundance of molluscs and crustaceans, and overall a more balanced community structure at the outer Burgas Bay stations, which also seems to indicate the lower anthropogenic impacts there.

The soft-bottom macroinvertebrate communities in the shallow coastal areas of the Burgas Bay were characterized by a lower number of taxa compared to the deeper areas (20-25m), and a clear numerical dominance of some species in the communities [5]. This phenomenon is not uncommon for the shallow coastal zone, where the patchiness of benthic communities is higher and the latter are subjected to higher variations in the environmental conditions such as temperature, salinity, and wave action, which could explain the observed patterns of biodiversity.

This study contributes to the characterization of the current state of the diversity of macrozoobenthic communities in the shallow coastal zone of the Burgas Bay (the southern Bulgarian Black Sea). Changes in the environmental conditions are reflected in the macrozoobenthic community parameters relatively quickly, and shallow soft-bottom macroinvertebrate communities are generally the first to be subjected to anthropogenic pressures from activities in the coastal zone. Therefore, shifts in their diversity and ecological state can serve as an early warning for long-term impacts on the vulnerable marine ecosystems.

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References:

1. Gomoiu M. T. In: Marine Coastal Eutrophication, Science of the Total Environment, Supplement 1992 (Vollendweider, R. A. et al., Eds.), 1992, 683-692.
2. Stoykov S. and Uzunova S. Proceedings of the Institute of Fisheries – Varna, 25, 1999, 153-174.
3. Stoykov S. and Uzunova S. Mediterranean Marine Science, 2/1, 2001, 27-35.
4. Todorova V. and Konsulova T., Mediterranean Marine Science, 1/1, 2000, 123-131.
5. Marinov T. The zoobenthos from the Bulgarian sector of the Black Sea, Publ. House of the BAS, Sofia, 1990, p. 195.

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BRYOPHYTE FLORA OF MEDITERRANEAN RIVERS IN BULGARIA

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Aim: The research presents a contribution to the knowledge of aquatic bryophyte flora. An overview was given about the relationship between the species' relative abundance and the abiotic habitat factors (flow velocity, shading, substrate type) in the Mediterranean Rivers in Bulgaria.

Materials and Methods: Aquatic bryophytes were studied at the beginning of the growing season (mid May 2014) at 9 sampling sites in nine rivers in South Bulgaria. Bryophyte composition was presented together with site descriptions (flow velocity, shading, and substrate type). The Nomenclature accepted in Grolle and Long [1] for liverworts and Hill et al. [2] for mosses was followed. The bryophyte relative abundance was quantified based on the percent of frequency of occurrence in the sampling sites.

Results: The majority of the studied rivers sites were sunny, with moderate velocity and stony bottom. The list of aquatic bryophytes (Bryophyta) included 14 taxa of witch 11 mosses and 3 liverworts. The recorded bryophytes species referred to 7 families and 12 genera. The most frequently distributed species was *Leptodictyum riparium* (Hedw.) Warnst. (registered at 7 sites,

relative abundance 78%), followed by *Cratoneuron filicinum* (Hedw.) Spruce and *Platyhypnidium riparioides* (Hedw.) Dixon (registered at 4 sites; relative abundance 44%), *Brachythecium rivulare* Schimp. and *Hygroamblystegium tenax* (Hedw.) Jenn. (registered at 3 sites; relative abundance 33%). Based on the calculated ratio between bryophytes and vascular plants in the studied rivers, Manastirska and Melnishka Rivers showed highest proportion of bryophytes (about 40%). The species whose distribution was most restricted to shaded, fast flowing habitats with coarser substrata were *Cratoneuron filicinum* (Hedw.) Spruce, *Hygroamblystegium tenax* (Hedw.) Jenn., *Aneura pinguis* (L.) Dumort., *Bryum pallens* Sw. ex anon. and *Brachythecium rutabulum* (Hedw.) Schimp. The rest of the species were relatively tolerant to the tested abiotic factors. The most of the presented sampling sites fulfilled the thresholds established for benchmark sites for the common European Mediterranean river type after Feio et al. [3].

Conclusions: Fourteen taxa were registered at the studied 9 highly seasonal rivers in Bulgaria during 2014, the most distributed bryophyte was *Leptodictyum riparium*. Bryophytes represented between 8-43% from the aquatic macrophyte communities at the studied rivers (median 25%).

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References:

1. Grolle R., Long D. G. Journal of Bryology, 22, 2000, 103–140.

2. Hill M. O., Bell N., Bruggeman-Nannenga M. A., Brugués M., Cano M. J., Enroth J., Flatberg K. I., Frahm J-P, Gallego M. T., Garilleti R., Guerra J., Hedenäs L., Holyoak D. T., Hyvönen J., Ignatov M. S., Lara F., Mazimpaka V., Muñoz J., Söderström L. Journal of Bryology, 28, 2006, 198–267.

3. Feio M. J., Aguiar F. C., Almeida S. F. P., Ferreira J., Ferreira M. T., Elias C., Serra S. R. S., Buffagni A., Cambra J., Chauvin C., Delmas F., Dörflinger G., Erba S., Flor N., Ferréol M., Germ M., Mancini L., Manolaki P., Marcheggiani S., Minciardi M. R., Munné A., Papastergiadou E., Prat N., Puccinelli C., Rosebery J., Sabater S., Ciadamidaro S., Tornés E., Tziortzis I., Urbanič G., Vieira C. Sci Total Environ., 476-477, 2014, 745-756.

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CONDITION AND THREATS TO THE POPULATIONS OF FIVE BALKAN ENDEMIC PLANTS ON MT FALAKRON, NORTH-EASTERN GREECE

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Introduction: Mt Falakron (2232 m) is situated between the Mt Rhodope and the Aegean Sea, North-Eastern Greece. The basic rocks are predominantly calcareous (marbles), soils are predominantly rendzinas. The Calcareous terrain is the reason for the especially rich flora. The climate is Transitional Mediterranean. The North slopes are affected by the Temperate Continental climate, while the south slopes are affected by the Mediterranean climate [1], which is a reason for great plant and habitat diversity.

Occurrence of endemics, relicts and rare species as *Abies borisi-regis* Mattf., *Achillea ageratifolia* (Sm.) Boiss., *Anthyllis aurea* Welden, *Echium russicum* J. F. Gmel., *Fritillaria drenovskyi* Degen & Stoj., *Gymnadenia conopsea* (L.) R. Br., *Haberlea rhodopensis* Friv., *Lathraea rhodopea* Dingl., *Ophrys cornuta* Steven, *O. mammosa* Desf, *Ostrya carpinifolia* Scop., *Petkovia orphanidea* (Boiss.) Stef., *Saxifraga ferdinandi-coburgi* Kellerer & Sünd., *S. stribrnyi* (Velen.) Podp., *S. sempervivum* C. Koch., *Sideritis scardica* Griseb., *Viola delphinantha* Boiss., *V. perinensis Becker* etc., some of them with a narrow ecological niche, is an indication of the great conservation importance of Mt Falakron, which can be defined as a natural museum and a treasury of world wealth.

The north slopes are covered by *Quercetum mixtum* and beech forest.

The south slopes are covered by pseudomaquis dominated by *Quercus coccifera* L., *Quercetum mixtum*, fragmented beech forests and relict forest of *Pinus nigra* Arnold, and *Ostrya carpinifola* Scop.

A typical subalpine-alpine grassy vegetation is found in the high belt, above 1500-1700 m, presented by *Sesleria rigida* Heuffel ex Rchb., *Asperula aristata* L. f., *A. purpurea* (L.) Ehrend., *Dianthus gracilis* Sm., *Festuca varia* Haenke, *Bromopsis cappadocica* Boiss., *Thymus thracicus* Velen., *Trifolium pretense* L., *T. campestre* Schreb, *Vincetoxicum hirundinaria* Medicus ssp. *nivale* (Boiss. & Heldr.) Markdr., *Thymus praecox* Opiz, *Achillea millefolium* L., *Potentilla detomasii* Ten., *P. cinerea* Chaix ex Vill., *Scabiosa ochroleuca* L., *Festuca valesiaca* Schlkeich. ex Gaudin etc. [2].

Above 1600-2000 m, can be found subalpine heath - vegetation dominated by chamaephytes dwarf shrubs *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. and fragments of *Juniperus communis* L. var. *saxatilis* Pall. and *Juniperus sibirica* Burgsd.

The investigated area lies between the passes of Prosotsani-Volakas and Drama-Livadero.

The Anthropogenic activities are very strong - animal husbandry, livestock grazing, marble quarries, collecting of plants, felling, building, ski and tourism.

A Mt Falakron is part of the Rodopi Mountain-Range national park and NATURA 2000.

The aim of this study is to investigate the populations of 5 Balkan endemics: *Achillea ageratifolia, Fritillaria drenovskyi, Haberlea rhodopensis, Petkovia orphanidea* and *Viola delphinantha*.

Materials and Methods: Field trips were performed in the period 2011-2015. The methods of NATURA 2000 for monitoring and assessment of protected plants were used: the count of generative, vegetative and juvenile individuals per square meter (density), a list of accompanying species, habitat specifications, threats. In addition the method of Peev was used [3] based on the reproductive potential, count of individuals, the size of the populations and threats. He divides population in 3 groups: provisionally endangered, partly endangered and critically endangered. This method is suitable for primary data, especially when referent data is missing.

Results: *Achillea ageratifolia* makes a fragmented population on the rocks and stones terrains, on the higher parts and the *Pinus* belt. Many fragments are threatened by pasture and trampling. The population is provisionally threatened. The perspective for the survival of this species is positive.

Fritillaria drenovskyi make a fragmented population, structured by 3 basic and several smaller fragments or single individuals.

1) In the circus on the south and west slopes of the Anonomi peak and west slopes of the Klisserdzic peak at 1750-1850 m. Part of the fragments are destroyed by the building of the ski center.

2) On a meadow in the *Pinus nigra* forest, on the east slope of Kouri peak at 1380-1395 m. Part of the fragment is destroyed by house building and growing in the courtyard.

3) On a meadow in the *Pinus nigra* forest, on the east slope of the Mikri Korti peak, at 1340 m.

A part of the fragments are destroyed by the ski center and threatened by pasture, stamping and hew. The population is partly endangered. The perspective for the survival of this species is positive.

Haberlea rhodopensis. One population was investigated, situated in the canyon of the rivulet Soushitsa (floating through Pirgi village). The population has mosaic structure and big density, growing in the cracks in the rocks. Part of the population is destroyed by constructing of two small basins and a marble quarry. The population is provisionally endangered. The perspective for the survival of this species is positive.

Petkovia orphanidea: it makes vast mosaic population from 1400-2200 m. Part of the fragments are destroyed by the ski center and threatened by pasture and trampling. The population is provisionally endangered.The perspective for the survival of this species is positive.

Viola delphinantha. Two fragments were found:

1) On the North slope of Profitis llias, at 1900-2000 m. A part of the population is destroyed by a marble quarry, which is not in use nowadays.

2) On the west and south slopes of Choros peak, at 1740-1800 m. Part of the fragment is threatened by herds.

The Population is provisionally threatened. The perspective for the survival of this species is positive.

Keywords: endemic plants, Greece, Mt Falakron, populations, threats

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References:

1. Kitanov B. In: Annual of Sofia University, Phys.-Mat. Fac., 3 (Nat. Hist.), 39: Sofia, 1943, 169-292 (In Bulgarian with German summary).
2. Tsiftis S., Tsiripidis I., Vidakis C. Orchids of Rodopi Mountain-Range National Park. Publisher: Management Body of Rodopi mountai-range national park, Mesohori, Paranesti, Drama, Greece, 2012, 1-199.
3. Peev D. Reports of Reg. Symp. 8-MAB, UNESCO, Sofia, 1981, 306-313 (In Bulgarian).

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ДОКЛАДИ/REPORTS
Тематично направление:
АНТРОПОГЕННИ ВЪЗДЕЙСТВИЯ ВЪРХУ ЖИВАТА ПРИРОДА

Topic:
ANTROPOGENIC IMPACT ON THE LIVING NATURE

MICROBIAL PROCESSES AND BIODIVERSITY OF MICROORGANISMS IN HOST ROCKS FOR GEOLOGICAL DISPOSAL OF RADIOACTIVE WASTE

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Abstract

This report summarizes the state of knowledge on the microbial diversity and the impact of microbial activities on the geological stable formation (i.e. clay or organic host-rocks), which is considered the key solution for the long-term disposal of radioactive waste and a generally accepted international concept to manage high level and long-lived radioactive waste. The observation of an important microbiological diversity in these extreme conditions consider the impact of these microorganisms of the rate of processes implicated in the metal

corrosion, transformation of clay minerals and radionuclide migration. All these processes might affect the safety of geological repositories compromising its isolation and containment functions.

Keywords: Nuclear waste, geological disposal, microbial activity, biocorrosion

Introduction

Nuclear power generation, nuclear fuel cycle and the use of radioactive materials in the industry and medicine produce radioactive waste that are classified according to the radioactivity content and its half-life: very low, low, intermediate and high level nuclear waste (HLNW) and to the half-life (short- or intermediate-lived: < 30 years; long-lived:> 30 years) [1]. Compared to other kinds of industrial waste, the radioactive waste has the advantage to be self-degrading due to the radioactive decay process which allows to decrease their toxicity with time. Moreover, the volume of waste is small, and thus confinement strategy can be used to isolate them from the environment. The generally accepted strategy to manage the HLNW is the disposal of waste packages in a deep underground repository (about 500 m deep) in stable geological formations (e.g. clay or granitic medium) [2]. This concept is known as a multi-barrier system which has been designed to prevent the migration of radionuclides that could be harmful to ecosystems and human health. It involves the use of several natural and/or engineered barriers capable of confining the radioactivity for several thousand years. Each of these barriers provides a unique and stand-alone level of protection if any of the barriers deteriorate, the next one will come into play. Multi-barrier systems have been widely considered in a number of countries in Europe, Asia and North America and the approach of the concept differs depending upon the type of nuclear waste and the geology of the available sites. The long-term behavior of HLNW depends on the properties of the waste repository, but also on the nature of the HLNW itself. The multi-barrier system aims at minimizing water migration around the metallic packages (tubing, over pack and container) because corrosion in water-saturated conditions is the major vector liable to alter the waste container. Note that materials selection for the packages is based on their properties, behavior and predictable nature of their corrosion processes [1].

The natural barrier (geological formation) presents a key part of the disposal system due to the intrinsic properties that provide good confining properties, such as low porosity and permeability, low hydraulic conductivity, high radionuclide sorption capacity, and swelling of clay minerals. A number of potentially suitable host rock types, such as granite, claystone and salt deposits, have been identified. Granitic rock environments have been extensively studied in Finland, Sweden and Canada as potential host rocks for a repository [3, 4]. The USA has proposed salt deposits [5]. In Europe, mainly in Germany, France, Belgium and Switzerland, clay formation is a candidate host rock for HLNW [6].

Microbial diversity and activity in deep geological formations

Several studies demonstrate that bacteria and archaea the present in most of the deep geological formations [7, 8, 9, 10, 11]. Therefore, the biochemical activity of either indigenous

to the repository host rock or introduced during the construction of a repository could effect the biocorrosion of metallic packages, and radionuclides migration [12, 13, 14, 3, 4].

A microbial process can affect the geochemistry of clays through three different mechanisms: (i) microbial reduction of Fe (III) in clay minerals; (ii) alteration of mineral surfaces by production of siderophores and small-organic acids, (iii) formation of a biofilm in the clay mineral surface [15]. In addition, microbial activities may interfere with the migration of radionuclides through geological formations by different processes: (i) biosorption to the cell surface [16], (ii) intracellular accumulation [17], (iii) biomineralization [18] or biotransformation [17, 19].

Occurrence of viable indigenous microbes, including sulphate-reducing bacteria and also some isolated strains belonging to the genus *Sphingomonas*, was evidenced in Opalinus Clay at the Mont Terri Underground Research Laboratory by culture-based methods [9, 10], as well as a representative of *Firmicutes*, *Paenibacillus* strain was recovered from the clay sample under anaerobic conditions [20]. A multidisciplinary approach was performed to study the microbial diversity in Boom clay formation, a deep-subsurface clay deposit in Mol, Belgium [21]. In the Meuse/Haute-Marne Underground Research Laboratory located at Bure (300 km east of Paris) the Callovo-Oxfordian argillite formation was evaluated for its use as a potential host rock for a high-level radioactive waste repository in France [22] and its microbial diversity was studied by culture methods by Urios et al. [11]. The bacterial diversity found in the French formations was dominated by *Firmicutes, Actinobacteria* and *Proteobacteri*a [11]. Culture-dependent analysis of microbial diversity from Spanish clay formations demonstrated the dominance of representatives from *Proteobacteria, Firmicutes, Actinobacteria,* as well as a pigmented yeast strain namely *Rhodotorula mucilaginosa* BII-R8 was isolated [23].

The microbial diversity was studied in the so-called Bitumen-Nitrate-Clay interaction (BN) *in situ* experiment, which was installed in the Opalinus clay (OC) in the Mont-Terri Underground Research Laboratory (St. Ursanne, Switzerland) by the RISA clone libraries construction [24, 25]. The aim of this experiment was to estimate the risks of release of NO_3- and organic molecules into the OC due to the weathering of the bitumen containers, foreseen for disposal of HLNW [25]. Our studies demonstrated that the addition of nitrate, nitrite and acetate to OC pore water (PW) strongly influence the intrinsic bacterial community. The prevalence of *Firmicutes* in the pore water samples before the injection was changed with the strong propagation of *Bacteroidetes* and *Betaproteobacteria* after the addition of nitrate. In case, when the samples were treated with nitrate and acetate, changes of the bacterial community structure occurred: *Firmicutes* and *Betaproteobacteria* were strongly reduced and overgrown by *Gammaproteobacteria*, mainly *Pseudomonas stutzerii* and *Alphaproteobacteria* mostly *Brevundimonas* sp.

Microbially Influenced Corrosion (MIC)

The observation of an important microbiological diversity in these conditions considered the impact of these microorganisms in terms of corrosion, since the introduction of exogenic materials (metal containers) in the geological medium is also likely to significantly modify

the geochemical conditions and to provide chemical compounds that will enable the microbial activities. Moreover, a significant amount of dihydrogen (H_2) will be produced due to the corrosion of metal containers and/or radiolysis of water. H_2 will constitute an energetic substrate for anaerobic bacterial communities, especially in this environment containing only small amounts of organic matter, and will strongly stimulate reducing reactions involving Fe (III) and sulphates [26,14].

It is widely accepted that microorganisms can affect the corrosion process due to their influence (directly or indirectly) on the chemical and physical characteristics of the metal/environment interface.

Corrosion by sulfate-reducing bacteria

Sulfate-reducing bacteria (SRB) have been reported to be the main group responsible for anaerobic corrosion, especially in environments with high sulfate concentration (e.g. seawater). SRB perform dissimilatory reduction of sulfur compounds, such as sulfate, sulfite and thiosulfate to sulfide [1]. Certain SRB (i.e. *Desulfovibrio* sp.) are able to use H_2 as electron donor for sulfate reduction under reducing conditions, and although SRB are often considered to be strictly anaerobic, it has been demonstrated that some genera tolerate O_2 [1]. The presence of H_2S is obviously by its characteristic smell and black precipitation of ferrous sulfide (FeS) when iron minerals are present. Some studies about the impact of SRB on steel corrosion under geological disposal conditions have pointed out that the corrosion rate increases 2-3 times compared to control experiments [1]. Corrosion was less severe in aerobic conditions compared to strict anaerobic conditions due to the formation of magnetite as a corrosion product [1].

Corrosion by iron-reducing bacteria (IRB)

IRB can couple oxidation of organic (e.g. lactate and acetate) or inorganic (e.g. H_2) substrates with Fe (III) reduction. In the environment, IRB may outcompete both SRB and methanogenic bacteria for H_2 as an electron donor. The role of IRB on corrosion processes is still under discussion. Several studies have demonstrated inhibitory or enhancing effects [27, 28]. The enhancing effect is associated with the destabilization/dissolution of the passivating oxide layers (i.e. magnetite (Fe_3O_4), a corrosion product) by structural Fe (III) reduction under anoxic conditions, which in turn exposes the metal surface to the corrosive environment and reactivates corrosion [29]. On the other hand, under aerobic conditions, Fe (II) produced by Fe(III) reduction can be oxidized by O_2, which forms a protective way against O_2 attack on the metal surface [27]. Recently, it was evidenced that depending on the medium composition IRB can inhibit corrosion by formation of a vivianite layer on carbon steel surface [30]. Cote et al. [30] evidenced that only in the presence of *Geobacter sulfurreducens* a protective vivianite layer was formed in a way that could stabilize the corrosion potential even after intrusion of air in the system (medium with 5 mM of phosphate).

Regarding the MIC studies and molecular understanding of Fe(III) reduction, the

Shewanella oneidensis strain MR-1 is considered as a model organism because its genome has been completely sequenced [27].The bacterium can be used in a wide range of compounds as terminal electron acceptors under anoxic conditions (fumarate, dimethyl sulfoxide (DMSO), trimethylamine N-oxide (TMAO); elemental sulfur, thiosulfate, nitrate (NO_3-) and nitrite (NO_2-); metal ions, Fe(III), Mn (IV) and Cr (VI); and radionuclides, U (VI). It has been shown that the *Shewanella oneidensis* strain MR-1 can use different mechanisms to access Fe (III). In the case which involves contact with Fe (III) from insoluble substrates, the bacteria may produce fimbriae, pili or nanowires (cellular appendages) as conductors for electrons [31].

Bioreduction of Fe (III) (hydr)oxides

The Fe (II)-Fe(III) redox cycling represents the major energy flux on Earth's surface. However, Fe (III) (hydr)oxides are characterized by a low solubility. In this way, dissolution mechanisms must take place for the cycling of Fe, such as: (i) dissolution promoted by surface protonation; (ii) ligand-promoted dissolution; and (iii) reductive dissolution mainly by biological processes [1].

The mechanism by surface protonation involves the chemisorption of protons at the Fe(III) (hydr)oxide surface, which weakens the Fe-O bonds by polarization promoting the detachment of structural Fe(III) from the mineral. The rate of dissolution by protonation is highly dependent on pH conditions (higher dissolution at acidic conditions). This mechanism can be further enhanced by organic acids (e.g. oxalic and citric acid) or anions (e.g. Cl^-) which also adsorb to the Fe (III) (hydr)oxide surface [32]. Under anaerobic conditions, reductive dissolution is the most prominent mechanism [33]. In this case, the microbial activity takes an important role. Indeed, the IRB species are considered to be the primary agent in the reductive dissolution in sedimentary environments [34].

Microbial reduction (or bioreduction) is a complex process which involves bacterial cells, Fe (III) (hydr)oxides, aqueous-phase components and secondary mineral phases that can be formed as a result of Fe(III) bioreduction. Moreover, the rate and extent of bioreduction depend on several conditions, such as the bacterial strain and cell density, order to improve the understanding of how different biogeochemical parameters may influence bioreduction.

Kostka and Nealson [26] were the first to demonstrate the Fe (III) bioreduction from synthetic magnetite coupled to growth and respiration (glucose as electron donor). Their study showed that the optimal conditions for dissolution of magnetite are pH 5-6.2 at 37°C in the presence of *Shewanella putrefaciens* strains MR-1 and MR-4. The dissolution rate varied according to the initial cell concentration; the optimum dissolution was reached with 10^5 cells mL^{-1}. They argued that dissolution can be limited by the saturation of magnetite surface with cell densities above 10^5 cells mL^{-1}.

Conclusions: The survival capability and adaptation of microorganisms is remarkable even in the extreme conditions that will prevail during the lifetime of nuclear waste repository. It is widely accepted that microbial activities might affect the safety of geological repositories compromising its isolation and containment functions. Therefore, it is important to control

and to limit microbial activity in order to improve the safety of a nuclear waste deposition. One of the possibilities is to create unfavorable conditions for microbial growth such as lack of space. “Space restriction” is an essential safety requirement to minimize microbial activity, because transport of electron donors and acceptors and other nutrients (e.g. P, Se) is limited by diffusion in compact clay materials. Another opportunity for microbial activity restriction is the lack of water, if water activity is < 0.6 the microbial activity stops because of osmotic effects in the cell induced by desiccation. Microbial activity also stops at pH > 12-12.5 of the cementitious materials that are used as backfill of the repository. An essential safety requirement for the backfill material is the limitation of voids and their pore size in order to prevent microbial development and its potential detrimental effects in the repository [35].

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References:

1. Schutz M. PhD thesis 2013.
2. Bennett D. G., Gens R. Journal of Nuclear Materials, 379 (1-3), 2008, 1-8.
3. Stroes-Gascoyne S., Sargent F. P. Journal of Contaminant Hydrology, 35(1-3), 1998, 175-190.
4. Pedersen K. In: Interactions of microorganisms with radionuclides (M. Keith-Loach, F. Livens, Eds.) Elsevier, 2002, 279-311.
5. Frederickson J. K., Zachara J. M., Balkwill D. L., Kennedy D., Li S.-M. W., Konstandarithes H. M., Daly M. J., Romine M. F., Brockman and F. J. Appl. Environ. Microbiol., 70, 2004, 4230-4241.
6. Stroes-Gascoyne S., Schippers A., Schwyn B., Poulain S., Sergeant C., Simonoff M., Altmann S., Nagaoka T., Mauclaire L., McKenzie J., Daumas S., Vinsot A., Beaucaire C., Matray J. M. Geomicrobial. J., 24, 2007, 1-17.
7. Boivin-Jahns V., Ruimy R., Bianchi A., Daumas S., Christen R. Appl. Environ. Microbiol., 62 (9), 1996, 3405-3412.
8. Mauclaire L., McKenzie J. A., Schwyn B., Bossart P. Physics and Chemistry of the Earth, Parts A/B/C 32 (1-7), 2007, 232-240.
9. Poulain S., Sergeant C., Simonoff M., Le Marrec C., Altmann S. Geomicrobiol. J., 25 (5) 2008, 240-249.
10. Stroes-Gascoyne S., Sergeant C., Schippers A., Hamon C. J., Neble S., Vesvres M. H., Barsotti V., Poulain S., Le Marrec C. Appl. Geochemistry, 26 (6), 2011, 980-989.
11. Urios L., Marsal F., Pellegrini D., Magot M. Appl. Geochemistry, 27 (7), 2012, 1442-1450.
12. Behrends T., Krawczyk-Barsch E., Arnold T. Appl. Geochemistry, 27 (2), 2012, 453-462.
13. Keith-Roach M. J., Livens F. R. In: Interactions of microorganisms with radionuclides (M. Keith-Loach, F. Livens, Eds.) Elsevier, 2002, 383-391.

14. Libert M., Schütz M. K, Esnault L., Feron D., Bildstein O. Bioelectrochemistry, 97, 2014, 162-168.

15. Meleshyn A. 2011. Gesellschaft fuer Anlagen-und Reaktorsicherheit (GRS) mbH, Germany.

16. Lloyd J. R., Macaskie L. In: Environmental Microbe-Metal Interactions (D. R Lovely, Ed.), ASM Press, Washington, D. C., 2000, 277–327.

17. Brookshaw D. R., Pattrick R. A. D., Lloyd J. R., Vaughan D. J. Mineral. Mag., 76 (3), 2012, 777–806.

18. Merroun M. L., Nedelkova M., Ojeda J. J., Reitz T., López-Fernández M., Arias J. M., Romero-González M., Selenska-Pobell S. J. Hazard. Mater., 197, 2011, 1–10.

19. Lovely D. R., Widman P. K., Woodward J. C., Phillips E. J. P. Appl. Environ. Microbiol., 59, 1999, 3572–3576.

20. Lutke L., Moll H., Bachvarova V., Selenska-Pobell S., Bernhard G. Dalton Trans. 42, 2013, 6979-6988.

21. Wouters K., Moors H., Boven P., Leys N. FEMS Microbiol. Ecol., 86, 2013, 458–473.

22. Cormenzana J. L., García-Gutiérrez M., Missana T., Alonso U. Phys. Chem. Earth, 2008, DOI: 10.1016/j.pce.2008.05.006

23. Lopez-Fernandez M., Fernandez-Sanfrancisco O., Moreno-Garcia A., Martin-Sanchez I., Sanchez-Castro I., Merroun M. L. Appl. Geochemistry, 49, 2014, 77-86.

24. Selenska-Pobell S., Radeva G., Geissler A., Moors H., Leys N. Annual Report, Institute of Resource Ecology, HZDR, 2011, p. 20.

25. Selenska-Pobell S., Radeva G., Geissler A., Moors H., Leys N. Annual Report, Institute of Resource Ecology, HZDR, 2012, p. 29.

26. Kostka J. E., Nealson K. H. Environ. Sci. Technol., 29 (10), 1995, 2535-2540.

27. Dubiel M., Hsu C. H., Chien C. C., Mansfeld F., Newman D. K. Appl. Environ. Microbiol., 68 (3), 2002, 1440-1445

28. Mehanna M., Basséguy R., Delia M.-L., Girbal L., Demuez M., Bergel A. Electrochim. Acta. 54 (1), 2008, 140-147.

29. Esnault L., Jullien M., Mustin C., Bildstein O., Libert M. Physics and Chemistry of the Earth, Parts A/B/C 36 (17–18), 2011, 1624-1629.

30. Cote C., Rosas O., Sztyler M., Doma J., Beech I, Basseguy R. Bioelectrochemistry, 2013, http://dx.doi.org/10.1016/j.bioelechem.2013.11.001.

31. Gorby Y. A., Yanina S., McLean J. S., Rosso K. M., Moyles D. et al. PNAS, 103 (30), 2006, 11358-11363.

32. Panias D., Taxiarchou M., Paspaliaris I., Kontopoulos A. Hydrometallurgy. 42 (2), 1996, 257-265.

33. Cornell R. M., Schwertmann U. The Iron Oxides: Structure, Properties, Reactions, Occurrences and Uses, Wiley-VCH, Wienheim, Germany, 2003, p.571.

34. Lovley D. R. In: Origins: Genesis, Evolution and Diversity of Life (J. Seckbach Ed.) Springer Netherlands, 2005, 299-313.

35. De Cannière P., Eurosafe Forum, 2013.

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SHORT-TERM AND LONG-TERM EXPERIMENTS IN ENVIRONMENTAL STUDIES

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The aim of this study was to compare genotoxic potential of polluted soil samples from KCM Plovdiv in short term and long term experiments using CFGE.

Materials and Methods: The wild type *Chlamydomonas reinhardtii* 137C was used. Five soil samples from different plots were studied. Extraction was performed with 0.01M $CaCl_2$ solution. The strain 137C was cultivated on liquid TAP medium under standard conditions in the growth camera Phytotron GC 400. The cell suspension was treated for 2 and 72 hours with soils samples. DSBs induction was measured by constant field gel electrophoresis.

Results: The results revealed that soil samples/extracts did not show genotoxic effect after 2h treatment. DNA damaging effect of soil samples was obtained when cells were treated for 72 h.

Conclusion: Our results demonstrated that long term treatment could be considered as more reliable approach for revealing genotoxicity of environmental samples.

Keywords: soil samples, DSBs, CFGE, test-system, *Chlamydomonas reinhardtii*

Introduction

Knowledge concerning occurrence and bioavailability of heavy metals and metalloids in soils are of major importance for environmental health, crops and livestock production, food and water quality and ecotoxicology [1].

Heavy metals are environmental pollutants, and their toxicity is a problem of increasing significance for ecological, evolutionary, nutritional and environmental reasons. There are different sources of heavy metals with different origin released in the environment - natural, agricultural, industrial, domestic effluent, atmospheric etc. Heavy metals could be accumulated in cells or tissues and could damage DNA in a different way, inducing frame shift mutations, cross-links, base modifications, single and/or double strand breaks in DNA and etc.

Last two decades *C. reinhardtii* was considered as a robust model in environmental toxicity investigations because several main reasons:

• Widely spread photosynthetic unicellular eukaryote;

• Cell structure and genome organization are typical for plants so the results could be easily extrapolated to higher plants;

• Short live cycle;
• Haploid genome- recessive mutations could be revealed in M_1 generation;
• Routine inexpensive laboratory cultivation techniques;
• Suitable for molecular methods or/or techniques etc.

C. reinhardtii has been also recommended for short-term and long-term experiments in modern eco/genotoxicology [2].

The aim of this study was to evaluate DNA damaging effect of soil samples collected at the area of KCM Plovdiv depending on the experimental design: short-term and long-term exposure.

Materials and Methods

Soil samples: KCM Plovdiv is situated in the area between Plovdiv and Asenovgrad on 900 ha. Five soil samples were collected from different plots in the region of KCM Plovdiv. The extraction was performed with 0.01M $CaCl_2$ solution 48 hours [3].

Strain: The wild type strain *Chlamydomonas reinhardtii* 137C maintained in our lab more than 20 years was used as a test system. All clones of this strain are offspring of a single zygote isolated from soil in Massachusetts, USA, by Smith in 1946.

Cultivation: The strain 137C was cultivated in the growth camera Phytotron GC 400, on liquid TAP medium at the t= 23°C ± 0.3 and continuous light of 5000-5500 lx.

Experimental conditions: Five days old cell suspension in the end of exponential and the beginning of stationary growth phase was used. Cells with a density of $1x10^6$ cells per ml were treated for 2 and 72 hours with soil extracts under continuous light on a shaker and t=23°C ± 0.3 following the requirements of ISO 8692:2004.

Methods: DSBs induction was measured by Constant Field Gel Electrophoresis (CFGE) as described by [4, 5]. Electrophoresis conditions were: 40 h at a constant field of 20 V and 10 mA. The level of induced DSB was evaluated as the fraction of DNA released (FDR) from wells by measurement of ethidium bromide fluorescence with GeneTool Analyser G:Box Syngene. FDR (fraction of DNA resealed) was calculated:

$$FDR = \frac{\text{DNA released}}{\text{DNA in well} + \text{DNA released}}$$

Data analysis: The experiments were repeated at least three times using independently grown algae culture. One-way analysis of variances (ANOVA) with Dunnett`s Multiple Comparison test was used to calculate statistically significant differences between the effects of soil samples after 2h and 72 h treatment. Two-way ANOVA with Bonferroni post test was applied to determine how the treatment time, soil extracts and interaction between them (GraphPad Prism 6.04) could affect the response.

Results

DNA damaging capacity of soil samples was evaluated by CFGE, measuring the level of DSB induced. Data in a Fig. 1 illustrate that soil extracts do not increase levels of DSBs comparing with spontaneous DSBs in the control. One way ANOVA data show no statistically significant differences among levels of DSBs in untreated cells, treated with $CaCl_2$ and soil extracts when short-term (2h) treatment ($p>0.05$) was applied. These results provide good information for the absence of DNA damaging capacity of soils samples after short-term exposure.

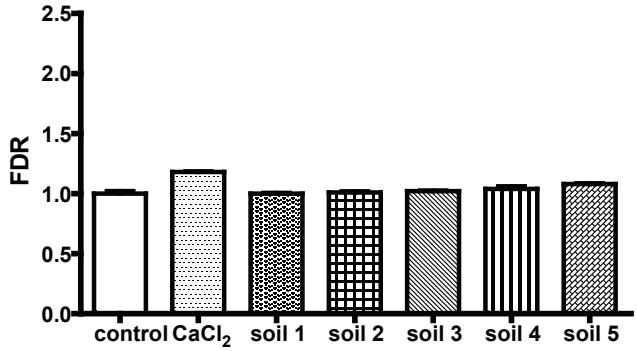

Fig. 1. DNA damaging potential of soil extracts when 2h treatment is applied. The error bars represent the standard error of the mean values. Where no error bars are evident, the errors are equal to or less than the symbols.

Data concerning 72h long-term treatment reveal some DNA damaging capacity of soil extracts. The most pronounced DSBs induction - more than 2-fold higher vs control (Fig. 2) was obtained after the treatment with soil extracts 2, 3 and 4 ($p>0.01$).

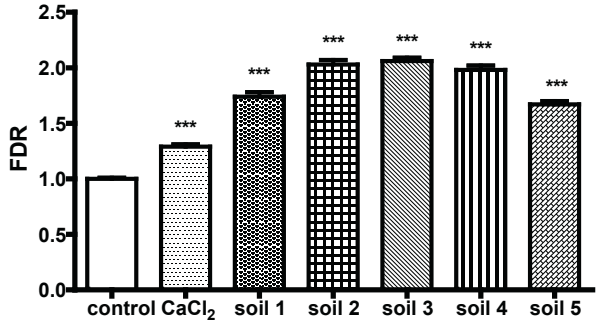

Fig. 2. DNA damaging potential of soil extracts after 72h treatment. The error bars represent the standard error of the mean values. Where no error bars are evident, the errors are equal to or less than the symbols.

Two-way ANOVA with Bonferroni post test demonstrate that the induction of DSBs depends on the effect of the treatment time (57.46%, $p<0.0001$), type of soil extracts

(18.44%; p<0.0001) and interaction between them (22.39%; p<0.0001). Statistically significant increase of the DSBs levels after 72h treatment with soil extracts (more than 2-fold increase) comparing with the induction of DSBs after 2h was found. The soil extracts 2, 3 and 4 are found to possess slight DNA damaging effect inducing DSBs (Fig. 3).

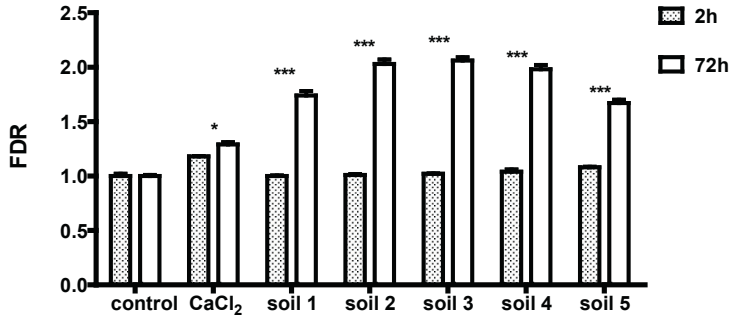

Fig. 3. Comparing DSBs induced after 2h and 72h treatment with soil extracts. The error bars represent the standard error of the mean values. Where no error bars are evident, the errors are equal to or less than the symbols.

Discussion

In this study we compared DNA damaging capacity of soil samples collected nearby KCM Plovdiv in short-term and long-term experiments. In a very clear way the role of exposure time was manifested - statistically significant differences were calculated, comparing DSBs measured after 2 and 72 hours treatment. Our finding contributes to the present understanding that 72 h experiments reveal in a better way genotoxic capacity of natural samples. On the other hand data obtained by two-way ANOVA with Bonferroni post tests show that the levels of DSBs were significantly dependent not only on the exposure time, but also on the type of soil extracts and the interaction between them.

Conclusion:

It is a well-known fact that DSBs are the most crucial DNA damage, because they are fatal for the cells in the case that are not repaired.

Our results demonstrate that:

1. Long term treatment could be considered as a more reliable approach for the revealing genotoxicity of environmental samples.

2. Soil samples extracts labeled as 2, 3 and 4 are shown to possess slight DNA damaging effect inducing DSBs.

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Reference:

1. Brian J. Heavy Metals in Soils Trace Metals and Metalloids in Soils and their Bioavailability, Third Edition Springer, 2008, p.11
2. Dimitrova M., Dimova E., Mitrovska Z., Kapchina-Toteva V., Chankova S., Algol. Stud. 123, 2007, 111–121
3. Houba V. J. G, Temminghoff E. J. M., Gaikhorst G. A., & van Nark W. Communications in Soil Science and Plant Analysis, 31 (9-10), 2000, 1299-1396.
4. Chankova S. G., Bryant P. E., Radiat. Biol. Radioecol. 42, (6), 2002, 600-603.
5. Chankova S. G., Matos J. A., Simões F., Bryant P. E., Int J Radiat Biol 81, 2005, 509-514.

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КРАТКИ СЪОБЩЕНИЯ/SHORT COMMUNICATIONS

Тематично направление:
АНТРОПОГЕННИ ВЪЗДЕЙСТВИЯ ВЪРХУ ЖИВАТА ПРИРОДА

Topic:
ANTROPOGENIC IMPACT ON THE LIVING NATURE

SENSITIVITY OF GREEN ALGAE AND CYANOBACTERIA TO PHENYLUREA HERBICIDES

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Introduction: The extensive use of herbicides in agriculture has become a serious environmental problem. Phenylurea herbicides are known to inhibit photosystem II (PSII) and induce oxidative stress that results in damage of the photosynthetic apparatus [1]. The PSII complex is the most sensitive place in the photosynthetic machinery to many abiotic stress factors.

Aim: The aim of the present study is to assess the sensitivity of the green alga *Chlorella vulgaris* and the cyanobacterium *Synechocystis salina* to isoproturon (3-(4-isopropilphenyl)-

1,1dimethylurea) and DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea). These phenylurea herbicides interact with PSII and influence its activity. The herbicides displace the secondary plastoquinone acceptor of PSII complex from Q_B site of D1 protein and block the electron transport in PSII.

Materials and Methods: The effects of different concentrations of phenylurea herbicides on the functions of PSII were studied by pulse amplitude modulated (PAM) chlorophyll fluorescence measurements and the determination of oxygen evolution by polarographic oxygen electrodes (Joliot-type and Clark-type). For characterization of the influence of herbicides on the PSII activity, we used the following parameters: the maximum quantum efficiency of PSII photochemistry in dark-adapted state (Fv/Fm); the maximum amplitude of the flash-induced oxygen yields (Y), which is released from fast operated centers [2]; the oxygen burst amplitude (A) under continuous illumination, which is released from fast and slow operated centers [2]; the oxygen evolution in the presence of benzoquinone ($H_2O \rightarrow BQ$). In order to compare the sensitivity of different parameters, which characterize the oxygen evolution we introduced the parameter RS50. RS50 represents the herbicide concentration causing 50% inhibition of the initial parameter value (i.e., of the control parameter value without herbicides). The cells were treated with different concentrations (0-300 nM) of herbicides for 30 min in the dark.

Results: The interaction of the studied herbicides with the cells of the green alga and cyanobacterium leads to inhibition of the maximum quantum efficiency of the PSII photochemistry in the dark-adapted state (Fv/Fm) with about 15% at 300 nM of the studied herbicides in both green alga *Chlorella vulgaris* and cyanobacterium *Synechocystis salina*. The parameter Fv/Fm for the untreated cells was higher for the green alga than the cyanobacterium. The lower value of the Fv/Fm ratio of *Synechocystis salina* compared to that of *Chlorella vulgaris* could be a result of the contribution of phycobiliprotein fluorescence in F_0, which lead to a decrease of the Fv/Fm ratio in the cyanobacterium [3].

The results show that the herbicide-induced structural changes in PSII influence the oxygen evolution, as the degree of inhibition increases with increasing the concentrations of DCMU and isoproturon. The oxygen evolution is more strongly influenced in comparison to the maximum quantum efficiency of PSII photochemistry in the dark-adapted state (Fv/Fm). The comparison of the relative sensitivity (RS50) of the parameters of the oxygen evolution of the cyanobacterium and the green alga to the phenylurea herbicide shows:

(i) similar inhibition of the oxygen evolution without an exogenous acceptor (parameters A and Y) for *Chlorella vulgaris* and *Synechocystis salina*;

(iv) stronger inhibition of the oxygen evolution in the presence of BQ in the cyanobacterium in comparison to the green alga;

(ii) stronger influence on the flash-induced oxygen yields (Y) in comparison to the oxygen evolution under continuous illumination (A), which suggests an influence of the studied herbicides on the fast-operated oxygen-evolving centers;

(iii) smaller influence on the oxygen evolution for *Chlorella* cells in the presence of exogenous acceptor BQ (93 nM and 129 nM for DCMU and isoproturon, respectively) than to the oxygen evolution without an exogenous acceptor (48 nM and 90 nm for DCMU and isoproturon, respectively), while for *Synechocystis* cells the inhibition is similar with and without an artificial acceptor (≈ 50 nM and ≈ 100 nM for DCMU and isoproturon, respectively).

Conclusion: Data revealed that the effects of phenylurea herbicides on the photosynthetic oxygen evolution of *Synechocystis salina* and *Chlorella vulgaris* can be used for assessing the biological impact of these herbicides on aquatic ecosystems.

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References:

1. Rutherford A. W. Rieger-Liszkay A., Trends Biochem Sci., 26 (11), 2001, 648-653.
2. Dobrikova A., Domonkos I., Sözer Ö., Laczkó-Dobos H., Kis M., Párduc Á., Gombos Z., Apostolova E.L., Physiol. Plantarum 147 (2), 2013, 248-260.
3. Campbell D., Hurry V., Clarke A. K., Gustafsson P., & Oquist G., Microbiology and Molecular Biology Review, 62 (3), 1998, 667–683.

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ECOLOGICAL STATUS OF ATANASOVSKO LAKE (BULGARIA) BASED ON THE MACROZOOBENTHOS COMMUNITY

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Introduction: The Atanasovsko Lake is a part of the Burgas Lake complex. It is a coastal lagoon, which is characterized by saline, brackish and fresh waters as well it's a part of the European ecological network NATURA 2000 and it has status of the maintained reserve. Macrozoobenthic invertebrates have main importance for fishes and for waterfowl birds as trophic resource.

Aim: The aim of the hydrobiological research is to present actual data on the ecological status of The Atanasovsko Lake according to the biological quality element macrozobenthos. The main tasks of this study were the establishment of the taxonomic composition of macroinvertebrate fauna, identification and analysis of some physicochemical parameters (in accordance to Regulation N-4/2012 for the characterization of the surface waters).

Materials and Methods: Macrozoobenthic invertebrates were sampled in October and November 2014 from 16 sites located in different water bodies (i.e. freshwater swamps and

surrounding canal, brackish and hypersaline basins). A hand-held net (EN-ISO 10870:2012) was used for collecting the samples. The ecological status was determined in accordance with Regulation N-4/2012. The taxonomic composition, the degree of dominance and the presence of some indicator groups of benthic organisms can get an idea of the ecological conditions of each water body. The Biotic index (for freshwater bodies) and AMBI and M-AMBI biotic index (for salt water bodies) were used for the evaluation of the status. Physicochemical parameters which following are: water temperature, oxygen content ($mg.ml^{-1}$), oxygen saturation (%), pH (pH), electrical conductivity (μS/sm), resistance (om/sm), TDS-salts (g/L - mg/L), ORP (mV).

Results: The abundance, taxonomic composition, newly found species and species with conservation importance of the macrozoobenthos were established. We found 46 taxa belonging to 18 different groups. Fifteen taxa were record for the first time in the Atanasovsko Lake. They represent 33% of all species. The new species in this study are: *Helobdella stagnalis* (Linnaeus, 1758); *Bittium reticulatum* (da Costa, 1778); *Bythinella* sp.; *Rissoa splendida* Eichwald, 1830; *Halacaridae* g. sp.; *Hydryphanthes* sp.; *Hydryphanthes* (Polyhydryphanthes) sp.; *Orchestia* bottae Milne Edwards, 1840; *Gomphus* sp.; *Corixa panzeri* (Fieber, 1848); *Hesperocorixa linnaei* (Fieber, 1848); *Agabus* sp.; Leptoceridae g. sp.; Tipuliidae g. sp. According values of Biotic index, all freshwater bodies had "moderate"ecological status (BI=2.5 or 3). According to the values of the M-AMBI index, the brackish water bodies were characterized by a "good" or "high" ecological status (M-AMBI=0.62 – 0.90), while the hypersaline water bodies were determined by a "bad" ecological status (M-AMBI=0.31).

Conclusions:

1. Following the analysis of the physicochemical parameters, it was found that the environmental conditions are suitable for the development of invertebrates.

2. The ecological state in the Atanasovsko Lake was mostly "moderate" in freshwater bodies and mostly "good" in the brackish water bodies according to the biotic indices.

3. In general, we may conclude that the summarized ecological status of the lake is appropriate and acceptable with respect to the environmental conditions. The only case of deterioration is not due to anthropogenic pollution but mainly to the high salinity of the water, which limits the occurrence even of saline invertebrates.

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STUDY ON THE MICROSTRUCTURE OF LEAF SURFACE, LIPIDS COMPOSITION, PIGMENTS SPECTRUM AND HORMONE STATUS OF *ERYNGIUM MARITIMUM* L.

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Abstract

The aim was to examine the microstructure of the leaf surface, lipids composition, pigment spectrum and hormone status of the halophyte *Eryngium maritimum* L.

Materials and Methods: The plants were collected near the Pomorie Lake (Bulgaria). The leaf surface was studied with scanning electron microscope (JEOL JSM-6060 LA). The isolation of the main lipid classes and the analysis of their fatty acid composition were performed using thin-layer and gas chromatographic techniques. The spectra of the photosynthetic pigments were recorded using a spectrophotometer Spekord M-40. The qualitative and quantitative analysis of ABA and IAA were performed using high performance liquid chromatography (HPLC).

Results and Conclusion: It was shown that its existence in saline and dry soils is provided among others adaptive mechanisms by the specific microstructure of its leaf, adaxial and abaxial surfaces of which have well-developed cuticle and stomata slit placed below the surface of the epidermis. The presence of a large amount of saturated fatty acids provides a decrease of the membrane permeability and better resistance against soil salinity. The key role in its photosynthetic activity plays chlorophyll *a*. The high level of active ABA is correlated with salt tolerance of *E. maritimum*. The high level of conjugated form of IAA is an indication of the limited activity of the hormone.

Keywords: *Eryngium maritimum* L., halophyte, lipids, phytohormones, surface ultrastructure

Introduction

Saline soils occupy 25% of the Earth's surface. Under optimal amounts salt functions as a mineral nutrient, while at high concentrations it becomes a stress factor. High concentrations

of salts have a toxic effect; adversely affecting the water balance of the plant. In the roots outer cells are damaged, which are in direct contact with the salt. In the stem the cells conduction system is damaged, in which the solution of salts moves to aboveground organs. Halophytes – plants that are evolutionarily formed in saline soils and are adapted to living in such conditions, can withstand salinity level up to 300 mmol. Unlike glykophytes – plants from unsalted places, halophytes are adapting to life in excess salt concentration. The aim of our study was to examine the microstructure of the leaf surface, lipids composition, pigment spectrum and hormone status of the halophyte *Eryngium maritimum* L., which is one of the most salt-tolerant plants that can accumulate in its vacuoles up to 10% of the water content of the whole plant.

Materials and Methods

Eryngium maritimum L. is a typical perennial plant of the coastal dunes: in open sand at the tops of shores, on the strandline, in foredunes, in more stabilized open sandy turf. It occurs also on shingle and sometimes on coastal wasteground. It is a member of the carrot family (*Apiaceae*, formerly *Umbelliferae*), but the flowers are aggregated into a dense, rounded inflorescence rather than the flat-topped umbels so typical of the family [1]. It shows a number of xeromorphic adaptations to survive water-loss, including its spiny leaves and bracts to deter grazing animals and its thick, waxy cuticle. The root system is able to grow down a meter or more into the sand. The cuticle probably also protects the plant from the erosive effects of blown sand. Like some other psammophytes (plants of open, sandy habitats) it is able to grow up through accreted sand, the shoot system being stimulated into renewed growth by burial. Samples of *E. maritimum* were collected in August 2014 from the sand dunes near the Pomorie Lake (soil salinity 330-350 mg salts in 100 g soil).

The leaf surface of *E. maritimum* was studied by means of a scanning electron microscope (JEOL JSM-6060 LA). Plant material was fixed in 70° ethyl alcohol. Fixed samples were dehydrated in ethyl alcohol solution with increasing concentrations. Following the treatment with absolute alcohol, samples were placed on brass objective tables and adhered there using adhesive tape, kept there for some time to reach air-dry condition and covered with a layer of gold in the ion coating chamber to provide conductivity. Structure dimensions on micro-photos were measured using the program UTHSCSA Image Tool 3.0, involving a scale bar set up by the instrument on picture. For lipid analysis small particles from the aerial parts were extracted with chloroform-methanol (2:1) to obtain the total lipophilic extract [2]. The isolation of the main lipid classes and the analysis of their fatty acid composition were performed using thin-layer and gas chromatographic techniques [3]. Photosynthetic pigments were extracted with 80% aqueous acetone [4]. Spectra were recorded using a spectrophotometer Spekord M-40. Extraction of hormones and determination of free and conjugated forms of abscisic acid (ABA) and indole-3-acetic acid (IAA) were performed according to the method [5]. Qualitative and quantitative analysis of ABA and IAA were performed using high performance liquid chromatography (HPLC) on a liquid chromatograph Agilent 1200 LC system with diode array detector G 1315 B (USA), column Eclipse XDB-C 18, with the parameters 4.6 x 150 mm, size of particles - 5 microns. IAA and ABA were determined at 280 and 254 nm respectively. Elution of hormones was performed at a rate 0.5 ml/min in the solvent system methanol:

water: acetic acid (59: 40: 1) in online regime. We use un-labeled IAA and (±) cis-, trans-ABA (Sigma, USA) and the standard addition method of quantification. Chromatograms were calculated using the software ChemStation (version 3.1 V.) in offline mode. Experiments were carried out in three biological and five analytical replicates. Digital materials were processed statistically using the programs MS Excel 2003 and Origin 6.0. Significant differences were assessed by Student's criterion, using a 5% level of significance ($P \leq 0.05$).

Results and Discussion

The leaf surface of *E. maritimum* is flat, leathery and thorn-toothed. The abaxial (lower) surface is covered with stomata having a simple structure. The stomata openings are below the epidermis level (Fig. 1). The number of openings per 1 mm^2 reaches 128±4.5. An average diameter of the stoma opening is 11.9±0.74 μm. The cuticle cells on the leaf abaxial surface are covered with a considerable layer of loose wax that produces an effect of mat surface (Fig. 2). The epidermis of the upper (adaxial) surface is covered with a layer of loose wax that, along with a considerable thickness of the cuticle layer, produces an effect of a glossy surface of the leaf lamina (Fig. 3). Stomata are less embedded in the epidermis surface than it occurs on the abaxial side (Fig. 4). The number of stomata per 1 mm^2 is 123±2.5, the diameter of the stoma opening – 12.1±1.7 μm. So, the stomata number and their dimensions on the abaxial and adaxial leaf lamina surface of *E. maritimum* do not practically differ. According to the literature data stomata occur on the both leaf surfaces in many angiosperms. In xenomorphic plants the morphological adaptation to dry conditions is achieved through the arrangement of sunken stomata [6]. Thus, the existence in saline and dry soils of true halophytes *E. maritimum* provided among others adaptive mechanisms by specific microstructure of leaf, adaxial and abaxial surface of which have well-developed cuticle and stomata slit placed below the surface of the epidermis.

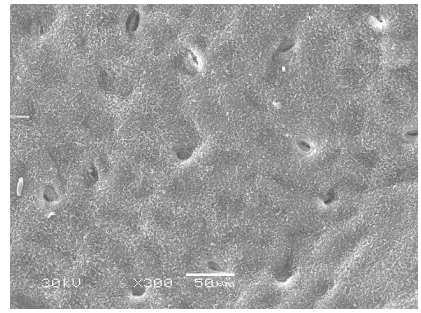

Fig. 1. The abaxial leaf lamina surface of *E. maritimum*

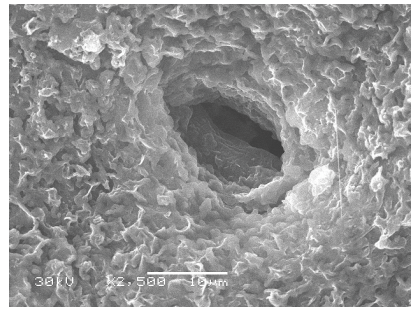

Fig. 2. Stoma on abaxial leaf lamina surface of *E. maritimum*

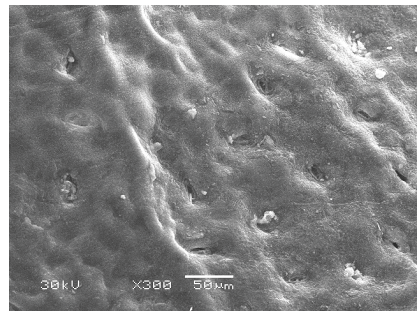

Fig. 3. The adaxial leaf lamina surface of *E. maritimum*

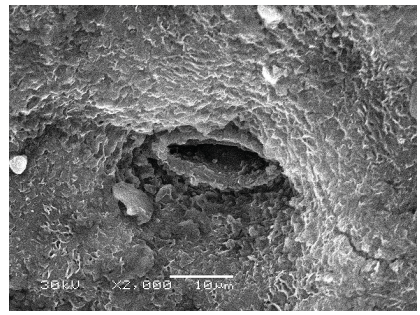

Fig. 4. Stoma on adaxial leaf lamina surface of *E. maritimum*

It was shown that the main fatty acids are palmitic, linoleic and linolenic, with the linoleic 18:2 predominating (Table 1). The content of 16:1 acid is very low. The amount of the saturated fatty acid is high in comparison with other terrestrial plants. The same was observed in other halophyte plants [7, 8] and seems to be typical for the salt stressed plants.

Table 1. Fatty acid profiles of main lipid classes of *Eryngium maritimum*

Lipids	Fatty acids (wt % of total)								
Lipid class	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	22:0
Triacylglycerols (TAG)	0.6	26.5	1.8	3.5	14.1	39.0	9.4	2.5	2.5
Monogalactosyl diacylglycerols (MGDG)	2.0	17.1	0.4	10.9	11.1	19.1	39.3	-	-
Digalactosyl diacylglycerols (DGDG)	3.5	28.4	0.2	5.1	8.6	35.1	19.2	-	-
Sulphoquinovosyl diacylglycerols (SQDG)	3.3	27.5	0.5	5.4	5.1	30.6	27.4	-	-
Phospholipids (PL)	4.4	35.2	0.7	5.2	6.7	39.5	8.2	-	-

The triacylglycerol (TAG) content is very high (60% of total) TAG are not membrane constituents, but their content is relatively high in some other halophyte plants from this region [8] (Fig. 5). The main glycolipid class is sulphoquinovosyl diacylglycerols (SQDG) – 12%, followed with digalactosyl diacylglycerols (DGDG) – 9.3% and monogalactosyl diacylglycerols (MGDG) – 8%. Contrary to the terrestrial plants, the content of phospholipids (PL) – 10.7 %, is relatively low. Almost half of the fatty acids in PL are saturated. PL are the main lipid constituents in lipid membranes, so the high content of saturated acids leads to the decrease of the membrane fluidity and to the better resistance to harmful environment. Long-chain fatty acids (20:0 and 22:0) were found only in the TAG. These acids are frequently found in the lipids of roots and probably have a preventive role for the plants.

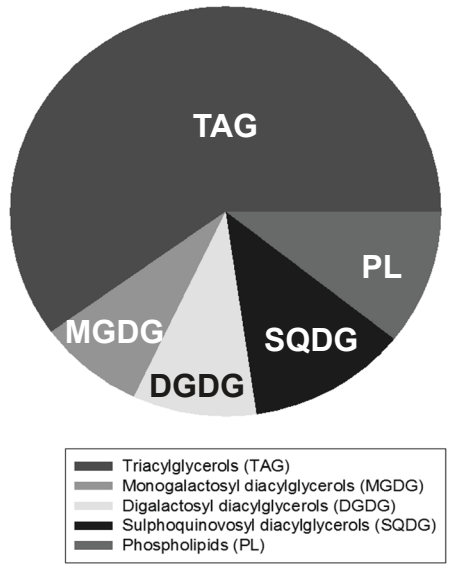

Fig. 5. Main lipid classes (% of total) in *Eryngium maritimum*

The higher plant pigment complex includes chlorophylls and carotenoids that transfer an additional energy to chlorophylls (light-harvesting function) and remove the excessive energy from chlorophylls as well (light-protecting function) [9]. It was shown that in the leaf of *E. maritimum* the level of chlorophylls was more lower as compared with the same in mesophyte plant *Triticum aestivum* L. (Fig. 6).

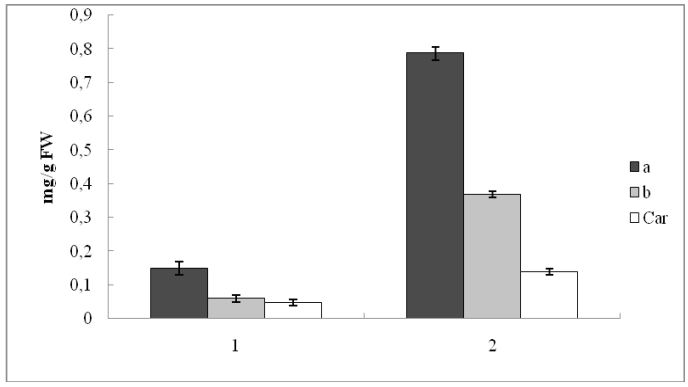

Fig. 6. Pigments content in leaves of *E. maritimum* (1) and leaves of winter wheat seedlings (2) [mg/g FW]

Also the content of carotenoids was twice lower than in winter wheat leaf [10]. On the whole the ratio between pigments was similar to that in winter wheat, but the amounts of chlorophylls and carotenoids were lower (Fig. 7). Thus, the key role in the photosynthetic activity of true halophyte *E. maritimum* plays chlorophyll *a*. At the same time a high amount of carotenoids (as compared with the amount of chlorophylls) points out that these pigments have a light-collecting function and could transfer an additional energy to chlorophylls [11].

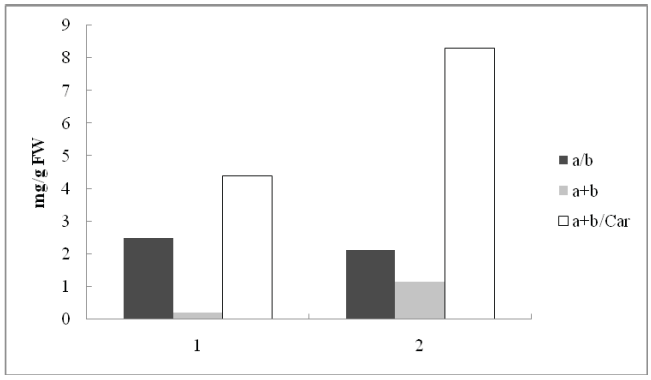

Fig. 7. The ratio of pigments classes in leaves of *E. maritimum* (1) and leaves of winter wheat seedlings (2) [mg/g FW]

Phytohormones play a key role in the regulation of growth, development and resistance of plants [11]. ABA is one of the major plant hormones involved in plant adaptation [12]. In plant tissues, it is present in free and conjugated forms [13]. IAA– a natural auxin, the major function of which is the regulation of growth processes. In the bound state IAA loses its activity [14]. We have shown that the pool of endogenous ABA was higher than that of IAA and the free form of ABA and conjugated form of IAA prevailed (Fig. 8).

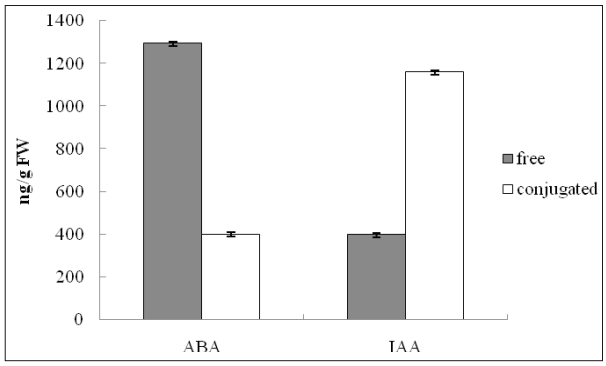

Fig. 8. The content of free and conjugated ABA and IAA in *Eryngium maritimum* L. leaves [ng/g FW]

The high level of active ABA is correlated with the salt tolerance of *E. maritimum,* its ability to survive and grow in stress conditions. The high level of conjugated form of IAA demonstrated that the activity of the hormone is limited.

Thus, the existence in saline and dry soils of the true halophyte *E. maritimum,* provid among others adaptive mechanisms by the specific microstructure of the leafs, adaxial and abaxial surfaces, which have well-developed cuticle and stomata slit placed below the surface of the epidermis. The presence of a large amount of saturated fatty acids provides the decrease of the membrane permeability and better resistance against soil salinity. Although the key role in the photosynthetic activity plays chlorophyll *a*, a high amount of carotenoids (as compared with the amount of chlorophylls) points out that these pigments have a light-collecting function and could transfer an additional energy to chlorophylls. The high level of active ABA correlates with the salt tolerance of *E. maritimum*, its ability to survive and grow in stress conditions. The high level of conjugated form of IAA demonstrates that the activity of the hormone is limited.

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References:

1. Minasiewicz J., Borzyszkowska S., Żółkoś K., Bloch-Orłowska J., Afranowicz R. Biodiv. Res. Conserv., 24, 2011, 39–48.

2. Blight E. G., Dyer W. J. Can. J. Biochem. Physiol. 37, 1959, 911-917.
3. Christie W. W. Lipid analysis. Pergamon press. Oxford, 1973, p. 352
4. Wellburn A. J. Plant Physiol., 144, 1994, 307-313.
5. Kosakivska I. V., Voytenko L. V., Likhnyovskiy R. V., Ustinova A. Y. Genetics and Plant Physiology., 4 (3-4), 2014, 117-125.
6. Rudall P. J. Anatomy of flowering plants, Camb. Univ. Press. Cambridge, 2007, p. 159.
7. Ivanova A., Nechev J., Stefanov K. Compt. rend. Acad. bulg. Sci., 53, 2000, 83-86.
8. Ivanova A., Khozin-Goldberg I., Kamenarska Z., Nechev J., Cohen Z., Popov S., Stefanov K. Z. Naturforsch. 58c, 2003, 783-788.
9. Hormaetxe K., Becerril J.M., Fleck I., Pintó M., García-Plazaola J. I. J. Exp. Bot., 56, 2005. 2629-2636.
10. Babenko L. M., Kosakivska I. V., Akimov Yu. A., Klymchuk D. O., Skaternya T. D. Genetics and Plant Physiology., 4 (1–2), 2014, 117-125.
11. Andersone U., Druva-Lūsīte L., Ieviņa B., Karlsons A., Ņečajeva J., Samsone I., Ievinsh G. J. Coast Conserv., 15, 509-522.
12. Peleg Z., Blumwald E. Current Opinion Plant Biol., 14, 2011, 290–295.
13. Wilkinson S., Davies W. J. Plant Cell Environ., 25 (1), 2002, 195-210.
14. Zheng-Yi Xu, Yun-Joo Y., Inhwan H., In: Abscisic acid: Metabolism, Transport and Signaling (Da-Peng Zhang, Ed.) Springer, Dordrecht, 2014, 77-87.
15. Del Pozo J. C., Lopez Mataz M. A., Ramirez-Parra E., Gutierrez C. Physiol. Plant., 123, 2005, 173-183.

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BIOCHEMICAL MARKERS FOR ASSESSMENT OF OXIDATIVE STRESS IN TWO GENOTYPES *PHASEOLUS VULGARIS* L.

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Abstract

Aims: to evaluate the level of PEG induced oxidative stress in two common bean (*Phaseolus vulgaris* L.) genotypes using well known biochemical endpoints; to identify the most reliable biochemical marker/s for drought tolerance.

Materials and Methods: *P. vulgaris* L. the cultivar Dobrudjanski 2 (D2) and its mutant line 4 (L4) derived by the methods of chemical mutagenesis were used. Germination was performed at standard conditions in a

phytocamera. Seedlings, grown on Sager-Granick medium to the phase of the third leaf, were split into three groups: untreated and treated with 8% and 16% polyethylene glycol (PEG - MW 10 000) for 24h. Three biochemical endpoints were used: malondialdehyde (MDA), total hydrogen peroxides (H_2O_2) and proline (Pro).

Statistical Analysis of Data: The statistical assessment of the results was calculated by Students t-test and one-way ANOVA analysis (GraphPad Prism version 6.04).

Results: Our data demonstrate no differences between the constitutive levels of MDA, H_2O_2 and Pro contents and well expressed variation between H_2O_2 and Pro levels after PEG treatment depending on the genotype and concentration. In stress conditions the higher H_2O_2 was measured for mutant line 4 and Pro overproduction for cultivar D2.

Conclusions: Cultivar D2 is characterized with the higher level of induced Pro, lower H_2O_2 content and could be recommended as more drought tolerant in comparison with its mutant line 4. Pro is reliable and sensitive marker for the distinguishing of drought tolerance even in genetically closely related genotypes. Pro accumulation could be used in short-term experiments for the rapid diagnose of the presence of oxidative stress.

Keywords: biochemical markers, drought stress, *Phaseolus vulgaris* L.

Introduction

One of the major sources of environmental stress is drought because it can induce the excessive accumulation of ROS [1]. Overproduction of ROS could cause serious damages in proteins, lipids, carbohydrates, DNA etc. As a result, drought can affect growth rate and can result to the reduced crops yield production [2].

As a model organism *Phaseolus vulgaris* L. (common bean) was used. Common bean is a convenient model in environmental mutagenesis studies because a lot of cultivars and mutant lines are available. On the other hand, common bean is a very important, widespread food legume, important agro - economic product cultivated on the Balkan Peninsula and Bulgaria with a rich content of proteins, vitamins, minerals, fibers etc. One of the major questions open till now concerns approaches used in a process of evaluation of a genotype's susceptibility to environmental stress and markers. As a common strategy genotypes that differ in their susceptibility to environmental stress are applied [3, 4, 5].

Here we hypothesize that genetically closely related genotypes would have approximately similar response to oxidative stress and could be used as an appropriate model for the identification of very sensitive and reliable markers in order to distinguish their stress response.

This study aims: to evaluate the level of PEG induced oxidative stress in two common bean genotypes (*P. vulgaris* L.) using well-known biochemical endpoints;

to identify the most reliable biochemical marker/s for drought tolerance of two closely related genotypes *P. vulgaris* L.

Materials and Methods

P. vulgaris L. the cultivar Dobrudjanski 2 (D2) and the mutant line 4 (L4) derived by the methods of chemical mutagenesis [6] were kindly provided by prof. Svetleva from

the Plovdiv Agricultural University. Germination was performed at standard conditions (t = 23±0.2°C, moisture 70%, in continuous light) in a phytocamera (Growth Chamber GC 400). After that, seedlings were grown on Sager-Granick (SG) medium [7] to the phase of the third leaf. The composition of the SG medium is similar to that of the Knop medium commonly used for growing of higher plants.

Plants were split into three groups: untreated and treated with 8% and 16% polyethylene glycol (PEG - MW 10 000) for 24h. Untreated plants were kept on SG medium at the same experimental conditions. Two concentrations of PEG - 8% and 16%, respectively, were applied as an inductor of drought stress because of several main reasons: PEG molecules are inert, non-ionic; PEG maintains an uniform water potential throughout the experimental period; PEG causes oxidative damage to plant growth through excessive generation of reactive oxygen species.

Several well-known biochemical endpoints were used – malondialdehyde (MDA) [8], total hydrogen peroxides (H_2O_2) [9] and proline (Pro) [10].

Statistical Analysis of Data: The statistical assessment of the results was calculated by the Students t-test and one-way ANOVA analysis (GraphPad Prism version 6.04).

Results and Discussion

In the present work, we hypothesize that on the basis of genetically closely related genotypes of *P. vulgaris* L. very sensitive and reliable markers could be successfully identified.

Till now, two main strategies have been proposed to assess organisms' resistance/ susceptibility to environmentally induced stress: the first one involves mutant genotypes that differ in their resistance/sensitivity to oxidative stress [11, 12, 13, 14, 15, 16, 17] and the second one - transgenic plants with preliminary selected "resistant" to some environmental stimuli genes [18, 19, 20, 21].

The general approach used in our previous investigations was the characterization of genotypes with different susceptibility to oxidative stress by a complex of biochemical and molecular markers [3, 4, 5, 17].

Here a new approach is proposed: using genetically closely related genotypes of *P. vulgaris* L. we hope to identify very sensitive and reliable markers.

The oxidative stress is commonly associated with different changes at the physiological, biochemical and molecular level and depends on the duration and magnitude of the stress stimulus, experimental design, genotype and etc. [3]. In this study two genotypes of *Phaseolus vulgaris* L. and three different markers - MDA, H_2O_2 and Pro we used to assess the level of oxidative stress.

Plants have evolved many defence mechanisms against different stress factors. Such defences can be broadly classified into two categories: permanent, constitutive defences mechanisms and temporary, inducible defences mechanisms. The constitutive defences are present before stress action, while induced defences are activated only when stress occur. Constitutive levels are not dependent on the presence of external triggering factors like abiotic stress. That is why our first step was to measure the constitutive levels of MDA,

H_2O_2 and Pro. Interestingly, no statistically significant differences in the constitutive levels of MDA, H_2O_2 and Pro were found for both genotypes possibly because mutant line 4 has been derived from cultivar D2 (data not shown).

1. *Malondialdehyde (MDA) content.*

It is well known that MDA is an indicator of lipid peroxidation. As a biochemical marker for oxidative stress MDA is routinely used to assess the degree of oxidative damage induced by different stress stimuli in plants [22]. In our experiments, the induced levels of MDA were around 20-30% higher in treated samples comparing to those in controls. A very strong similarity was obtained between induced MDA levels for both genotypes - D2 and its mutant line 4 (Fig. 1). Student's t-test data demonstrate that these minor differences between MDA levels measured in both genotypes after 8% and respectively after 16% PEG are statistically significant.

This observation that MDA content could increase after the treatment with PEG was in a good agreement with results obtained by Yin et al. [23] that MDA induced contents in *Phaseolus radiatus* varieties was increased with increasing the processing time of PEG drought stress. Unfortunately, data in literature are contradictory and experiments have been performed at different experimental conditions. For example, Pandey and Chacraborty [24] found that a drought sensitive variety of *Vigna mungo* L., responded to drought stress with higher level of lipid peroxidation in comparison with tolerant varieties.

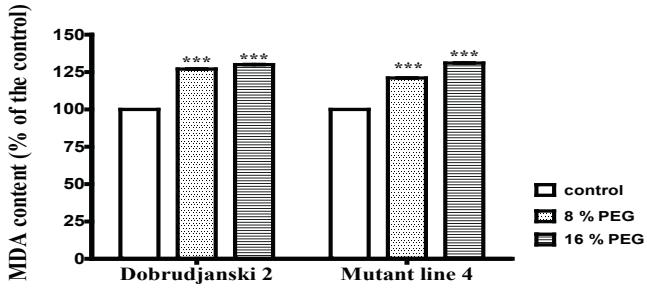

Fig. 1. MDA content depending on the PEG concentrations and *Phaseolus vulgaris* L. genotype. Data presented are mean values ± SE from at least three independent experiments. Where no error bars are evident, errors are equal or smaller than the symbols. One-way ANOVA analysis revealed statistically significant differences between: 8% treatment vs. control; 16% treatment vs. control; 8% vs. 16% treatment (*, p<0.001)**

In short, no effects of the concentration and the genotype were obtained. How we can explain this fact? At first, the similar stress responses of D2 and mutant line 4 could be explained by the fact that mutant line 4 has been derived from the cultivar D2 and both genotypes are very closely related [6, 25]. Secondly, our data are in agreement with a conclusion drawn by Yin et al. [23] that MDA content could not be used as identification index.

2. *Total hydrogen peroxides (H_2O_2) content.*

Total hydrogen peroxide was used as a second biochemical marker for oxidative stress because it is well known that H_2O_2 could play a role as a signal molecule and its higher

content could indicate occurrence of oxidative stress [22, 26]. Data are available in the literature that the magnitude of induced levels of H_2O_2 could depend on the severity of drought stress and the genotype [27, 28, 26].

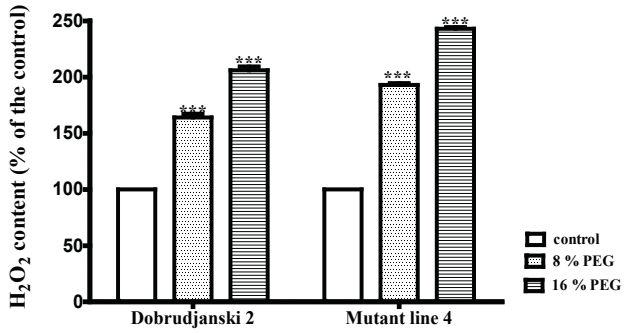

Fig. 2. H_2O_2 content depending on the PEG concentrations and *Phaseolus vulgaris* L. genotype. Data presented are mean values ± SE from at least three independent experiments. Where no error bars are evident, errors are equal or smaller than the symbols. One-way ANOVA data - statistically significant differences between: 8% treatment vs. control; 16% treatment vs. control; 8% vs. 16% treatment (*, p<0.001)**

In our experiments, the induced levels of H_2O_2 after PEG treatment were around 2 fold higher comparing with that in control samples (Fig. 2). Both effects of concentrations and genotype were found. Results indicated that an oxidative stress was induced at experimental conditions used by us in studied genotypes. About 30-35% higher content of H_2O_2 was obtained for mutant line 4 comparing with that for the cultivar D2. Based on the statement of [27] that the most increased production of H_2O_2 was more likely typical for sensitive cultivar, than for the drought tolerant cultivars we can speculate that mutant line 4 was with higher susceptibility to drought stress than the cultivar D2.

3. *Proline (Pro) content.*

It is well known that Pro is one of the most commonly known osmolytes in drought stressed plants that allows plants to survive [31, 5, 13]. Pro has been proposed as a biochemical marker for environmental stress, including drought stress [29, 30]. Our next step was to measure Pro contents induced by PEG treatment. An increasing of Pro contents was found as a result of PEG treatment for both genotypes (Fig. 3). These results are in a good agreement with results obtained by [24, 30] that an enhancement of Pro content has been obtained at different experimental conditions, using various genotypes and plant developmental stages.

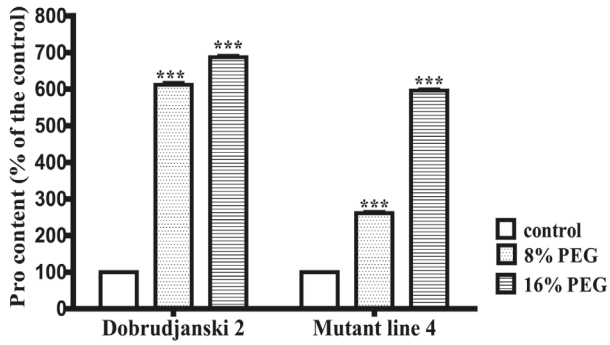

Fig. 3. Pro content depending on the PEG concentrations and *Phaseolus vulgaris* L. genotype. Data presented are mean values ± SE from at least three independent experiments. Where no error bars are evident, errors are equal or smaller than the symbols. One-way ANOVA analysis revealed statistically significant differences between: 8% treatment vs control; 16% treatment vs control; 8% vs. 16% treatment (*, p<0.001)**

In our experiments, both concentrations of PEG were found to induce Pro accumulation in cultivar Dobrudjanski 2 and mutant line 4. A well-expressed relationship between concentrations and Pro accumulation was found. The most pronounced overproduction was measured for D2 after the treatment with both concentrations. Pro accumulation is commonly associated with its defense functions [28]. Because higher Pro accumulation has been observed in drought tolerant plants in comparison with sensitive plants [32, 24], Pro accumulation has been advocated as a parameter for the selection of a drought tolerant genotype of black gram and green gram [30].

Based on these statements it could be speculated that D2 is more tolerant to PEG induced stress than mutant line 4.

Conclusions

1. Proline is a reliable and sensitive marker for the distinguishing of drought tolerance even in genetically closely related genotypes.

2. Proline accumulation could be used in short-term experiments for rapid diagnose of the presence oxidative stress.

3. Cultivar Dobrudjanski 2 is characterized with higher induced level of Proline accumulation, lower H_2O_2 content and could be recommended as more drought tolerant in comparison with its mutant line 4.

Acknowledgements: This study was funded by the projects: DDVU_02/87 "Complex morphometric, physiological, biochemical and molecular assessment of drought tolerance in Bulgarian common bean genotypes (*Phaseolus vulgaris* L.)", "Biochemical and molecular markers of drought tolerance in Bulgarian common bean genotypes" – scientific cooperation between RAS and BAS and „Ecological and genetic risk: methods and strategies for overcoming"– BAS.

References:

1. Boughalleb F. and Mhamdi M., Agricultural Journal, 6 (6), 2011, 378-391.

2. Asfaw A., Wageningen University, The Netherlands, 2011, p.187.

3. Chankova S. G., Kapchina V. M. and Stoyanova D., Rad Biol & Radioecology, 2000, 40 (5): 535-543.

4. Chankova S., Mitrovska Z., Miteva D., Oleskina Y. P. and Yurina N. P., Gene, 516, 2013, 184–189.

5. Chankova S. G., Dimova E. G., Mitrovska Z., Miteva D., Mokerova D. V., Yonova P. A. and Yurina N. P. Ecotoxicology and Environmental Safety, 2014, 101, 131–137.

6. Svetleva D., Krastev V., Dimova D., Mitrovska Z., Miteva D., Parvanova P. and Chankova S., JCEA, 13 (2), 2012, 349-361.

7. Harris E. The Clamydomonas sourcebook. Second edition. Introduction to Chlamydomonas and its laboratory use. Academic press is an imprint of Elsevier, 1, 2009, 242-248. 444 pp

8. Dhindsa R. S., Plumb-Dhindsa P. and Jhorpe T. A., J. Exp. Botany, 32, 1981, 93-101.

9. Heath R. L. and Packer L., Arch. Biochem. Biophys., 125, 1968, 189-198.

10. Bates L. S., Waldren R. P. and Teare I. D., Plant and Soil, 29, 1973, 205-207.

11. Teoule R., Cadet J. / Eds J. Hutterman, W. Kohnlein, R. Teoule, A. Bertinshamps. Berlin, Heidelberg: New York: Springer-Verlag, 1978, 171-203.

12. Von Sountag C., Schulte-Frochlinde D. / Eds J. Hutterman, W. Kohnlein, R. Teoule, A. Bertinshamps. Berlin, Heidelberg: New York: Springer-Verlag, 1978, 204-226.

13. Shevchenko V. A. Radiation Genetics of Unicellular Algae (in Russ.) Moskva: Nauka, 1979.

14. Foyer C. H., Harbinson J. / Eds. C. H. Foyer, P. M. Mullineux. Boca Raton, Fl.: CRS Press, 1994, 1-42.

15. Verslues P. E., Sharp R. E., Plant Physiology, 119, 1999, 1349-1360.

16. Chankova S. G., Kapchina V. M. and Stoyanova D. Rad Biol & Radioecology, 2000, 40 (5), 535-543.

17. Chankova S. G. and Yurina N. P., Radiobiology and Environmental Security, NATO Science for Peace and Security, Series C: Environmental Security, Eds. Mothersill C., Korogodina V., Seymour C.B., 1st Edition 2011, VII, 2012, pages 413, 235 illus, ISBN 978 94 007 1938 5.

18. Ouvrard O., Cellier F., Ferrare K., Tousch D., Lamaze T., Dupuis J. M. and Casse-Delbart F., Plant Mol. Biol., 31, 1996, 819–829.

19. Khomdram S., Barthakur S. and Devi G. A. S., Ann. Agric. Res. New Series, 34 (3), 2013, 189-196.

20. El Rabey, H. A., Khan, J. A, Abulnaja, K. O. and Al-Malki, A. L., African Journal of Biotechnology, 11(40), 2012, 9527-9533, online at http://www.academicjournals.org/AJB, DOI: 10.5897/AJB12.856

21. Yooyongwech S., Cha-um S., Supaibulwatana K. POJ, 5(6), 2012, 597-603, ISSN:1836-3644.

22. Labudda, M. E-wudawnictwo,Poland, 2013.
23. Yin Z.C., Liang J., Hao X.Y., Lu H., Hao J. J. and Yin F. X., American Journal of Plant Sciences, 6, 2015, 785-798. http://dx.doi.org/10.4236/ajps.2015.65084
24. Pandey S. and Chakraborty D., Plant Science Today, 2 (2), 2015, 60-64. doi:10.14719/pst.2015.2.2.99
25. Apostolova E., Krastev V., Yahubyan G., Svetleva D., Parvanova,P., Mitrovska Z. and Chankova,S., International Journal of Agricultural Sciences and Natural Resources., 1 (4), 2014, 50-57.
26. Yang H. Y., Zhang C. H., Wu W. L., Li W. L., Wei Y. L., and Dong S. S., Russ. J. Plant Physiol., 62 (4), 2015, 472–479, ISSN 1021-4437.
27. Shi Sh., Fan M., Iwama K., Li F., Zhang Z. and Jia, L., IJPP, 9 (2), 2015, ISSN: 1735-6814 (Print), 1735-8043 (Online), www.ijpp.info
28. Siddiqui M. H., Al-Khaishany M. Y., Al-Qutami M. A., Al-Whaibi M. H., Grover A., Ali H. M., Al-Wahibi M. S. and Bukhari N. A., Int. J. Mol. Sci., 16, 2015, 10214-10227; doi:10.3390/ijms160510214.
29. Routley D. G., Crop Sci., 6, 1966, 358-361.
30. Baroowa B. and Gogoi N., ILNS, 42, 2015, 1-12, doi: 10.18052/www.scipress.com/ILNS.42.1
31. Shinde B. P. and Thakur J., International Journal of Current Microbiology and Applied Sciences, 4 (1), 2015, 809-821; ISSN: 2319-7706; http://www.ijcmas.com/.
32. Turkan I., Bor M., Ozdemir F., and Koca H., Plant Science, 168, 2005, 223–231. doi: 10.1016/j.plantsci. 2004.07.032

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HIGH-LIGHT INDUCIBLE PROTEINS HLIA/HLIB ARE ESSENTIAL FOR LIGHT STRESS-ADAPTATION IN CYANOBACTERIUM *SYNECHOCYSTIS* PCC 6803

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Abstract

Aims: Hlip (high-light inducible proteins) are important in the protection of the photosynthetic apparatus of cyanobacteria from light stress. However, the interaction of these proteins with chlorophyll-protein complexes of thylakoids remains unclear.

Materials and Methods: the association of HliA/HliB stress proteins with photosystem 1 (PS1) complexes of the cyanobacterium *Synechocystis* PCC 6803 has been studied for understanding of their function. HliA/HliB are determined using gel-electrophoresis and Western blotting.

Main results: it has been shown that stress-induced HliA/HliB proteins are associated with the PS1 trimers in wild type cells grown at moderate light condition (40 μmol photons $m^{-2}s^{-1}$). The content of these proteins increased 1.7-fold after light stress (150 μmol photons $m^{-2}s^{-1}$) for 1 h. In the absence of PS1 trimers (ΔpsaL mutant) the HliA/B proteins are associated with PS1 monomers and photosystem 2 (PS2) complex. The HliA/HliB proteins are associated with the PS1 monomers but not with the PS1 trimers in the *Synechocystis* PS2-less mutant grown at 5 μmol photons $m^{-2}s^{-1}$; the Hli proteins content associated with PS1 monomers increased 1.2 times after light stress. The HliA/HliB proteins have not been detected in wild type cells of cyanobacterium grown in glucose-supplemented medium at 5 μmol photons $m^{-2}s^{-1}$, but light stress induces the synthesis of stress proteins associated with PS1 trimers.

Conclusion: thus, for the first time, the association of the HliA/HliB proteins not only with PS1 trimers, but also with PS1 monomers is shown, that presupposes the universal role of these proteins in protecting of the photosynthetic apparatus from excess light. The characterization of stress-responsive proteins will provide new insights into the understanding of adaptation mechanisms.

Keywords: high-light inducible proteins HliA/HliB, light stress, cyanobacteria

Introduction

Light is the source of energy for photosynthetic organisms; however intense light is dangerous for the photosynthetic apparatus because of the formation of reactive oxygen species [1, 2]. During the evolution the phototrophs have created various acclimation responses to high-light conditions. Among the defense mechanisms, the important role belongs to photoprotective proteins, the family of high light-induced proteins (Hlip's), the widespread short-lived proteins in thylakoid membranes of cyanobacteria. These proteins are the evolutionary ancestors of the light-harvesting complexes (LHC) of higher plants [3, 4].

Although currently accumulated data implies the importance of Hlip's proteins for survival under high light, the data about the function and localization of Hlip's are highly controversial [5].

The aim of this work was to study the localization of the HlipA/B proteins in the trimers and monomers of photosystem 1 (PS1) of the cyanobacterium *Synechocystis*.

Materials and Methods

Growth conditions. The object of the study were the cyanobacterium *Synechocystis* sp. PCC 6803: wild-type cells, PS2-less mutant (ΔpsbDI, ΔpsbDII, ΔpsbC) and PS1 trimers–less mutant (ΔpsaL) [6].

Cells were cultivated in BG-11 medium [7] at 28°C. The culture was bubbled with air under normal light conditions (40 μmol photons $m^{-2}s^{-1}$) and under stress conditions (150 μmol photons $m^{-2}s^{-1}$, 1 h). PS2-less mutant cells were grown at low light intensity (5 μmol photons $m^{-2}s^{-1}$) and supplemented with 5 mM glucose, and antibiotics (chloramphenicol 20 mg/ml, erythromycin 15 mg/ml, spectinomycin 20 mg/ml).

Thylakoid membrane preparation and fractionation of membrane protein complexes. Thylakoid membranes from the different *Synechocystis* strains were isolated as described by Shubin et al. [8] with modification. Briefly, the cells were disrupted using a French-press (three times for each sample), or mechanically with quartz sand in a medium A (50 mM 3-(N-morpholino)propanesulfonic acid, pH 7.0; 0.4 mM sucrose; 10 mM NaCl; 1 mM phenylmethylsulfonyl fluoride) for the isolation of thylakoid membranes. The homogenate was centrifuged at 5000 g for 10 min to remove unbroken cells, cellular debris, and quartz sand. The membranes in the supernatant were precipitated by centrifugation at 50 000 g at 4°C for 60 min. After washing with medium A to remove any remaining phycobilisomes, the membranes were resuspended in medium A to a chlorophyll *a* concentration of 1 mg/ml. To fractionate the membrane protein complexes 10% dodecyl maltoside was added to the thylakoid membranes to achieve a detergent to chlorophyll ratio of 15:1. The membrane was solubilized at 4°C for 30 min. After incubation the lysate was centrifuged at 18 000 g for 10 min. The supernatant was applied to a column of DEAE-Toyopearl 650M (1.5 x 20 cm) pre-equilibrated medium A. The column was washed with medium A and elution of chlorophyll-protein complexes with a linear gradient of NaCl (0 - 300 mM) in the medium A. The absorbance of fractions was measured at 678 nm. PS2 complexes and PS1 trimers and monomers fractions were pooled and proteins were precipitated with cold acetone at -20^{0}C.

Proteins fractionation by polyacrylamide gel electrophoresis, Western blotting. Proteins were fractionated by PAGE in the presence of SDS-Na. The protein content was determined by the method of Bradford [9]. Before applying, the samples were heated at 95^{0}C for 10 min and then centrifuged at 18 000 g for 10 min. SDS-PAGE wad performed as described [10]. Electrophoresis was performed in a Tris-glycine buffer (25 mM Tris, 250 mM Gly, 0.1% SDS, pH 7.5). Proteins were separated, transferred onto nitrocellulose membrane (pore size 0.45μm) in a blotting Tris-glycine transfer buffer (25 mM Tris, 250 mM Gly, 20% ethanol, 0.02% SDS, pH 7.5) for 1h at 200 mA. Then the membrane was placed in a blocking buffer TBST (50 mM Tris/HCl, 200 mM NaCl, 0.1% Tween 20, pH 7.5) supplemented with 5% dry milk for 1h at 4°C and added to the primary antibody against HliA/B at 1/4000 dilution (Abcam, USA). The membrane was incubated with the antibody overnight at 4°C and with constant stirring. A secondary antibodies were used goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10 000) (AgriSera, Sweden). 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

Association HlipA/B with PSI trimers in wild-type cells of *Synechocystis*

Cells of wild-type and the PS2-less mutant (ΔPsbDI, ΔPsbDII, ΔPsbC) of *Synechocystis* were grown at normal light (40 μmol photons $m^{-2}s^{-1}$) and stress light (150 μmol photons $m^{-2}s^{-1}$). Then the PS1 trimer fraction and a fraction containing the PS1 monomers and PS2 complexes were isolated (Fig. 1).

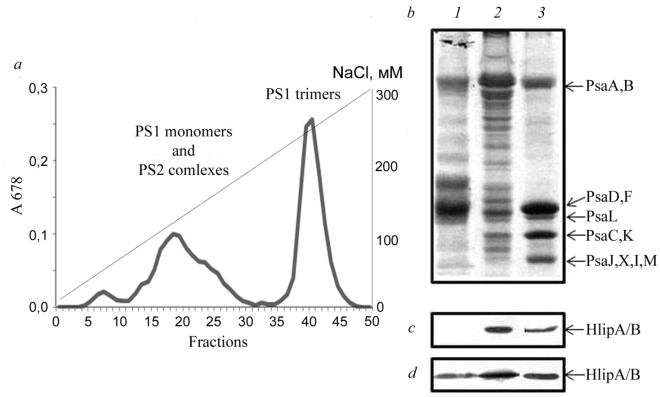

Fig. 1. Association of proteins HlipA/B with PS1 trimers and monomers isolated from wild-type cells *Synechocystis*; a - chromatographic profile of chlorophyll-protein complexes on the anion exchange column (DEAE-650M Toyoperl); b - electrophoregram of proteins: 1 – fractions 15-20, 2 – fractions 22-26, 3 - fractions 39-44; Western blot analysis HlipA/B protein content before (c) and after (d) light stress

It was shown that the content of HlipA/B in the cells had been increased 1.5 fold after exposure to stress light. In wild-type cells grown under normal light conditions, HlipA/B proteins were associated with the PS1 trimer fraction predominantly. Under stress conditions HlipA/B proteins were associated not only with PS1 trimers but also with PS1 monomers. This conclusion was confirmed by the data obtained with the PS2-less mutant of the cyanobacterium *Synechocystis*.

Association HlipA/B with PS1 monomers in the PS2-less mutant

The PS2-less *Synechocystis* mutant containing only PS1 in the thylakoid membranes was used in order to show the association of HliA/HliB with PS1. The HlipA/B proteins were identified in the fraction of the PS1 monomers but not in the PS1 trimers (Fig. 2).

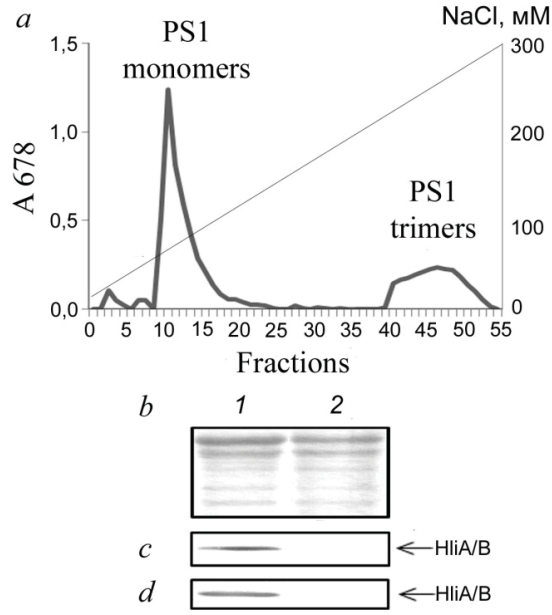

Fig. 2. Association of the proteins HlipA/B with the PS1 monomers of the mutants cells *Synechocystis*, deficient in PS2; a - profile of the fractionation of the photosystem complexes of the thylakoid membranes of the cells the mutant *Synechocystis*, b - electrophoregram of the proteins stained with Coomassie R-250: 1 – fractions 10-15, 2 – fractions 44-49. Western blot analysis of the HlipA/B protein content before (c) and after (d) exposure to light stress

This may be due to the fact that the structure of thylakoid membranes was changed by deleting the PS2; the structure of PS1 trimer could be changed too. Apparently, these changes lead to inability of HlipA/B to be associated only with the PS1 trimers.

Association of HlipA/B with the photosystems of the mutant not containing PS1 trimers

The PS1 trimers-less *Synechosystis* mutant was studied in order to establish whether the HliA/HliB proteins associated with the monomers PS1 and PS2 complex in the absence of trimers PS1. After light stress it has been shown that the HlipA/B proteins were found not only in the PS2 complex (fractions 26-30), but also in the PS1 monomers (fraction 20-24) (Fig. 3).

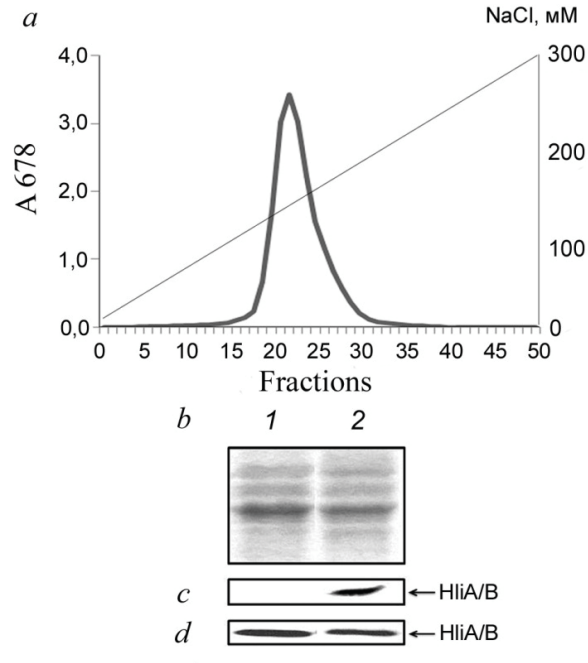

Fig. 3. Association of the HlipA/B proteins with PS1 monomers and PS2 complexes of mutants cells *Synechocystis*, which do not contain PS1 trimers; a - profile of the fractionation of the photosystems of the thylakoid membranes of cells mutant *Synechocystis*, b - electrophoregram of proteins stained with Coomassie R-250: 1 – fractions 20-24, 2 – fractions 26-30. Western blot analysis of HlipA/B protein content before (c) and after (d) light stress

The absence of PS1 trimers does not affect the association of HlipA/B proteins with the PS1 monomers and PS2 complexes.

Thus, we can conclude that the HliA/HliB proteins can contact with the main chlorophyll-protein complexes of the thylakoid membranes of cyanobacteria: with the PS1 monomers, with the PS2 complex and the PS1 trimers. The association HliA/HliB as PS1 and PS2 indicates their universal functions in the protection of the photosystems of cyanobacteria.

Conclusions:

• the HlipA/B proteins were associated with the PS1 trimer fraction predominantly;

• under stress conditions the HlipA/B proteins were associated not only with the PS1 trimers but also with PS1 monomers;

• the lack of PS1 trimers does not affect the HlipA/B protein content in PS1monomers and PS2 complexes.

• thus, for the first time, the association of HliA/HliB proteins not only with PS1 trimers, but also with PS1 monomers is shown, that presupposes the universal role of these proteins in protecting of the photosynthetic apparatus from excess light. The characterization of stress-responsive proteins will provide new insights into the understanding of adaptation mechanisms.

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References:

1. Karapetyan N. V. Photosynth. Res., 7 (2), 2008, 195-204.

2. Yurina N. P., Makerova D. V., Odintsova M. S. Russ. J. Plant Physiol., 60 (4), 2013, 577-588.

3. Sturm S., Engelken J., Gruber A., Vugrinec S., Kroth P. G., Adamska I., Lavaud J. BMC Evolutionary Biology, 2013, 13:159.

4. Heddad M., Adamska I. Comp. Funct. Genom., 3 (5), 2002, 504-510.

5. Wang Q., Jantaro S., Lu B., Majeed W., Bailey M., He Q., Plant Physiol., 147 (8), 2008, 1239-1250.

6. Shen G., Boussiba S., Vermaas W. F. J. Plant Cell, 12 (10), 1993, 1853-1863.

7. Rippka R., Deruelles J., Waterbur, J. B. Herdmann M., Stanier R. Y. J. Gen. Microbiol., 111 (1), 1979, 1-61.

8. Shubin V. V., Bezsmertnaya I. N., Karapetyan N. V. FEBS Lett., 309 (3), 1992, 340-342.

9. Bradford M. M. Anal. Biochem., 72 (3), 1976, 248–254.

10. Laemmli U. K. Nature, 227 (3437), 1970, 680-685.

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DOES WATER LEAF EXTRACT OF *PAPAVER RHOEAS* L. POSSESS ANY PRO-OXIDATIVE CAPACITY?

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Abstract

Aim: The aim of this research is to measure the pro-oxidative capacity of *Papaver rhoeas* L. (corn poppy) water leaf extract depending on the concentration's range and endpoints used.

Materials and Methods: *Chlamydomonas reinhardtii* strain 137 C+ was used as a model organism. Water leaf extract of poppy at the concentration's range of 1% to 5% was applied. Cells were treated for 1h at room temperature in the light. The amounts of MDA and H_2O_2 were determined spectrophotometrically. The values were compared with those measured in control sample - DMSO. The statistical assessment of the results was performed using t-test and one way ANOVA (GraphPad).

Results: Both concentrations 2.5% and 5% of the water leaf extract were obtained to possess slight pro-oxidative capacity measured as increased levels of H_2O_2 comparing with that in a control DMSO sample. Further, no pro-oxidative effect of the same concentrations was revealed using MDA amounts as an endpoint – the measured values were approximately equal to or lower than that of the control DMSO.

Conclusions: As a result of our experiments two main conclusions could be drawn: water leaf extract of *Papaver rhoeas* L. possess very slight or no pro-oxidative capacity depending on the concentrations applied; at least two endpoints ought to be used for such evaluation.

Keywords: pro-oxidative/anti-oxidative capacity, water leaf extract, *Papaver rhoeas* L.

Introduction

Papaver rhoeas L. (corn poppy) is an annual herb widely spread in numerous regions around the world. It belongs to the Papaveraceae family which is known as a family of flowering plants. The flowers of poppy are chiefly employed as a mild pain reliever and as a treatment for irritable coughs; it also helps to reduce nervous over-activity. The flowers and petals are anodyne, expectorant, hypnotic, slightly narcotic and sedative. *Papaver rhoeas* L. plant extract contains a lot of flavonoids: kaempferol, quercetin, luteolin and hypolaetin and glycosides 3-O-β-D-glucopyranosylquercetin (isoquercetin), 3-O-β-D-glucopyranosyl kaempferol (astragaline), 3-O-β-D-galactopyranosylkaempferol (hyperoside) [1].

These antioxidants could protect cells from free radicals, induced oxidative damage via modulation of the antioxidant enzymes activity, preventing the DNA damage and inhibiting biomolecule oxidation. They are found to provide the phenolic hydroxyl group to react with the free radicals and consequently inhibit the oxidative mechanisms that cause diseases [2].

Biochemical markers for oxidative stress

Following the present state of knowledge it is well accepted that oxidative stress could be considered as a consequence of an imbalance between oxidative and reductive processes

in the cell. Oxidative stress is induced when the physiological antioxidant defense system can no longer counteract the elevated ROS levels or as a result of the cellular inability to repair oxidative damage [3, 4]. These circumstances are manifested by an increase of lipid hydroperoxides, which are estimated through malondialdehyde (MDA) quantification. The contents of malondialdehyde (MDA) are an important marker for different types of stress, associated with metabolic changes occurring due to various stimuli that affect the bodily functions. Lipid peroxidation could result in cell toxicity, mutagenicity, and inhibition of the activity of various enzymes, DNA replication, transcription and breathing [3, 5, 6]. Oxidative stress could damage DNA comprising: breaking single or double strand base modifications, deoxyribose modifications and crosslinks formation [6]. When DNA damage is not repaired before or during replication this can lead to cell death mutation, replication errors and instability of the genome each of which is associated with the process of carcinogenesis [5, 6].

Hydrogen peroxide (H_2O_2) is a product of the peroxisome, chloroplast and oxidation reactions. That's why H_2O_2 is commonly used as another marker for oxidative stress. H_2O_2 may inactivate various macromolecules directly, but its transformation into hydroxyl radicals, with the participation of transition metals, is the main reason for its toxicity. Hydroxyl radicals react with proteins, lipids and DNA, causing cell death [7].

Aim: The aim of this research is to compare pro-oxidative capacity of *Papaver rhoeas* L. water leaf extract depending on the concentrations range and endpoints used.

Materials and Methods

Strain 137 C+ was kindly provided in our lab from St. Petersburg collection 25 years ago. The strain was isolated in 1946 by D. Smith from soil in Massachusetts, USA.

Chlamydomonas reinhardtii was chosen as a model test system because of the following basic reasons:

- widely spread photosynthetic unicellular eukaryote;
- typical plant cell structure and genome organization so that results could be extrapolated easily to higher plants;
- single cell organism so that the response of a single *Chlamydomonas* cell is equivalent to the response of an individual organism;
- haploid genome so that the induced recessive mutations could be revealed in a F_1 (first generation), that is not possible in diploid eukaryotic plant cells;
- short life cycle and routine inexpensive laboratory and cultivation techniques.

Cultivation: Strain 137 C+ was cultivated on liquid TAP medium under standard conditions [8] in a growth chamber Phytotron GC 400 – (under continuous light of 5000–5500 lx and t = 23°C ±0.1°C), which is optimal for the cultivation of *C. reinhardtii* strains. Cell suspension was allowed to grow 5-7 days to the end of the exponential and the beginning of the stationary growth phase.

Methods for water extract preparation: Plants *P. rhoeas* L. were collected around Banska Bystrica – Slovakia in May 2008. Plant leaves (weight – about 60 g) were dried at room temperature,

cut up into small pieces (2–3 cm) and transferred into a small Erlenmeyer flask. Material from the leaves was pre-extracted three times with hexane to remove chlorophyll. The plant's material was extracted four times with 100 ml of water (50^{O}C). Water was evaporated by distillation on vacuum rotary evaporator (20 torr, 50°C). The remaining water was removed by azeotropic distillation with toluene. Plant extracts kindly provided by prof. Miadokova were stored at 4°C.

Cells treatment with plant extract: Appropriate concentrations of the extract were prepared before every experiment from a stock solution of 10 mg/ml. For the preparation of the stock solution DMSO and distillated water were used. Several concentrations of poppy water leaf extract in the range of 1% to 5% were applied. 100 ml cell suspensions with density – 1x10^{6} cells/ml were prepared and treated for 1h in the light [9].

MDA measurement: After the treatment cells were centrifuged at 400 x g at 4°C and cell pellets were grinded with 3ml 0.1% TCA on ice and liquid nitrogen and harvested at 13 000 x g for 20 min at 4°C. The supernatant was used for the assays. MDA was measured by the method of Dhindsa et al. [10], which is based on the reaction of MDA, formed by the oxidation of unsaturated fatty acids (mainly linoleic and linolenic) with thiobarbituric acid (TBA). 0.5 ml 0.1M phosphate buffer pH 7.5 and 1 ml of 0.5% TBA in 20% TCA was added to 0.5 ml of the supernatant. The tubes were incubated for 30 min. in a boiling water bath, and then were quenched by placing them on ice. After tempering the extinction of the color compound formed was measured at a wavelength of 532 nm and 600 nm. MDA quantity was calculated in μmol/g F by the formula:

$$MDA=\frac{(E532-E600)a.b}{\varepsilon.FW}[\mu mol/gFW]$$

wherein:
ε is the molar extinction coefficient equal to 155 Mm / sm;
and a = V_{TCA}/V_{sample}; b = V_{cell}/V_{sample}; V is the volume (ml). FW - fresh weight in g

Measurement of H_2O_2: To 0.5 ml of the supernatant were added 0.5 ml 0.1M phosphate buffer pH 7.8 and 1 ml of 1M KI.

The tubes were incubated in the dark at room temperature for 60 min. The absorbance was measured at a wavelength of 390 nm spectrophotometrically [11]. The amount of the H_2O_2 was determined by standard curve of known concentration of H_2O_2, and calculated by the formula:

$$H_2O_2=\frac{A390\ [dilution].10}{tg\alpha.FW.1000}\ [\mu mol/g\ FW]$$

tgα 0.003 of standard curve
FW - fresh weight in g
The values have been compared with those measured in the control sample - DMSO.

Statistics: Student's t-test and chi-square method were used for statistical analysis. Standard deviation (SD) of each treatment was calculated. The significant differences were determined by the Student's t-test.

Results

MDA quantity as a biomarker for lipid peroxidation and intracellular hydrogen peroxide quantity are both measured by the methods of Dhindsa et al. [10], and Heath and Packer [11]. The control levels in untreated cells are taken as 100%.

Both concentrations 2.5% and 5% of water leaf extract were obtained to possess slight pro-oxidative capacity measured as increased levels of H_2O_2 comparing with that in a control DMSO sample (Fig. 1).

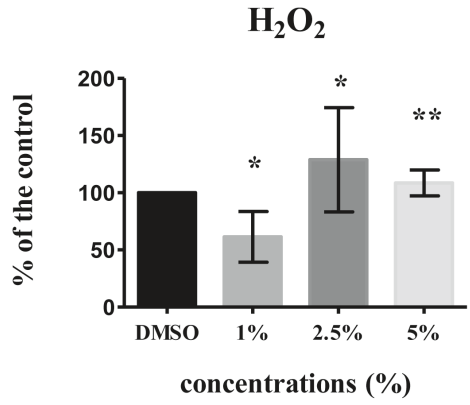

Fig. 1. Levels of intracellular hydrogen peroxide after the treatment with water leaf extract of *Papaver rhoeas* L., * p<0.05; ** p<0.01

Further, no pro-oxidative effect of the same concentrations was revealed using MDA amounts as an endpoint – the measured values were approximately equal to or lower than that of the control DMSO (Fig. 2).

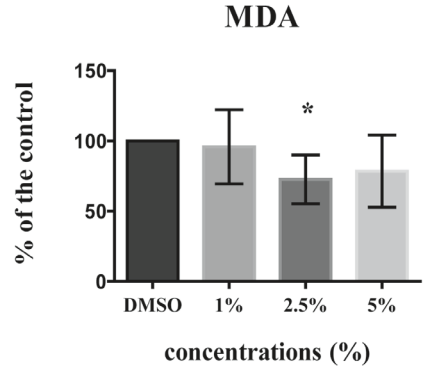

Fig. 2. Lipid peroxidation of the membranes after the treatment with water leaf extract of *Papaver rhoeas* L., * p<0.05

Conclusions: As a result of our experiments two main conclusion could be drawn: water leaf extract of *Papaver rhoeas* L. possess very slight or no pro-oxidative capacity depending on the concentrations applied; at least two endpoints ought to be used for such evaluation.

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References:

1. Zargari A. Medical Plants, 1, 1994, 91–102.
2. Charles J. D. Antioxidant Properties of Spices, Herbs and other Sources 2013 New York 9-11.
3. Valavanidis A., Vlahogianni T., Dassenakis M., Scoullos M., Ecotoxicol. Environ. Saf., 64, 2006, 178–189, doi: 10.1016/j.ecoenv.2005.03.013.
4. Kelley E. E., Khoo N. K., Hundley N. J., Malik U. Z, Freeman B. A., et al., Free Radic. Biol. Med., 48, 2010, 493–498, doi: 10.1016/j. free rad. biomed. 2009.11.012.
5. Sciskalska M., Zalewska M., Gizelak A., Milnerorowicz H., Bio Trace Elem Res, 159 (1-3), 2014, 59 – 68.
6. Klaunig J. E., Wang Z., Pu X., Zhou S., Toxicol. Apll. Pharmacol., 254, 2011, 86 - 99 doi 10.1016 /j.toap.2009.11.028 [Pub.Med [Cross Ref].
7. Lee Y. W., Ha M. S., et al Neurochem Res., 26 (4) l, 2001, 337 - 343.
8. Harris E., In: The Chlamydomonas sourcebook. Second edition. Introduction to Chlamydomonas and its laboratory use (ed. E. Harris), San Diego, Academic Press – Elsevier, 2009, 241–252.
9. Angelova O., Miadokova E., Parvanova P., Gregan F., Chankova S. Comptes rendus de l'Academie bulgare des Sciences, 67, (2), 2014, 243 – 250.
10. Dhindsa R. S., P. Plumb-Dhindsa, T. A. Yhorpe, J. Exp. Botany, 32, 1, 1981, 93-101.
11. Heath R. L., L. Packer. Arch. Biochem. Biophys, 125, 1, 1968, 189-198.

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ENERGY FLOW AND BALANCE OF BIOGENIC ELEMENTS THROUGH TWO HOUSE MICE SPECIES *MUS MUSCULUS MUSCULUS* AND *MUS SPICILEGUS*

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Abstract

Aim: The purpose of the study is to investigate the energy needs of two house mice (*Mus musculus musculus* and *Mus spicilegus*), and to estimate the amount and the flow of the main biogen elements Nitrogen (N), Phosphorus (P), Potassium (K) and Calcium (Ca).

Materials and Methods: Food preferences have been provided by stomach contents analyses. The measurements and calculations of the daily energy flow were made on the base of formulas, standard for this type of investigations.

The biogen elements N, P, K and Ca were determined.

Results: On the base of the laboratory experiments for the food preferences of the two investigated species the basic bioenergetic characteristics, such as the energy flow through mice's bodies were established. The calculations of the balance of the main biogen elements N, P, K and Ca were also done. The results show that for both mice species about 19% of the total food intake (C) is excreted as feces and urine (F+U) and therefore the assimilation (A) was around 80%. It incorporates also the metabolic energy (R), which is spent mainly as costs for respiration to maintain the animals' basic vital functions. From the total of 0.092 g of nitrogen in the consumed food, *Mus spicilegus* returned to nature 78.2% and *Mus m. musculus* 83.1% by excreta. The main nitrogen amount (69.4%) in the total excretion belongs to urine. Therefore, the amount of nitrogen that's included in body metabolic processes is within 26.9 to 31.8% for both rodent species. The assimilated quantities of the other three biogenic elements are significantly higher than those of the nitrogen. *Mus spicilegus* returns 34% of the phosphorus, 15.8% of the potassium and 10.5% of the calcium. There were no statistically significant differences between them and the results obtained for the semi synantropic *M. m. musculus*.

Keywords: house mice, energy flow, biogen elements

Introduction

In North Bulgaria, *M. spicilegus* (Petenyi, 1882) and *M. m. musculus* (L., 1758) live in the conditions of synthopy, and with restricted distribution to the north of the Stara Planina Mountains [1, 2]. *M. spicilegus* is a steppe species, an entirely outdoor inhabitant which gathers "hillocks" for the winter [3]. These mounds serve as food reservoirs during the winter period, especially for young, immature individuals that are born in the summer of the same year [1, 4, 5, 6]. *M. m. musculus* is a semi-synanthropic and widely distributed mouse species in Europe [7, 2].

The result obtained on the diet of the investigated mice species are the basis for a laboratory modeling investigation on energy flow and matter turnover through the animal's body. Such a type of investigations was the milestone for further determination and calculations on the food preferences so as the energy flow. When estimating the flow of matter through populations or

through trophic levels it is essential to determine the contents of different biogenic elements in the animals' body. To calculate the balance of the main biogen elements N, P, K and Ca it is necessary to pre-identify their amounts in different types of offered foods.

Such kind of investigations on *Mus musculus musculus* and *Mus spicilegus* are unique and extremely important for evaluating the influence of small rodents on the primary productivity of ecosystems.

Materials and Methods

Because of the close analogy in the morphology of both investigated *Mus* species, and their synthopic distribution in the periods from spring to autumn the animals included in the experiments were caught from regions where only one of the investigated mice species resided. The mound building mouse *M. spicilegus* is not widespread south of the Stara Planina Mountain and no hybridization zone with *M. m. musculus* exists [1]. Thus, mice, included in tests for food choice, were caught from North Bulgaria (43^{O}22′35.55″ N and 24^{O}27′15.28″ E) directly from "hillocks", while *M. m. musculus* were caught from the south of Stara Planina Mountain (42^{O}48′15.8″ N and 23^{O} 14′05.5″ E).

The food spectrum was determined by the stomach content analyses using the method of [8]. For *Mus spicilegus* seed supplies from "hillocks" were also analyzed.

The method of the "food preferences" has been described by [9] and presents perfect results in tests with mice and voles. The mice were fed with seeds of three weed species predominating in the animals' diet and food supplies: bluesteam (*Andropogon halepensis*), setaria (*Setaria viridis*), and amaranth (*Amaranthus* sp.).

All experiments were carried out during the winter season (from January to March) when animals were not sexually active and therefore do not take into account the energy losses for accumulation and formation of biomass which are practically near to zero. 15 mice of *M. spicilegus* and 11 mice of *M. m. musculus* took part in the 7 days duration tests.

The measurements and calculations of the daily consumption and quantity of the excrement for each animal were recorded.

In keeping with this terminology, [10] presents the total food transformation in the population as follows:

$$C = P + R + F + U$$
$$D = C - F \text{ or } D = A + U$$

Where: C is consumption, D – utilization, A – assimilation, P – production, R – respiration, F – excrements, U – urine and (F + U) – excretion

In caloric equivalents this equation is:

$$K1C = K2P + R + K3F + K4U$$

The energy value of food and excretory products of both mice species were determined by the method of bomb calorimetry using the water calorimeter type KL-10.

The relative content of the main biogens (Nitrogen - N, Phosphorus - P, Potassium - K and Calcium - Ca) in the offered food, food remains and excreta were based on previously conducted "food preference" experiments. The total nitrogen in the reconstituted solution was determined according to Kiejdahl [11]. The determination of the phosphorus was provided colorimetrically with a photoelectro-colorimeter "Specol 1", with a wavelength of 680 nm. Potassium and calcium were determined by a flame photometer "Flapho".

The mean values, SD and reliability of the differences, determined by the t-test, were calculated using ANOVA.

Results and Discussion

The obtained results were based on the hypothesis about the synthopic distribution of both mouse species in North Bulgaria in the spring-summer period. The results confirm the well-known concept that the two house mice are predominantly seeds-eating species [12]. Green vegetable parts and insects were found only in very small quantities. Mice switch to heterogeneous or herbivorous feeding only in periods when seed productivity practically lacks in the field.

The animals' diet strictly depends on the plant species composition and its changes influence the mice food spectrum. The stomach content analyses of the animals from the field show that the mice diet includes mainly weeds like *Setaria viridis, Amaranthus* spp. and *Andropogon halepensis* (Fig. 1). The results of [13] were confirmed, also, a clear preference for *Setaria* spp. for the populations in Central Europe was established.

Analyzing the mice diet in seasonal and perennial aspect, it is possible to conclude that the food preferences of the animals are mostly due to the phytocenosis' composition and its change over time and effects on the composition of their food. According to [14], the demand and accumulation of food supplies relates to a minimum energy consumption, i.e. animals gather food closely near to their shelters. From the energetic point of view those who occupy good places in terms of food habitats are in better energetic position. The lack of sufficient nutritious food forces them to make bigger hikes or to collect large amounts of low calorie foods.

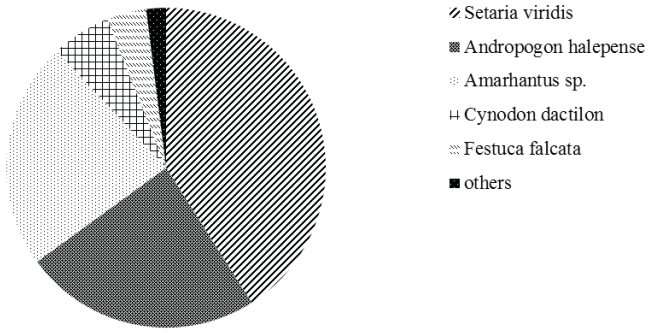

Fig. 1. Diet of the two investigated mice species

The food experiment was conducted with weed seeds, commonly found in the mice diet. Daily amounts of the weed seeds consumed by mice are presented in Table. 1.

Table 1. Values of mice food intake in g/animal/day (x±Sd) for different types of weed seeds

species		*Andropogon halepensis*	*Setaria viridis*	*Amaranthus* sp.	total
Mus spicilegus n=91	x	1.01	0.88	0.89	2.77
	±Sd	0.34	0.32	0.59	0.76
	min	0.25	0.03	0	1.53
	max	1.75	1.37	2.47	4.30
Mus musculus n=77	x	1.07	0.93	0.87	2.87
	±Sd	0.33	0.20	0.35	0.35
	min	0.88	0.64	0.48	2.35
	max	1.65	1.15	1.27	3.24

The table presents data on the average food consumption (C) of the three offered weeds. Statistical calculations show that both mouse species did not show preference for any of the seeds. No significant differences between the two rodent species or between their affinities with respect to the weeds were recorded. All three offered foods incorporated into the daily animals' diet were in approximately equal amounts - average by 33%.

Statistically significant differences in terms of the average assimilation (A) did not manifest between the two investigated species. Reliable differences were reported, however, in the average food consumption (C) ($p \leq 0.1$) and the amount of excreta (F) ($p \leq 0.001$). The cause for such results is probably in the specifics of the offered seeds and the life mode of the animals. In the autumn and winter seasons *M. m. musculus* occupies human settlements and changed its feeding behavior. Relatively high content of cellulose (predominantly occurring in the seed coat) and other non-digestible components in weed seeds forced the animals to eat a large volume of food, as the main amount of ballast substances is brought back to nature in the form of excrement. In ecological studies related to energy flow and productivity, interest is drawn mainly to the total caloric value of the animals. It differs among species and varies due to their place in the trophic chains. The performed tests are the basis for calculating the daily energy costs of the two investigated mouse species.

The results of the calculations of the amounts of digested (D), assimilated (A) food and excretory products (feces (F) and urine (U) are presented in Table 2.

Table 2. Mean values (x ± Sd) the consumed (C) digested (D), assimilate food (A) and excretory products (F) and (U) in g/animal/day for both mice species

species		Initial body mass (g)	Final body mass (g)	C	F	D	U	A
Mus spici-legus n=91	x	15.4	15.4	2.77	0.49	2.28	0.05	2.23
	±Sd	1.7	1.4	0.76	0.15	0.21	-	0.37
	min			1.53	0.23	1.26	-	1.16
	max			4.30	0.78	3.71	-	2.39
Mus mus-culus n=77	x	15.3	15.5	2.87	0.49	2.38	0.05	2.33
	±Sd	1.6	1.8	0.35	0.06	0.26	-	0.34
	min			2.34	0.41	1.76	-	1.66
	max			3.24	0.56	2.99	-	2.87

It was found that for both mouse species about 19% from the total food intake (C) is excreted as feces and urine (F + U) and therefore the assimilation (A) was around 80%. The calculation incorporates also the metabolic energy (R), which is spent mainly as costs for respiration to maintain the animals' basic life functions.

Test for diet selectivity is a basis for further calculations of the daily energy costs in both mouse species. In research on the productivity and energy flow, the interest is directed to the total caloric value of the animals. It differs among species and depends on their position in the trophic chains.

Calorimetric measurements of the average seeds samples and food leftovers are presented in Tables 3.

Table 3. Energy value (kJ/g) of weed seeds and food leftovers (x ± Sd)

seeds	*Andropogon halepense*	*Setaria viridis*	*Amarhantus* sp.
offered food	18.3±0.03	18.3±1.2	18.6±0.2
Mus spicilegus			
leftovers	16.1±0.08	17.1±0.4	16.9±0.2
consumption	2.2±0.6	1.2±0.4	1.7±0.2
Mus m. musculus			
leftovers	15.4±0.06	17.5±0.6	17.1±0.2
consumption	2.9±0.5	0.8±0.9	1.5±0.2

The average energy consumed with weeds from *Mus spicilegus* and *Mus m. musculus* is as follows: *Andropogon halepense* - 2.2 kJ/g and 2.9 kJ/g; *Setaria viridis* - 1.2 and 3.8 kJ/g; *Amarhanthus* sp. - 1.7 and 1.5 kJ/g. Totally this makes respectively 5.1 kJ/g and 8.2 kJ/g. On

the base of Tables 3 and 4 it is possible to calculate that the daily amount of energy intake (C) with 2.8 g food is 14.1 kJ/g × day^{-1} for *Mus spicilegus* with an average mass of 15.4 g and respectively 23.5 kJ/g × day^{-1} for *Mus m. musculus* with average mass of 15.3 g.

Parallel measurements of the energy content of feces, and the whole body of adult animals with an average body mass of about 15.4 g and 15.3 g for the wild and for semi-synantropic species were also provided. The results of the calorimetric measurements are presented in Table 4.

Table 4. Energy values of whole body and feces of experimental animals in kJ/g (x±Sd)

species	*Mus spicilegus*	*Mus m. musculus*
body	22.0±0.40	22.5±0.50
faeces	1.8±0.04	1.8±0.04

No significant differences in the body and excreta energy values between the two investigated species were established. Therefore, the average energy value of the body of *Mus spicilegus* with body mass about 15.4 g amounts to 338.8 kJ/animal, and for excreta 0.9 kJ/animal x day^{-1}. These values for *Mus m. musculus* with body mass about 15.3 g amounts to 344.3 kJ/animal and 0.9 kJ /animal × day^{-1}.

Further calculations showed that from the total consumption (C) of an animal per day about 5% were returned to nature by feces (F) and 95% of the food energy was utilized as digestion (D). The energy balance in the body of the two mouse species is presented in Table 5.

Table 5. Energy balance in the body of *Mus spicilegus* and *Mus m. musculus* (x±Sd), in kJ/animal×day^{-1}

species	W(g)	C (g)	C	F	D	U	A
Mus spicilegus n = 91	15.4±1.5	2.77±0.76	14.1±1.6	0.8±0.1	13.3±0.4	0.86	12.4±0.3
Mus m. musculus n = 77	15.3±1.4	2.88±0.54	23.5±3.3	0.8±0.1	22.7±1.1	0.86	21.8±0.9

W – body mass in grams

The table demonstrates that of the total energy consumption (C) 9.9% for *Mus spicilegus* and 6% for *Mus m. musculus* return to nature with the excretory products. Therefore, about 90-94% of the energy of the food (C) is utilized for the assimilation (A). All animals included in the experiments were adult and sexually inactive and their body weight stayed unchanged during the experiments. Therefore all the metabolic energy goes as costs for respiration (R) and maintenance.

It is well known [15, 9, 14] that in sexually inactive adult individuals' energy amount intended for forming and accumulation of biomass and production is practically nil. Therefore, the total assimilated energy for oxidation and assimilation is equal to respiration (A = R).

When estimating the flow of matter through populations or through trophic levels it is essential to determine the contents of different biogenic elements in the animals' body. To calculate the balance of the main biogen elements N, P, K and Ca it is necessary to pre-identify their amounts in different types of offered foods. Sufficient information on the content of nutrients and their flow through the body and in the populations of small rodents practically is likely to be found only in the works of [16] for *Peromiscus polonatus* and in the study of [17] and [18] for *Clethrionomys glareolus* and *Apodemus flavicollis*. Studies on nitrogen balance in the body of *Microtus arvalis, Clethrionomys glareolus, Apodemus agrarius* and *Apodemus flavicollis* were also conducted by [9]. The author found that in adult sexually inactive individuals with an average weight of 25.0 g the daily amount of nitrogen taken with food is about 0.33 g, approximately 90% of it is returned to nature with excretory products and mostly with urine.

In parallel with food experiment average samples of seeds, food remains and excreta were analyzed for the quantitative content of the main biogen elements. All results were presented in % / g dry weight. The results of analyses for various types of weeds and food remains in the both types of house mice are presented in Table 6.

Table 6. Contents of N, P, K and Ca (%/g) in the food and food remains from the experiment with weeds

seeds	*Andropogon halepense*			*Setaria viridis*			*Amarhantus* sp.		
elements	seeds	remains	consumption	seeds	remains	consumption	seeds	remains	consumption
Mus spicilegus									
N	2.37	1.45	0.92	2.36	1.95	0.41	2.59	2.76	-0.17
P	0.30	0.19	0.11	0.39	0.29	0.10	0.49	0.48	0.01
K	1.71	0.41	1.30	2.17	0.83	1.34	2.37	0.81	1.56
Ca	1.15	0.21	0.94	1.26	0.39	0.87	2.04	0.56	1.48
Mus m. musculus									
N	2.38	1.61	0.76	2.36	2.00	0.36	2.59	2.81	-0.22
P	0.30	0.22	0.08	0.39	0.29	0.10	0.49	0.48	0.01
K	1.71	0.45	1.26	2.17	0.74	1.43	2.37	0.85	1.52
Ca	1.15	0.49	0.66	1.26	0.49	0.77	2.04	0.75	1.29

Data show that the amaranth seeds are richest in biogens. *Mus spicilegus* procure the greatest amount of Nitrogen and Phosphorus from seeds of the bluesteam and Potassium and Calcium from the amaranth seeds. *Mus m. musculus* selects the food similarly. The negative Nitrogen value in the consumed amaranth quantity is due to the fact that the basal Nitrogen amount is contained in the seed's coat which is not a food resource for the mice.

From the above table it can be estimated that the total amount of biogen elements (in %) for an animal per day with food income are as follows:

Precalculating in grams per day the daily amount of offered food per day (9.0 g) per animal generally gives 0.73 g Nitrogen, 0.012 g Phosphorus, 0.063 g Potassium and 0.045 g Calcium.

Based on the results from the N, P, K and Ca contents in both the food and excreta the biogen elements' balance in the body of animals was calculated. The results are presented in tables 7, 8, 9 and 10, where C is the consumption, D - digestion, A - assimilation, F - feces and U - urine.

Table 7. Nitrogen balance in the body of both mice species (in g/animal×day^{-1})

	Body mass	C	F	D	U	A
Mus spicilegus						
X	15.4	0.092	0.022	0.070	0.05	0.020
± Sd	1.7	0.019	0.007	0.016	-	0.016
Mus m. musculus						
X	15.3	0.089	0.015	0.065	0.05	0.015
± Sd	1.6	0.016	0.006	0.016	-	0.015

Table 8. Phosphorus balance in the body of both house mice species (in g/animal×day^{-1})

	Body mass	C	F	D	U	A
Mus spicilegus						
X	15.4	0.015	0.005	0.010	0	0.010
± Sd	1.7	0.003	0.002	0.003		0.003
Mus m. musculus						
X	15.3	0.015	0.0047	0.010	0	0.010
± Sd	1.6	0.002	0.001	0.002		0.002

Table 9. Potassium balance in the body of both house mice species (in g/animal×day^{-1})

	Body mass	C	F	D	U	A
Mus spicilegus						
X	15.4	0.019	0.003	0.016	0	0.016
± Sd	1.7	0.006	0.001	0.005		0.005
Mus m. musculus						
X	15.3	0.020	0.003	0.017	0	0.017
± Sd	1.6	0.004	0.001	0.004		0.004

Table 10. Calcium balance in the body of both house mice species (in g/animal×day^{-1})

	Body mass	C	F	D	U	A
Mus spicilegus						
X	15.4	0.020	0.021	0.018	0	0.017
± Sd	1.7	0.004	0.0010	0.004		0.004
Mus m. musculus						
X	15.3	0.013	0.0023	0.011	0	0.011
± Sd	1.6	0.004	0.0010	0.005		0.005

From the total of 0.092 g of nitrogen in the consumed food *Mus spicilegus* returned to nature 78.2% and *Mus m. musculus* 83.1% by excreta. The main nitrogen amount (69.4%) in the total excretion belongs to urine. Therefore, the amount of nitrogen that's included in metabolic processes is within 26.9% to 31.8% for both rodent species.

The assimilated quantities of the other three biogen elements are significantly higher than those of nitrogen. From the tables it might be calculated that *Mus spicilegus* returns 34% of the Phosphorus, 15.8% of the Potassium and 10.5% of the Calcium. It means that 66% P, 82.4% K and 89.5% Ca are included in the metabolic processes. These values for *Mus m. musculus* excretion are as follows: 34% P, 15.5% K and 17.7% Ca. The assimilated amounts of nutrients accounted for 66% P, 84.5% K, and 82.3% Ca.

The statistically significant differences between the two species are established only with respect to Calcium $p<0.001$ for the consumption and digestion of food and $p = 0.01$ for excrement.

Such kind of investigations on *Mus musculus musculus* and *Mus spicilegus* are unique and extremely important for evaluating the influence of small rodent populations on the primary productivity of ecosystems so as to the soil fertility.

References:

1. Orsini P., Bonhomme F., Britton-Davidian J., Croset H., Gerasimov S., Thaler L. Zeitschr. für Säugetierk., 48, 1983, 86-95.

2. Guenet J. L., Bonhomme F. Trends Genet. 19, 2003, 24 -31.

3. Berry R. J. Mammal Review., 11 (3), 1981, 91 – 136.

4. Garza J. C., Dallas J., Duryadi D., Gerasimov S., Croset H., Boursot PMol. Ecol., 6, 1997, 1009–1017.

5. Simeonovska-Nikolova D. Acta ethol., 6, 2003, 39–45.

6. Szenczi P., Banszegi O., Ducs A., Gedeon C. I., Marko G., Nemeth I., Altbacker V. J. Mammal. 92 (4), 2011, 852–860.

7. Auffray J. C., Vanlerberghe F., Britton-Davidian J. Biol. J. Linn. Soc., 41, 1990, 13–25.

8. Bomford MAustralian Wildlife Research., 14, 1987, 197- 206.

9. Drozdz A. ActaTheriol., 13, 1968, 367-389.

10. Petrusewicz K., Ryskowski L. Small Mammals: Their Production and Population Dynamics. Cambridge University Press, Cambridge, 1967, p. 204.

11. Kjeldahl J. Zeitschrift für analytische Chemie, 22 (1), 1883, 366-383 (in German)
12. Sokolov V. E., Kotenkova E., Lyalyukhina S. I. Biology of house and mound-building mice. Nauka, Moscow, Russia, 1990, p. 207, (in Russian).
13. Hölzl M., Krištofík J., Darolová A., Hoi H. Naturwissenschaften., 98 (10), 2011, 863-70.
14. Petrusewicz K., Macfadyen A. Productivity of terrestrial animals principles and methods. IBP Handbook No 3, 2nd edition, Oxford, Blackwell., 1971, p. 190.
15. Golley F. B. Mammals of Georgia. Athens. GA. University of Geogia Press 1962, p. 246.
16. Kaufman D. W., Kaufman G. A. In: Mineral cycling in Southeastern ecosystems (F. G. Howell, G. B. Gentry and M. H. Smith, Eds.), AEC Symposium Series, 1975, 518 - 527.
17. Górecki A., Szafranek L. Acta theriol., 26 (21), 1981, 341-347.
18. Malzahn E. Acta Theriologica, 26 (13), 1981, 231- 256.

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THE ACTIVITIES OF THE ASPARTATE AMINOTRANSFERASE, ALANINE AMINOTRANSFERASE AND ALKALINE PHOSPHATASE ENZYMES IN THE BLOOD SERUM OF RATS IN CONDITIONS OF CHRONIC LEAD POISONING

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Abstract

Aim: The objective of this study was to analyze the activities of the Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP) enzymes in the blood serum of rats in conditions of chronic lead poisoning.

Materials and Methods: Research included 40 female rats of the strain, weighing about 150-200 g, and 4-5 months of age and 10 young offspring of the above females, 1.5 months old and about 80-100 g of weight. Lead in the form of lead (II) acetate Pb $(CH_3COO)_2$ was given to female rats orally, by means of water.

Results: In the conditions of chronic lead poisoning of the female rats and their offspring a significant increase in the activities of AST, ALT and ALP enzymes in the blood serum of the experimental groups in relation to the control ones was determined. A higher dose of lead resulted in a more significant increase of ALP activity in the blood serum of the female rats.

Conclusion: The activities of AST, ALT and ALP enzymes in the blood serum of young rats were significantly increased in conditions of chronic lead poisoning of their mothers during periods of pregnancy and lactation, but no dependence on the dose of the received lead by their mothers was established.

Keywords: Lead, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), chronic lead poisoning.

Introduction

Lead is a toxic metal that can be found everywhere in the environment. Overexposure to lead continues to be an important worldwide problem. Food is an important source of lead and determination of lead in food can be used for the estimation of lead exposure. The level of lead in the Earth's crust is about 20 μg/g. In areas where leaded gasoline is banned, the major exposure pathways of nonsmoking adults are from food and water. Several studies were done to determine the concentration of lead in foods [1, 2] and to study its dangerous effects. Recently, the USA, all European countries and many developing countries have outlawed or strictly regulated the use of leaded petrol. In such countries, levels of lead in food and drinking water are closely monitored. Lead may reach and contaminate plants, vegetables, fruits and canned food through air, water and soil during cultivation and also during industrial processing and packaging. Fruits and vegetables grown in polluted soils may become contaminated as a result of plant uptake of lead from soils or direct deposition of leaded dust onto plant surfaces. Therefore, through these diverse mechanisms, lead deposited in soil becomes a persistent and long-term source of lead exposure for humans.

Lead is ubiquitous in the environment, persists indefinitely, and can be found at low levels in almost all living organisms [3]. Sources of lead contamination of air, water, and soil include internal combustion engines, oil burners, smelters, lead pipes, glass and alloy processing plants, incinerators, industrial effluents, and smokestack fallout [4]. Lead is found in the soil, plants and grains grown on contaminated soil, and tissues of animals that eat contaminated plants and feed grains [5]. Because of the widespread environmental exposure, low levels of lead can be demonstrated in tissues of clinically normal birds and animals [3] Lead poisoning occurs when an animal or a bird inhales or ingests a concentrated source of lead. Concentrated lead sources include lead-based paint, lead arsenate crop sprays, lead plates in automotive batteries, fishing sinkers, lead shotgun pellets, drapery weights, sewage sludge, and lead mine tailings [6].

Toxicity of lead is expressed *inter alia* by changes in the activities of certain enzymes. Namely, lead can directly destroy the enzyme structure by inter-reacting with bioelements contained in the metalloenzymes as in the case of aminolevulinic acid dehydratase (ALAD) which enables the process of HEM biosynthesis and subsequently results in the occurrence of anemia [7, 8, 9, 10].

In addition to this, when exposed to increased lead concentrations, the activities of certain enzymes can be disturbed as a direct consequence of damages to the tissues and organ cells [1, 9, 11, 12].

This study investigates the effects of chronic lead poisoning on the activities of alanine and aspartate aminotransferases (ALT and AST) and alkalinephosphatase (ALP) in the blood serum of female rats and their offspring.

The aim of this study was to analyze the activities of the AST, ALT and ALP enzymes in the blood serum of rats in conditions of chronic lead poisoning. The above aim was fulfilled by defining the activities of the above enzymes in the blood serum of female rats and their offspring in conditions of chronic lead intoxication and in relation to the control group, the period of intoxication and lead dose.

Aspartate aminotransferase (AST) is found in high concentrations in liver, heart, skeletal muscle and kidney. AST is present in both cytoplasm and mitochondria of cells. In cases involving mild tissue injury, the predominant form of AST is the one from the cytoplasm. Severe tissue damage results in predomination of the mitochondrial enzyme being released. High levels of AST can be found in cases such as myocardial infarction, acute liver cell damage, viral hepatitis and carbon tetrachloride poisoning. Slight to moderate elevation of AST is seen in muscular dystrophy, dermatomyositis, acute pancreatitis and crushed muscle injuries.

Alkaline phosphatase (ALP) is present in a number of tissues including liver, bone, intestine, and placenta. Serum ALP is of interest in the diagnosis of 2 main groups of conditions-hepatobiliary disease and bone disease associated with increased osteoblastic activity.

Materials and Methods

The survey used a total of 50 female rats of which 40 weighed 150-200 g at the age of 4-5 months and 10 young female rats weighing 80-100 grams and about 1.5 months old. The room where the experimental rats were kept was constantly monitored for temperature, humidity and illumination intensity. In terms of nutrition, the rules applying to feeding and breeding laboratory animals were respected.

Lead intoxication of the animals was performed using lead acetate Pb $(CH_3COO)_2$, orally, by adding it to drinking water. Each female was kept in a cage and the amount of water that was drunk was measured every day by changing the amount of water in the flask. Thus a specific concentration of lead acetate was received through drinking water. The selection of the lead acetate for chronic lead intoxication of experimental animals was made because of its good solubility and thus better absorption in the digestive tract in comparison to other species. The procedure of oral administration of lead was chosen because of its simplicity, as, besides breathing, a natural way of intoxication with lead from the external environment is digestion of polluted water and food. Lead in the form of lead acetate was given in concentration of 30 and 100 mg/kg body weight daily over a period of 60 days. These amounts of lead acetate are considered as sub-toxic doses. Regardless of the fact that they were being increased, they caused no toxic effects on experimental animals.

The experiment was conducted during a period of 60 days. Female rats were treated with lead acetate ten days before mating, seven days during mating, 21 days during pregnancy, and 21 days during breastfeeding. In addition to the determination of the activity of enzymes aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase in the blood serum, the survey was designed to determine the lead content in some tissues and organs as a result of absorption and deposition of lead.Young experimental rats were separated from their mothers after childbirth and put in separate cages. They have not been treated with

lead acetate because in them the lead content that they received from their mothers was determined during breastfeeding through milk and during pregnancy through the placenta.

Blood was taken from adult female experimental rats to determine the activity of enzymes - AST, ALT and ALP. The activity of the enzymes AST and ALT was measured by the spectrophotometric method (UV test), while the activity of ALP was determined by automated spectrophotometric analyzer - Hitachi 911.

The results obtained were statistically processed by the mean value (X), standard deviation, standard error, students't-test, and variance analysis (ANOVA).

Results

Table 1 shows the results of the activity of AST, ALT and ALP in the blood serum of female rats that were chronically intoxicated with lead acetate daily with a concentration of 100 mg / kg BM per day. Compared to the control group, the value of the three enzymes was increased.

The statistical processing of the results showed that the concentration of the administered dose of lead strongly affected ALP activity in the blood serum of the female rats. Namely, when female rats were treated with a higher concentration of lead acetate (100 mg / kg BM daily) it resulted in a higher increase in ALP activity in the blood serum of female rats in relation to the lower dose (30 mg / kg, BM daily) at equal intervals (20, 30, 40, 50 and 60 days) (Table 1 and Table 2)

Table 1. Activity of AST, ALT and ALP (mean value ± SD U/L) in blood serum of female rats treated with lead acetate (100 mg/kg BM per day) in relation to the control group and the period of intoxication

	AST	ALT	ALP
Control group	35.89	39.98	89.86
10 days	112.45	55.98	115.53
20 days	124.21	62.34	143.22
30 days	130.62	86.93	167.52
40 days	145.72	99.37	186.19
50 days	152.22	124.66	201.13
60 days	167.12	129.42	227.15

Table 2 shows the average values of AST, ALT and ALP in the blood serum of female rats that were treated with a lower concentration of lead acetate (30 mg / kg BM per day). Compared to the control group, there is a significant increase in the activity of the examined enzymes (AST, ALT and ALP), but these results are lower compared to the results that were obtained by the treatment of female rats with higher concentrations of lead acetate (100 mg / kg BM per day). Compared to the control group which was not treated with lead acetate, in the experimental groups there was an increase in the activity of AST, ALT and ALP from 2 to 5 times. This fact

indicates that there is a positive correlation between the level of the applied concentration of lead acetate in relation to the measured values of the activity of AST, ALT and ALP.

Table 2. The activity of AST, ALT and ALP (mean value ± SD U/L) in the blood serum of female rats treated with lead acetate (30 mg / kg BM per day) compared to the control group and the period of intoxication

	AST	ALT	ALP
Controlgroup	35.89	39.98	89.86
10 days	92.21	42.12	92.83
20 days	110.68	49.90	118.45
30 days	115.98	57.44	134.22
40 days	124.27	71.10	157.78
50 days	129.35	95.58	172.12
60 days	134.32	110.13	198.59

One of the aims of the experiment was to examine the activity of enzymes in the blood serum of those young rats whose mothers were exposed to chronic lead poisoning (100 mg / kg BM per day and 30 mg / kg, BM per day) during the periods of pregnancy and lactation. The results showed that the activity of the enzymes tested was significantly higher in the blood serum of young experimental groups compared to the control group, and the mentioned differences are statistically significant ($p > 0.05$) (Table 3 and Table 4).

Table 3. Activity of AST, ALT, and ALP in the blood serum of young rats (mean value U/L) whose mothers had been treated with lead acetate (100 mg/kg BM per day) during pregnancy and lactation compared to the control group

	AST	ALT	ALP
Control group	35.12	37.43	156.29
First analysis	75.88	66.59	328.14
Second analysis	83.21	83.90	355.15

Table 4. Activity of AST, ALT, and ALP in the blood serum of young rats (mean value U/L), whose mothers had been treated with lead acetate (30 mg/kg, BM per day) during pregnancy and lactation compared to the control group

	AST	ALT	ALP
Control group	35.12	37.43	156.29
First analysis	83.12	72.30	370.96
Second analysis	85.18	75.29	381.25

Discussion

In conditions of chronic lead poisoning of female rats and their descendants a significant increase in the activities of AST, ALT and ALP enzymes in the blood serum of experimental groups was established as compared to the control groups. No dependence on the lead dose for AST and ALT enzymes was established. However, for ALP it was found that the dose of lead significantly affected the level of increase in the ALP in the blood serum of female rats, but it was not the case with their descendants. Namely, the higher dose of lead resulted in a significant increase in ALP activity in the blood serum of female rats. The dependence on the period of intoxication was established with the extension of the period of exposure to lead influence. The activities of the aforementioned enzymes in the blood serum linearly increased.The increase in the activity of the enzyme AST was more significant as compared to the ALT enzyme, i.e. Ritis coefficient - which shows the relationship between AST/ALT activities). It was higher than 1. The **AST/ALT ratio** is the ratio between the concentrations of the AST and ALT in the blood of a human or animal. It is measured with a blood test and is sometimes useful in medical diagnosis to differentiate between causes of liver damage, or hepatotoxicity.

The increase of the activities of AST and ALT in the blood serum was probably a consequence of the hepatotoxic effects of lead, i.e. the appearance of toxic hepatitis. The above is supported by the fact that the De Ritis ratio was higher than 1. It is true that the liver, among its many other vital functions, also has a role in the accumulation and detoxification of foreign substances, and therefore of toxic metals. The lead that enters the body by ingestion is transferred to the liver via the vena portae hepatis, where most of it remains stored. Only a smaller portion of this toxic metal "crosses the barrier of the liver" and enters the blood. Lead accumulated in the liver can act by directly damaging hepatocytes, primarily by destroying the permeability of the cell membrane.

The influence of chronic lead poisoning on the activity of some enzymes in serum can be explained by the increased permeability of the cell membrane of hepatocytes, which results in an increased release of cytosol enzymes, such is AST and ALT in the blood. The exact mechanism of hepatotoxicity of lead remains unknown, although several researchers indicate that there is a tendency of lead to be linked particularly to mitochondria membranes. Furthermore, lead toxicity, at the molecular level, is explained with an interaction between lead and calcium. Lead reacts with calcium based on very similar ionic properties.These interactions are observed at the level of Ca^{2+} entering the cell, at the level of Ca^{2+} - binding proteins and receptors, and at the level of maintaining stability of the cell membrane with calcium. Osteoblasts, neurons, capillary endothelial cells and hepatocytes accumulate the excess of Ca^{2+} in mitochondria in the presence of lead. Interaction of lead and calcium is observed at the transport system level of plasma membranes, such as the Ca^{2+} - channel and Ca^{2+} - pump, during which lead disturbs the homeostasis of the intracellular Ca^{2+} and reacts with numerous Ca^{2+} - dependent mechanisms, such as: calmodulin (CaM), protein kinase C, etc.The most striking negative effect of lead is the impaired calcium homeostasis, which results in an increased intake of calcium into the cell. Ca^{2+} ions are very reactive and lead to the inhibition of the mitochondria function.When this is damaged, the release of energy in

the form of ATP is disrupted, and this is the most important factor for the maintenance of the normal function of plasma membranes. The result of this is the destruction of the integrity and permeability of the hepatocytes' membranes, which has the consequence of increased release of cytosol enzymes from the cell, as is the case with AST and ALT. It is well known that enzymes found in the cell cytosol are likely to be released into the extracellular space even with minimal damage to the cells or their membranes.While the process of damaging the cell and its membrane extends and develops, the amount of enzymes that are released into the circulation increases.The results of this research have proven this because with the extension of the lead intoxication period the activities of AST and ALT in the blood serum of female rats were linearly increased. On the other hand, the factor which contributes to the rapid release of enzymes from the liver cells is the high permeability of the capillaries in the tissue. Another possible mechanism for hepatotoxicity of lead is indirect - interference within metabolic pathways. Lead impairs the liver function by inhibiting the enzymes involved in protein synthesis [13, 14]. Regarding the dose of lead, some differences are observed in terms of AST and ALT activities in the blood serum of female rats. The higher dose of lead resulted in slightly higher activities of AST and ALT, but those differences did not show statistical significance. This can be explained by the fact that the enzymes AST and ALT do not belong to the "fine" indicators of liver damage at all levels, but only at the level of plasma membranes. It has already been pointed out that the dissolved enzymes in the cytosol are released easily and quickly from damaged cells, even in case of minimal damage to the plasma membrane (e.g. edema without disturbing the integrity of the membrane).

Similar results for the young rats were also obtained with mothers that were intoxicated with lead. Higher or lower doses were noticed during the periods of pregnancy and lactation. A significant increase in the activity of AST and ALT in the blood serum of experimental groups of young mice compared to the control group was found, but no significant differences were observed in terms of the dose of lead. Because of this, it is likely that the lead that entered the young organism through the placenta and milk has hepatotoxic effect. The results obtained in this study are consistent with the results of other studies, but the review of available literature showed no data on the effects of lead on young experimental animals. A significant increase in the activities of ALT and AST in conditions of chronic lead poisoning in humans and in experimental animals has also been established in literature. These studies explain the increase in the activities of these enzymes with the hepatotoxic effects of lead. However, other studies have emphasized the significant reduction in the activities of AST and ALT under the influence of lead.

The authors explain this with the possible inhibition of the synthesis of AST and ALT enzymes affected by this toxic metal [1, 2, 15]. A possible explanation for such diverse research results is that the studies were quite different in terms of their experimental design and the applied doses of lead, length of exposure, and manner in which lead entered the body of animals.

Regarding ALP, this enzyme may be an indicator of liver and bone damage that are associated with the increased activity of osteoblasts. Besides in the liver, lead can also be deposited in the bones. By increasing the time of lead intoxication, its concentration in bones increases linearly. In a culture of bone cells, it has been observed that this toxic metal reduces the synthesis of proteins and collagen fibers of type I. Reduced synthesis of osteonectin, the protein that binds calcium, has been discovered. Interaction between lead and calcium at

osteoblasts level has been observed. Namely, these cells accumulate the excess of calcium in mitochondria in the presence of lead and it is found that lead reacts with proteins and hormonal signals that normally regulate the status of intra- and extra-cellular calcium. As a result of the interaction between lead and calcium a significant reduction in the concentration of calcium in the bones in conditions of poisoning by this element may appear, as some studies have already shown. This is probably about heteroionic replacement of calcium from the crystalline hydroxyapatite - (group of phosphate minerals) Ca_{10} (PO_4) 6 (OH) $_2$) with the lead from the blood, due to their very similar electronic configurations. However, despite the stated negative effects that lead can cause when it is found in bones, most researchers still think that the bones are not the primary place where the toxicity of lead is shown. The fact is that pregnancy may affect the activity of ALP in terms of increasing its activity because through the placenta lead enters the young organism during this period. However, the control groups of pregnant female rats in the same stage of pregnancy that were not treated with lead denied this. In the light of the above mentioned facts, the noticed increase of ALP activity in the serum of experimental animals under the influence of lead in this research cannot be attributed to the increased release of this enzyme from the bones or from the placenta, but from the liver and within the already mentioned hepatotoxicity. ALP is an enzyme known as a biochemical marker of intrahepatic cholestasis that occurs in some forms of toxic hepatitis. While the pathological process develops over time, as the level of intrahepatic cholestasis increases, so the ALP activity increases in the blood serum, as shown by the results of this study.

ALP activity in the blood serum of female rats basically depended on the applied dosage of lead, which was not the case with AST and ALT enzymes. Namely, a higher dose of lead resulted in a significantly increased activity of ALP in the blood serum. This can be explained by the assumption that a higher dose of lead causes a higher level of intrahepatic cholestasis.The results obtained for the young rats were identical to the results obtained for their mothers. Through the placenta and milk lead enters the young rats and deposits in the liver and possibly causes toxic hepatitis followed by intrahepatic cholestasis, resulting in increased activity of ALP in the blood serum. Some previous studies provided results similar to those obtained in this study concerning the activity of ALP and lead poisoning, but some other studies also gave different results - decreased activity of ALP. The authors explain such results with interactions between lead and zinc and/or magnesium in the active center of the metalloenzyme ALP which resulted in the fall in enzyme activity [16, 17]. The available literature does not give any information about the impact of lead on young rats during periods of pregnancy and lactation. As for the impact of lead on the activity of ALP, various studies again provided different results which can be the consequence of different experimental designs, as in the case of AST and ALT.

Conclusions

1. The activities of AST, ALT, and ALP in the blood serum were significantly increased in conditions of chronic intoxication of female rats with lead. These activities were closely dependent on the period of intoxication. By increasing the period of intoxication, the activity of the above enzymes also linearly increased. The activities of AST and ALT were

not dependent on the administered dose of lead, and in the case of ALP, the increase in activity was basically dependent on the dose of lead.

2. The activities of AST, ALT and ALP enzymes in the blood serum of young rats were significantly higher in conditions of chronic lead poisoning of their mothers during periods of pregnancy and lactation, but dependence on the dose of lead obtained from their mothers is not established.

3. The increase in the activities of AST, ALT, and ALP in the blood serum of experimental animals is the result of the hepatotoxic effect of lead followed by intrahepatic cholestasis.

References:

1. Nilsson U., Attewell R., Christoffersson J. O., Schütz A., Ahlgren L., Skerfving S., Mattsson S. Pharmacol. Toxicol., 68 (6), 1991, 477-84.
2. Goering P. L., Neurotoxicol., 14 (2-3), 1993, 45-60.
3. Doganoc D. Z. Bull. Environ. Contam. Toxicol., 57, 1996, 932–937.
4. Karadjova I. Girousi S., Illiadou E., Stratis I. Microchem. Acta, 134, 2000, 185-191.
5. Brams E., Anthony W., Sci. Total. Environ., 28 (1-3), 1983, 295–306.
6. Ozores H. M., Hanlon E., Bryan H., Schaffer B. Compost. Sci. & Utiliz., 5 (4), 1997, 40-45.
7. Ischiba M., Tomokuni K., Toxicol. Lett., 40 (2), 1988, 159-63.
8. Angle C. R., Thomas D. J., Swanson S. A. iometals., 6 (3), 1993, 179-84.
9. Atef M., Youssef S. A., Ramadan A., Afifi N. A., Muity A. A. Dtsch Tieraiztl Wachenschr., 101 (5), 1994, 187-90.
10. Ambrogi C., Cardini G., Baldi S. B., Cini C. C., Buzzigoli G., Quinones-Galvan A., Ferrannini E. Vet. Hum. Toxicol., 38 (5), 1996, 336-9.
11. Nehru B., Kaushal S., Biol. Trace Elem. Res., 38 (1), 1993, 27-34.
12. Miyuhara T. et al., Calcif. Tissue Int., 54 (2), 1994, 165-169.
13. Fullmer C. S., Environ. Res., 72 (1), 1997, 45-55.
14. Schanne F., Long G., Rosen J., Biochim. et Biophys. Acta (BBA) - Molecular Basis of Disease, 1360 (3), 1997, 247-254.
15. Solliway B., Schaffer A., Pratt H., Yannai S., Pharmacol. Toxicol., 78 (1), 1996, 18-22.
16. Todorovic T., Vujanovic D., Magnes Res., 15 (3-4), 2002, 173-7.
17. Vujanovic D. et al., Arch. Pharmacol., 5-6, 1996, 285-306.

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Тематично направление:
МЕХАНИЗМИ НА АДАПТАЦИЯ НА ЖИВИТЕ СИСТЕМИ
Topic:
MECHANISMS OF ADAPTATION OF THE LIVING SYSTEMS

DNA DAMAGING EFFECT OF UV-B IN DIFFERENT *CHLORELLA* GENOTYPES

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Introduction: The growing extent of anthropogenic pollution has led to an ozone depletion resulting in an increased level of UV-B radiation [1]. Such factor negatively affects the biota. Some of the most impacted organisms are the algae - the basis of the food chain in aquatic ecosystems. This is due to the fact that they such as the other photoautotrophic organisms use solar irradiation as an energy source for their physiological processes such as photosynthesis and growth [1]. However, high levels of UV-B, could be considered as a very deleterious affecting many physiological processes as well as leading to DNA damage, inhibition of photosynthesis and inactivation of enzymes [1]. In short harmful effects of UV-B can negatively affect the diversity of algal communities [1] and to influence genotype resistance to different environmental stimuli. In the light of ecology such problem could be considered as very important because it is related to the formation of genetic elite of the population.

Chlorella species are cosmopolitan and could be found in a wide range of habitats such as in soil, freshwater lakes, snow as well as in hot springs [1]. *Chlorella* is one of the best-studied photoautotrophic eukaryotes. *Chlorella* is an ideal experimental organism used to clarify various research questions and in biotechnology.

Our hypothesis is that algal species isolated from habitats with extreme environmental conditions would have similar or/and even more efficient cellular defense mechanisms towards UV-B irradiation.

The aim of this study was to compare the capacity of *Chlorella* species isolated from various habitats to repair DSBs induced by UV-B irradiation.

Materials and Methods: Three *Chlorella* species were used in the present study - *Chlorella vulgaris*, *Chlorella vulgaris* 8/1 and *Chlorella кesslery*. The main difference among them is the temperature requirements due to differences in their habitats. *Chlorella vulgaris* is psychrophilic organism, originally isolated from the soil in island Livingston, Antarctic. *Chlorella vulgaris* 8/1 is thermophilic, isolated from thermal springs in the region of Rupite, Bulgaria. *Chlorella кesslery* is a mesophilic isolated from USA freshwater and obtained from Trebon collection.

Irradiation: Cells were irradiated with UV-B (λ= 312nm) in BLX-254, Life Technology, UV crosslinker. The irradiation was in a dose range of 50, 100, 250, 500J/m^2. In order to determine the role of photoreactivation two recovery times - 4 and 24 hours were given after the irradiation. Cells were kept in a continuous light at t=23°C in the growth camera Phytotron GC 400 and in a dark on ice to prevent DSBs repair.

Induction and repair efficiency of double-strand breaks (DSBs) was measured by constant field gel electrophoresis (CFGE). *Chlamydomonas reinhardtii* protocol [2, 3] was optimized for *Chlorella* species. Additional step of cell wall disruption with sonicator was added due to the differences in the cell wall composition of *Chlamydomonas reinhardtii* and *Chlorella*.

Results: Data obtained in our study revealed that *Chlorella* species could be characterized as DNA proficient.

New information was obtained concerning UV-B capacity to induce DSBs. Irradiation with increasing doses UV-B resulted in induction of DSBs in a similar manner for the three species.

An acceleration of DSBs rejoining was found when a recovery time in the range of 4 -24 h (RT / light) was given. No statistically significant difference was obtained between repair capacities depending on the duration of recovery time.

When cells were kept for 4 -24h RT/dark on ice some partial repair of DSBs was obtained for *Chlorella vulgaris* and *Chlorella vulgaris* 8/1. Both species were found to express similar repair capacity.

Intriguingly, the same experimental conditions were shown to prevent in a full extend the repair of DSBs induced by UV-B in *Chlorella kesslery*.

Conclusion: New evidence is provided that UV-B irradiation could induce DSBs. Our finding that *Chlorella vulgaris* (Antarctic) and *Chlorella vulgaris* 8/1 (Thermophilic) express similar capacity to repair DSBs induced by UV-B could contribute for better understanding of mechanisms of genotype resistance.

Keywords: *Chlorella*, DSBs, UV-B, repair efficiency, CFGE

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References:

1. Wong C. Y., Teoh M. L., Phang S. M., Lim P. E. & Beardall J. PloS one, 10 (10), 2015, e0139469.

2. Chankova S. G., Bryant P. E. Radiation Biology and Radioecology, 42 (6), 2002, 600-603.

3. Chankova S. G., Matos J. A., Simoes F., Bryant P. E. International Journal of Radiation Biology, 81 (7), 2005, 509–514.

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PROTEIN PROFILING AND ANTIOXIDANT ENZYME ACTIVITY OF *ARTEMISIA ALBA IN VITRO* CULTURES

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Aim: *Artemisia alba* Turra is a fragrant shrub distributed in the southern parts of Europe. The aerials of the plant are used in traditional medicine as a tonic and for treating intestinal disorders [1]. The aim of the present work is to assess the impact of exogenously applied plant growth regulators (PGR) on the total protein profile and the enzyme activities in *A. alba* shoot cultures.

Materials and Methods:

Plant material - *In vitro* shoot cultures were initiated as previously described [1]. PGR treatments were applied as follows: the control PGR-free medium (GAIP_0); 0.5 mg/L indole-3-butyric acid (IBA) (GAIP_1); 1.0 mg/L IBA (GAIP_2); 0.2 mg/L benzyl adenine (BA) + 0.5 mg/L IBA (GAIP_3); 0.2 mg/L BA + 1.0 mg/L IBA (GAIP_4); 0.2 mg/L BA (GAIP_5); 0.7 mg/L BA (GAIP_6); 0.2 mg/L BA + 0.8 mg/L IBA (GAIP_7); 0.7 mg/L BA + 0.5 mg/L IBA (GAIP_8); 0.7 mg/L BA + 1.0 mg/L IBA (GAIP_9) supplemented media. *A. alba* shoots were grown for 12 weeks at 25ºC and a 16/8 h photoperiod.

Fresh plant material (0.5 g) was frozen in liquid nitrogen and then homogenized in 1.5 ml 50 mM sodium phosphate buffer, pH 7.0, containing 0.05 g polyvinyl polypyrrolidone, 0.1 mM EDTA, 2 mM ascorbat, using a pre-chilled mortar and pestle. The homogenates were centrifuged at 15 000 rpm for 30 min at 4^{O}C. The supernatant was used for further analyses of the soluble proteins of aerial, root and callus samples of *A. alba*.

Electrophoretic profile studies

12% SDS-PAGE was used to determine the relative abundance of the major proteins in the samples and to estimate the relative molecular masses. The protein content of fresh leaf and roots of *A. alba* was measured according to Bradford [2].

Enzyme activity assays

Phenylalanine ammonia lyase (PAL, EC 4.3.1.24), glutathione reductase (GR, EC 1.8.1.7), ascorbate peroxidase (APX, EC 1.11.1.11); catalase (CAT, EC 1.11.1.6), guaiacol

peroxidase (GPOX, EC 1.11.1.7), markers of oxidative stress (H_2O_2) and lipid peroxidation (MDA), as well as total phenols and flavonoids were estimated spectrophotometrically as previously reported [3]. Enzyme activities were also determined by zymography using 8 % native PAGE according to the procedures described in [4].

Results and Discussion: The aerials of the plants are rich in protein content (~0.01g/g fresh leaf). In roots and callus the protein content is lower (~0.005 g/g fresh tissue). The PGR-free control, as well as plants treated with 0.5 and 1.0 mg/L IBA alone, were characterized by an elevated protein content, as well as presence of bands, corresponding to 24 and 26 kDa, absent when auxin and cytokinin combinations (IBA and BA, respectively) were applied. On the other hand, the combination of high IBA and BA concentrations, as well as high BA supplementation (GAIP_7, GAIP_8 and GAIP_9) led to a marked alteration of the electrophoretic profile of the aerial and callus samples as compared with the other PGR-treatments. Cytokinin treatment (0.2 and 0.7 mg/L BA alone) led to increase of the protein content in the calluses with content, exceeding all other underground samples (0.007 and 0.006 g/g fresh tissue for GAIP_5 and GAIP_6, respectively). As a general observation, samples from aerial, root and callus parts displayed marked differences in their electrophoretic profile.

Plants possess antioxidant defense systems, consisting of enzymatic and non-enzymatic components, which normally maintain reactive oxygen species (ROS) balance within the cell. Antioxidant enzymes include superoxide dismutase (SOD), which catalyzes the disproportion of superoxide radicals to H_2O_2 and dioxygen; catalase (CAT) and variety of peroxidases (POX), which remove H_2O_2. Unlike POX, CAT efficiently scavenges H_2O_2 and does not require a reducing substrate to perform the task. APX is the most important POX in H_2O_2 detoxification, catalyzing the reduction of H_2O_2 to water using the reducing power of ascorbate. Analyses by native PAGE enzyme activity staining revealed presence of antioxidant enzymes in *A. alba* shoot cultures. Data are in accordance with the activities of antioxidant enzymes obtained spectrophotometrically. In addition, superoxide dismutase (SOD EC 1.15.1.1), peroxidase (POX, EC 1.11.1.7) and polyphenoloxidase (PPO, EC 1.14.18.1) were detected on electrophoretic gels.

SOD activity was observed in both the aerial and the root parts of the plant. Four different isoforms of the enzyme were detected by zymography after fractionation of plant extracts by native PAGE. The inhibition of SOD activity of all bands by both, 10 mM H_2O_2 and 10 mM KCN, indicated a presence of Cu/Zn-SOD in the *A. alba* shoot cultures.

In general, the aerials of plants with a well developed root system (in PGR-lacking control, as well as in IBA rooting stimulated media) were characterized by elevated CAT, APX and GPOX levels, as compared with the aerials of the plants where combinations of IBA and BA led to suppressed rooting and to intensive callusogenesis. Thus, for example, these activities of plants with inhibited root system (0.5 mg/l IBA + 0.2 mg/l IBA treated plants) were 57% (for CAT), 88% (for APX) and 23% (for GPOX), of the ones for plants with stimulation of root development by 0.5 mg/l IBA. Nevertheless, a significant drop of MDA and H_2O_2 was observed (58.3% and 57.1%, respectively). Analyses of the levels of flavonoids and phenolics in these samples showed that in the

plants with suppressed rooting polyphenolics were considerably elevated (151% and 184%, for phenolics and flavonoids, respectively). In addition, electrophoretic profiles indicated distinctive difference in the enzymatic activities of the aerial, root and callus tissue samples. Noteworthy is the occurrence of bands with molecular mass of 24 and 26 kDa only in the aerials of these PGR-treatments, where both aerials and root system were well developed.

Conclusion: Application of PGR in *A. alba in vitro* leads to changes in the electrophoretic profile of samples of aerials and roots of the plant, which is confirmed by the results in determining the activities of antioxidant enzymes. Changes in polyphenol content and molecular markers of lipid peroxidation and oxidative stress have also been observed, implying for a correlation between enzymatic and non-enzymatic antioxidant protection of the plant *in vitro*. It was shown that the effect of PGR on the antioxidant defense of *in vitro* cultivated *A. alba* is related to the auxin:cytokinin ratio, as well as on the concentration of the regulator applied. As it is known, cytokinins and auxins strongly affect (synergistically or antagonistically) different patterns of plant growth and development. In addition, these two groups of PGR affect each other's metabolism as well. Further research is in progress to investigate the chemical structure of polyphenolic compounds produced by the different *in vitro* systems in order to understand their role in the antioxidant defense of the plant, as well as to utilize the obtained dependencies for the biotechnological delivery of bioactive compounds with antioxidant activity.

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References:

1. Danova K., Todorova M., Trendafilova A., Evstatieva L. Nat. Prod. Commun., 7, 2012, 1-2.

2. Bradford M. M., Anal Biochem, 72, 1976, 248–254.

3. Danova K., Nikolova-Damianova B., Denev R., Markovska Y., Plant Growth Regulation, 68, 2012, 447–457.

4. Manchenko G. P., Handbook of detection of enzymes on electrophoretic gels, CRC Press LCC, Boca Raton, Florida, 2003.

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DO ENDOGENOUS CYTOKININS REGULATE TERPENOID BIOGENESIS AND THYLAKOID MORPHOGENESIS *IN ARTEMISIA ALBA IN VITRO* MODEL SYSTEM?

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Aim: Terpenoids such as plant hormones, photosynthetic pigments, signal transduction components, defensive molecules and structural components of the plant cell wall play a major role for the survival of plants. The pharmacological activity of many terpenoids imposes the challenge of better understanding the fundamental aspects affecting their biogenesis in the plant organism. In our previous research it was established that terpenoid profile of the essential oils of *Artemisia alba* Turra depends on plant growth regulators (PGR) supplementation and morphological development of the plant *in vitro* [1]. These effects were further shown to be related to endogenous cytokinin levels [2]. In the present work, the essential oil bearing species *A. alba* has been chosen as an *in vitro* model system with the aim to understand possible interrelations between the biogenetic pathways of terpenoids and cytokinins, as well as the structural organization of the photosynthetic apparatus as a response of exogenous plant growth regulators treatments.

Materials and Methods: Shoot cultures of *A. alba* were initiated and maintained as previously described [1]. Different treatments with indole-3-butyric acid (IBA) and benzyladenine (BA) were applied as follows: the control PGR-free medium (GAIP_0); 0.5 mg/L IBA (GAIP_1); 1.0 mg/L IBA (GAIP_2); 0.2 mg/L BA + 0.5 mg/L IBA (GAIP_3); 0.2 mg/L BA + 1.0 mg/L IBA (GAIP_4). Thylakoid membranes (TM) were isolated [2] and examined by flow cytometry (FCM) and atomic force microscopy (AFM). Levels of endogenous cytokinins (CKs) were determined by LC/MS [3]. For a better comparative interpretation of the obtained results, data have been presented as a sum of the analyzed bioactive CK metabolites (*trans*-zeatin, *trans*-zeatin riboside, dihydrozeatin, dihydrozeatin riboside, *cis*-zeatin, *cis*-zeatin riboside, isopentenyl adenine, isopentenyl adenosine). Terpenoid profile was studied by GS/MS analyses of the essential oils [1].

Results and Discussion: As a general observation, in accordance to their aerial and root parts development, two major morphological groups could be distinguished in *A. alba* in the described experiment. The first type consisted of plantlets with clearly differentiated aerial and root parts (the plants of the PGR-free control, as well as plants where IBA was applied

alone - GAIP_0, GAIP_1 and GAIP_2). The second morphological type displayed intensive axillary shoots formation, inhibited rooting and intensive callusogenesis at the base of the plant clumps (GAIP_3 and GAIP_4). The essential oils of the first morphological type were characterized by domination of monoterpenoid components, and of the second type – by prevalence of the sesquiterpenoids in them. The sum of bioactive CKs was considerably higher in the plants of the first group, as compared with the plants of the second group (81.4 % - GAIP_1; 144 % - GAIP_2; 51.8 % - GAIP_3 and 63.3 % for GAIP_4, expressed as the values of the untreated GAIP_0, where the sum of bioactive CKs was accepted for 100%).

FCM revealed co-existence of at least two TM fractions differing in size and internal structure in all PGR-treatments. AFM further demonstrated that IBA induced the formation of small TM subfraction and small granas (plants with predominant monoterpenoid content in the oils and slightly reduced cytokinin levels), while the combined action of IBA and BA resulted in the formation of large thylakoids and featureless granas (plants with predominant sesquiterpenoids in the oils and a drop of bioactive cytokinins levels). As it is known, there are two common precursors of terpenoid biogenesis in higher plants, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP). There are two independent biosynthetic pathways leading to the formation of IPP: the cytosolic mevalonate pathway and the plastid-localized mevalonate-independent pathway. The alternative mevalonate-independent pathway is involved in the biosynthesis of plastidic terpenoids, including monoterpens, diterpens and carotenoids. On the other hand, isoprenylation is a key step in cytokinin biogenesis. The predominance of sesquiterpenoids (drop of monoterpenoids) in the essential oils of the *in vitro* cultured plants with impairment of chloroplast structure might possibly be explained by affecting the functionality of mevalonate-independent pathway and overlapping of cytokinin and terpenoid biogenesis in *A. alba in vitro*.

Conclusion: We demonstrate that PGR influence the endogenous cytokinins level which in turn plays a crucial role in the thylakoid morphogenesis. The fraction of "swollen" thylakoids can be interpreted as indicative of early stage of senescence-like response. The observed relations between the terpenoid profiles and endogenous cytokinin levels bring out the necessity to search for hints of possible overlapping of terpenoid biosynthetic pathways and isoprenylation of cytokinins as a factor affecting terpenoid profile of the essential oil in this species.

Keywords: *Artemisia alba in vitro* culture, terpenoid biogenesis, endogenous cytokinins, thylakoid membranes, flow cytometry, atomic force microscopy

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References:

1. Danova K., Todorova M., Trendafilova A., Evstatieva L., Natural Product Communications, 2012, 7, 1075-1076.

2. Krumova S., Motyka V., Dobrev P., Todorova M., Trendafilova A., Evstatieva L., Danova K., Bulgarian Journal of Agricultural Science, 2013, 19, 26–30.
3. Djilianov D. L., Dobrev P. I., Moyankova D. P., Vaňková R., Georgieva D. Ts., Gajdošová S., Motyka V., Journal of Plant Growth Regulation, 2013, 32, 564-574.

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SCREENING OF THE ANTIMICROBIAL ACTIVITY OF *IN VITRO* CULTIVATED MEDICINAL PLANTS ORIGINATING FROM BULGARIA AND TUNISA

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Aims: *Listeria monocytogenes* is a Gram-positive bacterium found in soil, water and some animals, including poultry and cattle. It might be also present in raw milk and raw milk products. Its occurrence in food processing facilities might also be the cause of contamination of processed meats. It causes infections of the central nervous system, and bacteremia in immunocompromised patients, pregnant women, newborns and elderly people. It might also cause gastroenteritis in healthy persons at exposure to large inoculum of the pathogen. *Bacillus cereus* is a type of Gram-positive bacteria producing toxins which can cause two types of ailments: one type characterized by diarrhea and the other, called emetic toxin, by nausea and vomiting. It is present in foods and multiplies quickly at room temperature [1]. Epidemiological studies worldwide have shown an increasing occurrence of food-borne pathogens resistant to conventional antibacterial agents. In addition, the risks to human health of chemical additives to prevent the contamination by food-borne bacteria, has brought the need of restriction of their application as food preservatives and have driven food industry and food research towards the search for natural antimicrobial compounds from plant origin [2]. The aim of the present study was to screen the antimicrobial activity of *in vitro* cultivated medicinal plants originating from the flora of Bulgaria and Tunisia.

Materials and Methods: Plant material of *Hypericum perforatum, H. richeri, H. rumeliacum* and *Inula britannica* was collected from the wild habitats in Bulgaria, and

Lavandula dentata, originated from the experimental field of Biological Agriculture Technical Center in Chott-Mariem, Sousse, Tunisia. *In vitro* cultures were initiated by surface sterilization of the stem explants of the plants and placement into benzyl adenine supplemented media. After *in vitro* growth induction stock shoots of the species were maintained in growth regulators free medium with a period of sub-culture adjusted to the growth patterns of the respective species. Air dried plant material of the plants was subjected to successive ultrasonic extraction with chloroform and methanol. Effect of the extracts on the growth of *Listeria monocytogenes* (Gr +) and *Bacillus cereus* (Gr +) were screened by the disc diffusion method.

Results: The highest activity was recorded regarding growth inhibition of *Listeria monocytogenes* by the chloroform extracts of *H. perforatum* and *H. richeri*, at 0.125 mg extract per disc (13 mm and 10 mm inhibition zone, respectively) followed by the chloroform extracts of *L. dentata* (0.25 mg/disc – 7 mm) and *I. britanica* (0.5 mg/disc – 6 mm). With the current disk diffusion method methanol extracts did not show satisfactory activity.

The representatives of the *Hypericum* genus are characterized by a wide array of secondary metabolites mainly flavonoids, phenolic compounds, phloroglucinols and naphtodianthrones which have been shown to possess notable antimicrobial activity. Different types of *Hypericum perforatum* preparations have been marketed successfully as antimicrobial remedies, due to the wide array of the described polar and non-polar chemical constituents in the plant [3]. The results of the current study indicate the prospective of also other representatives of the genus, which might also be further utilized as sources of biologically active compounds with antimicrobial activity. Leaves, flowers, whole plant and powder of *L. dentata* have been traditionally widely used at the countries of south Mediterranean region as a remedy against pathogens in the digestive system, respiratory tract and influenza [4]. While *L. dentata* essential oil composition and antibacterial activity have been studied in literature [5], showing marked differences between Tunisian and Moroccan accessions of the plant, scarce information is available on the chemical composition or antimicrobial potential of the extracts of the species. *I. britannica* is well known medicinal plant used as an antibacterial, carminative, diuretic remedy and for treating asthma, hepatitis and tumors in Chinese Traditional Medicine [6]. The total methanolic extract of aerial parts of the wild collected plant afforded sesquiterpene lactons pulchellin C, gailiardin, britannin, ivalin and 11,13-dihydro-inuchinanolide and Qualitative similarity of the sesquiterpene lactone profiles of the intact plant and *in vitro* derived material was shown by TLC using sulfuric acid spray reagent for visualization [7].

Conclusion: *In vitro* culture development of the studied medicinal plants could further be used as a constant and controllable source of raw material for the delivery of antimicrobial agent of the studied species. Further research is in progress to analyze the chemical composition of the various extracts as well as to broaden the study with enriched fractions obtained from these species.

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References:

1. Food safety. Food poisoning, bacteria and viruses, 2015, http://www.foodsafety.gov/index.html.
2. Bajpai V. K., Na M., Kang S. C., Food and Chemical Toxicology, 48, 2010, 1945–1949.
3. Saddiqe Z., Naeem I., Maimoona A., Journal of Ethnopharmacology, 131, 2010, 511–521.
4. Jamila F., Mostafa E.., Journal of Ethnopharmacology, 154, 2014, 76–87.
5. Soro N'D. K., Majdouli K., Khabbal Y., Zaïr T., International Journal of Innovation and Applied Studies, 7, 2014, 774-781.
6. Chinese Traditional Medicine
7. Todorova M., Trendafilova A., Genova V., Dimitrov D., Wolfram E., Danova K., 8th Conference on Medicinal and Aromatic Plants of Southeast European Countries (8th CMAPSEEC), May 19-22, 2014, Durres, Albania – poster.

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HOW EXPERIMENTAL DESIGN AFFECTS ADAPTIVE RESPONSE IN *SACCHAROMYCES CEREVISIAE*

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Adaptive response (AR) is a non-specific cell defense phenomenon. It is already known that the AR could be triggered by various agents in different organisms [1]. The AR requires low levels of damage that could trigger different defense systems for subsequent protection against similar or different high stress - "test" dose [1, 2]. AR has already been studied using different endpoints - increase in cell survival, DSB rejoining, decrease in the frequency of chromosome aberrations, micronuclei, mutations, etc. [reviewed in 1]. Even though, the molecular mechanisms involved in the formation of AR are not entirely clear.

In the present study, zeocin was chosen as a potential inducer of AR in *Saccharomyces cerevisiae*. Zeocin is a radiomimetic, member of the family of bleomycin antibiotics.

Zeocin could affect DNA by the induction of DSBs [2], as well as base substitutions. Currently little is known about the optimal conditions required for zeocin-induced adaptive response in *Saccharomyces cerevisiae*.

The aim of this study was to clarify the contribution of the experimental design for the development of AR in *Saccharomyces cerevisiae*.

Materials and Methods:

Test-system: *Saccharomyces cerevisiae* diploid strain D7ts1 (MATa/α; ade2-119/ade2-40; trp5-27/*trp5*-12; *ilv1*-92/ilv1-92; ts1/ts1) - for 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.

Experimental designs: The priming (10 μg/ml) and test (100 μg/ml) zeocin doses were chosen based on preliminary cell survival results. Single treatments with both concentrations were performed. To clarify the role of inter-treatment (ITT) and recovery time (RT) for the induction of AR split treatment was done:

- Role of ITT (the time between the priming and test dose) was evaluated based on the following parameters - cell survival, gene conversion, reverse mutation and mitotic crossing-over with Zimmermann's test. The experimental design was treatment with priming dose, inter-treatment time – 45, 60, 120, 180 or 240 min, treatment with test dose;
- Role of RT (the time given after the test dose) was evaluated based on double strand breaks (DSBs) induction and repair capacity with constant field gel electrophoresis (CFGE). The experimental design was treatment with priming dose, inter-treatment time – 45 min, treatment with test dose and recovery time – 30, 60, 90, 120, 180 and 240 min.

Zimmermann's test. Cells, grown in YEPD media to the beginning of stationary phase were harvested and resuspended in phosphate-buffered saline (PBS). The suspension was then treated with a priming dose of 10 μg/ml zeocin for 1 min on ice during centrifugation (825xg). Cells were resuspended and incubated for 45, 60, 120, 180 or 240 min at optimal for cell growth conditions (30^0C with aeration) or on ice (to prevent the repair capacity). Subsequent treatment with a test dose of 100 μg/ml zeocin for 1 min on ice during centrifugation was performed. The pellet was resuspended in PBS. Appropriate dilutions of cell suspensions were plated on solid complete medium for survival and total aberrations. Gene conversion was detected on selective media lacking tryptophan and selective media lacking isoleucine was used for reverse mutations. Five plates in each category were incubated for 5-7 days at t = 30^OC.

CFGE. 1 ml of cell suspension 1 ml with density $1x10^6$ cells/ml was treated with a priming zeocin dose 10 μg/ml for 1 minute on ice during centrifugation (825xg). Inter-treatment time of 45 min at optimal for the cell growth conditions (30^OC with aeration) was given. Cells were then treated with the test dose. After the pellet was resuspended in cold distilled water cells were given 30, 60, 90, 120, 180 and 240 min recovery time before embedding into agarose plugs. The following procedure is performed as described in [3].

Results:

The role of the inter-treatment time is evaluated based on the endpoints cell survival, mitotic gene conversion, reversion and mitotic crossing-over.

Differences are obtained among the endpoints when incubation is performed at optimal for cell growth conditions. The increase in cell survival and decrease in mitotic gene conversion are measured when inter-treatment time is 45, 180 and 240 min in comparison with single treatment with test dose zeocin. Inter-treatment time of 180 and 240 min leads

to cell survival higher than this of the untreated control. A possible explanation could be a switch from one to another growth phase. Additionally, when incubation is on ice, no statistically significant difference is observed for the effect of the 45, 180 and 240 min inter-treatment time.

Similar results are obtained for gene conversion - around 2-fold decrease when 45, 180 and 240 min inter-treatment time is given at optimal for the cell growth conditions.

Around 2-fold decrease is measured in reversion frequency when 45 and 180 min inter-treatment time is given. Even though, a statistically significant difference is calculated between them. On the other hand, samples with a 240 min inter-treatment time and incubation at optimal growth conditions and on ice show revertant frequency similar to that induced by the single test-dose treatment with zeocin.

Interesting results have been obtained for the third genetic event – mitotic crossing-over. The rate of total aberrations when a 45 min inter-treatment time is given is around 4-fold less than this after a single test dose treatment in samples incubated at optimal conditions. The inter-treatment time of 180 and 240 min results in levels of total aberrations similar to those induced after a single test dose of zeocin.

Taken into account the results from the microbiological test for the inter-treatment time, further experiments are carried out with a 45 min inter-treatment time and incubation at optimal conditions.

The role of the recovery time is evaluated as DSBs induction and repair capacity. Data reveal that the most pronounced adaptive response is observed when a 30 min recovery time is given (around 2-fold less DSBs). Increasing of the recovery time results in increase of the DSBs induced. When a 240 min recovery time is given the results from the split treatment demonstrates levels of DSBs higher than those measured after a single test dose treatment.

Conclusion: Here for the first time, from our point of knowledge, data are presented about zeocin induced adaptive response in *Saccharomyces cerevisiae.* The role of the experimental design in terms of inter-treatment and recovery time is revealed. The optimal experimental conditions for the most pronounced adaptive response are 45 min inter-treatment and 30 min recovery time. In these conditions, cells can overcome the damaging activity of the radiomimetic zeocin.

Keywords: adaptive response, CFGE, DSBs, yeast, zeocin

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References:

1. Dimova E. G., Bryant P. E., Chankova S. G., Genetics and Molecular Biology, 31, 2, 2008, 396-408.

2. Chankova S. G., Dimova E., Dimitrova M., Bryant P. E., Radiation and Environmental Biophysics, 46, 2007, 409–416.

3. Todorova T., Miteva D., Chankova S., Comptes rendus de I`Académie bulgare des Sciences, 68(1), 2015, 71-78.

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VARIATIONS IN ANTIOXIDANT CAPACITY DURING PEA VEGETATIVE GROWTH AT HIGH AIR TEMPERATURE

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Introduction: Reactive oxygen species (ROS) are oxygen species that are normally present in living tissues at low levels. The adverse environmental conditions enhance ROS production that can lead to oxidative stress. Plants possess enzymatic and non-enzymatic antioxidant mechanisms termed antioxidant system which underlies their antioxidant capacity (AOC). AOC can be used as a screening criterion for plant tolerance to unfavorable environment; methods to characterize AOC are considered [1, 2]. As a valuable food, pea cultivars are often used for the study of tolerance to unfavorable environment. Pea is a cool-season vegetable crop, and elevated temperatures affect its growth, photosynthesis and reproduction. Cultivar Ran 1 is a Bulgarian early spring-grown pea with a tolerant photosynthetic apparatus and male gametophyte viability at high air temperature that can relate to enhanced antioxidant defense.

The purpose of this study was to follow AOC variations during pea vegetative organs (roots, stem, leaves) formation at high air temperature. Three assays measuring AOC of different non-enzymatic antioxidants were used; earlier we showed that AOC measured by same assays, differed significantly between pea organs during their growth at optimal temperature conditions.

Materials and Methods: Pea (*Pisum sativum* cv. Ran) plants were grown in Hoagland-Arnon nutrient solution in a climatic room (22°C air temperature; 100 μmol.m^{-2}.s^{-1} light intensity; 12/12h day/night photoperiod). A part of the plants (in second true leaf stage) was transferred to grow at high air temperature (40°C; same light conditions) while the rest of plants remained to grow at 22°C. Growth analyses (fresh and dry weights, size, leaf area) of plants grown at 22°C and 40°C were made twice, after a short (two days) heat exposure, and after prolonged (eight days) one. At the same time intervals, samples for AOC assays were collected, fixed with liquid nitrogen, then lyophilized. Lyophilized samples were extracted

(acetone/water/acetic acid=70/29.5/0.5 v/v), the extracts were centrifuged and supernatants were used for HORAC (hydroxyl radical averting capacity), ORAC (oxygen radical absorbance capacity) and F-C (Folin-Ciocalteau) assays which assess different functions of certain non-enzymatic antioxidants [2]. HORAC assay is based on free hydroxyl radical generation during decomposition of H_2O_2 in the presence of CoF_2 at 37^0C and pH 7.4; gallic acid (GA) was used as a standard. In the ORAC assay, the peroxyl radical generation is achieved by the decomposition of AAPH at 37^0C and pH 7.4; TROLOX (water soluble vitamin E analogue) was used as standard. In both assays, fluorescein disodium salt was used as a fluorescent probe which monitored ROS generation and scavenging by pea antioxidants. The reactions were performed in quartz fluorescence cells (10 mm light path) of Perkin Elmer LS-5 fluorimeter equipped with thermostated cell holder. For both assays, sigmoidal fluorescence decay curves were obtained for blank (sodium phosphate buffer), standards and pea samples; their net areas under curves (AUC) were calculated; AOC was evaluated by formulae using blank, standard and pea sample AUC. The F–C assay (known also as total phenolics assay) is based on the reaction of phenolic compounds with the F-C reagent; GA was used for standard curve construction. The assay results (from two experiments) were expressed as μmole standard (GA or TROLOX) equivalents per gram sample dry weight.

Results: During the studied vegetation period at 22^0C pea plants developed all leaf stages before flower formation, root and shoot (leaf, stipule, stem) biomasses enhanced. Later on (out of the experiment) the plants developed reproductive organs and produced seeds. One week-exposure to 40^0C affected the pea vegetative and reproductive growth; leaf, stem and root biomasses were diminishing, and at the end of the exposure pea plants were smaller in size, did not expand sufficiently the upper leaf stages, and did not develop reproductive organs. Shortly, exposure to heat (40^0C) slows down pea growth that is regarded as adaptive strategy; due to this ability the plant can conserve energy for defense [3]. Pea growth progress at 22^oC was accompanied by AOC increase in the whole plant; the increase was expressed in a different extent by the used assays, probably due to the different endogenous levels of the responsive antioxidants. The highest AOC possessed the green organs (leaves, shoot apex) while the AOC of stem and roots were lower. Exposure to heat alters pea growth by affecting different mechanisms; strong negative effect has the oxidative stress generated by the growing ROS level and decreasing AOC of heat-treated tissues. Sustaining or increasing AO level/activity protects the plant and guarantees its heat tolerance in certain limits [3].

Changes of opposite pattern (established by the used assays though in different extent) occurred in the AOC during exposure to 40^oC. After short exposure, the whole plant AOC increased about twofold as measured by the HORAC and ORAC, and remained relatively stable as measured by the F-C assay. Prolonged exposure influenced negatively the AOC of the green organs; leaf and especially shoot apex AOC values were reduced in half while stem and root ones decreased slightly. The pea shoot apex produces leaves and flowers, and its weakening disturbs leaf and generative development. As plant part important for survival and reproduction, pea shoot apex contains high level of potent antioxidants such as flavonoids, tocopherols (TOCs), ascorbic acid (AsA). Elevated temperatures as well as

most environmental constraints enhance ROS formation in the plant. Plant reaction is dose-dependent - low ROS level mediates protective responses that re-establish redox homeostasis while high level leads to oxidative stress that can provoke cellular disturbances [3]. The heat tolerance is associated with AOC delivered by ROS scavenging and detoxification systems. The levels of endogenous non-enzymatic antioxidants and quenchers along with antioxidant enzyme activities usually correlate positively with the heat tolerance. Metabolites with antioxidant function such as flavonoids, AsA, glutathione (GSH), TOC, carotenoids, protect the plant against heat-induced oxidative stress [3]. Flavonoids act as lipid peroxidation inhibitors and metal chelators, and participate in two antioxidant pathways such as direct reaction with free radicals, and chelating of the metal ions contributing to ROS production. These functions are reflected by HORAC and ORAC assays. HORAC assay estimates capacity of phenolics (flavonoids and phenolic acids) to chelate metal ions; thus they prevent hydroxyl radical formation which can occur during decomposition of H_2O_2 by certain transition metal ions [2]. ORAC assay measures hydrophilic chain-breaking capacity against peroxyl radicals; the assay indicates capacity of traditional chain-breaking antioxidants such as flavonoids, AsA, TOCs, GSH, β-carotene [2]. Flavonoids accumulate in surface plant organs, especially pea leaf guard and epidermal cells contain flavonol glycosides and anthocyanins, among them quercetin glucoside prevails. Quercetin forms complexes with various metal ions that can be stronger antioxidants than quercetin alone. Many flavonoid biosynthetic genes are induced, and flavonoid biosynthesis is enhanced mostly due to raising oxidative stress [3]. At limited CO_2 assimilation rate, chloroplast ROS detoxifying enzymes may be strongly reduced that upregulates the biosynthesis of ROS scavenging flavonoids. Due to effective reducing functions most flavonoids can be better antioxidants than AsA and α-TOC. Essential for ROS detoxification are interdependent TOCs, AsA, GSH; together with flavonoids they are underlying ORAC assay. TOCs are lipid soluble antioxidants, scavengers of ROS and lipid radicals that are responsive for membrane stability; TOCs are present in all cell compartments [3]. TOC composition and content differ along the plants; especially pea contains only α-TOC (possessing highest AOC) mostly in shoot apex. Genes for TOC synthesis in higher plants are also activated at oxidative stress; especially α-TOC alters in two phases, the initial increase provides reducing of ROS and inhibiting of lipid peroxidation, followed by loss when the stress is severe and TOC degradation prevails. TOC protection against lipid peroxidation is enhanced by AsA-GSH cycle where TOC recycling proceeds. AsA donates electrons in enzymatic and non-enzymatic reactions, protects membranes by direct scavenging of certain ROS, and by regeneration of α-TOC. At normal conditions the leaves maintain AsA and GSH in high reduction state, especially pea leaves possess large AsA pool and roots contain it as well. Besides regeneration of AsA via AsA-GSH cycle, GSH can directly scavenge H_2O_2 and other ROS, thus acting as preventive antioxidant [3]. GSH content also declines at intensifying the stress when redox state becomes more oxidized that leads to disturbances in AsA-GSH-TOC system [3]. Most probably the reduced AOC at stressful high temperature is due to denaturation and inhibition of regenerative processes in this system.

In conclusion, by means of three different *in vitro* assays we established that normally highest AOC possessed green pea organs (leaves, shoot apex). The obtained AOC

values reflect the impacts of certain endogenous non-enzymatic antioxidants. The high air temperature affected negatively mostly AOC of upper leaf stages (shoot apex) and disturbed pea growth and development. The AOC is dynamic index; it varies depending on developmental and environmental conditions, the used assays are capable to detect the variations.

References:

1. Gillespie K. PhD Thesis, Urbana, University of Illinois, 2010.
2. Huang D. et al. Agric. Food Chem. 53, 2005, 1841-56.
3. Hasanuzzaman M. et al. Int. J. Mol. Sci., 14, 2013, 9643-84.

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ASSESSING THE IMPACT OF SALT STRESS ON THE PHOTOSYNTHETIC APPARATUS OF *PAULOWNIA* LINES

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Aim: *Paulownia* trees are native to China. They can be used for timber, biomass yield and biofuel production. Species belonging to the genus *Paulownia* are characterized with high tolerance to various environmental conditions, therefore in the last years they are used for remediation of the soils. There are few studies related to growth, development, biochemical and physiological processes of these plant species under salt stress, which is a main abiotic environmental stressor. The aim of the present study is to characterize the functional activity of the photosynthetic apparatus and pigment composition of two lines of *Paulownia* (*Paulownia tomentosa x fortunei*, *TF* and *Paulownia elongata x elongata*, *EE*) under salt stress.

Materials and Methods: Pigment composition, pulse amplitude modulated (PAM) chlorophyll fluorescence and redox kinetics of P_{700} were used for characterization of two hybrid lines of *Paulownia*: *Paulownia tomentosa x fortunei (TF)* and *Paulownia elongata x elongata (EE)* grown on non-saline (type 0) and two saline soils (types 1 and 2) with different salt content. The electrical conductivity of the soils is as follows: type 0 - 1.60 mS

cm^{-1}, type 1 - 6.3 mS cm^{-1} and type 2 - 14 mS cm^{-1}. The plants were grown in a greenhouse under controlled conditions (temperature 30 °C day/15 °C night, relative humidity 40% -65%). Details for the cultivation of the plants are given in Ivanova et al., [1]. For the experiments mature leaves from two-year-old plants were used. Seven replications (plants) were used for each treatment. The pigments were extracted from leaves with 80% acetone and were determined spectrophotometrically according to the equations developed by Lichtenthaler [2]. PAM chlorophyll fluorescence measurements on leaf discs were made by PAM fluorimeter (H. Walz, Effeltrich, Germany, model PAM 101-103). The measurements were made as in Dankov et al., [3]. The leaves were dark adapted for 30 min. The initial fluorescence level (*Fo*) was measured at instrument frequency of 1.6 kHz and measuring beam set at 0.02 $\mu mol.m^{-2}.s^{-1}$ PFD. For evaluation of maximal fluorescence level (*Fm*), saturating flashes of 2500 $\mu mol.m^{-2}.s^{-1}$ PFD with duration of 0.8 s were provided by Schott lamp KL 1500 (Schott Glaswerke, Mainz, Germany). The saturating flash gives *Fm* in dark-adapted state and *Fm'* in light-adapted state. The interval between two consecutive flashes was 60 s. The actinic light illumination (250 $\mu mol.m^{-2}.s^{-1}$ PFD) was provided by second Schott lamp KL 1500 for the induction of the photosynthesis. The redox state of P_{700} was investigated on leaf discs with a dual wavelength (820 nm) unit (Walz ED 700DW-E) attached to a PAM 101E main control unit [3].

The results were mean values from seven independent experiments. The statistical differences between the means were determined using ANOVA. Values of $p<0.05$ were considered as significant differences.

Results: Analysis of the pigment composition reveals that in *TF* line the amount of the chlorophyll increases (about 40%) in the plants grown in saline soils in comparison to those grown in non-saline soil, while in the other studied line *(EE)* the changes in the chlorophyll content are not registered. The increase of the chlorophyll in *TF* is similar in both soils (type 1 and type 2) with different salinity. In addition, the data reveal that salt stress does not influence the carotenoid (*Car*) content in both studied lines of *Paulownia*. The variation in the chlorophyll content in *TF* line is accompanied with an increase of the *Chl a/b* ratio (2.91 in non-saline soil and ≈ 3.13 in saline soils, $p<0.05$) and decrease of the *Car/Chl* ratio (0.38 - non-saline soil and ≈ 0.24 - saline soils, $p<0.05$).

The effect of the salinity on the studied lines of *Paulownia* (*TF* and *EE*) was examined by PAM chlorophyll fluorescence, which is a sensitive method for detection of changes induced in photosynthetic apparatus. The analysis of the PAM chlorophyll fluorescence curves shows that the maximum quantum yields of primary photochemistry in dark-adapted state (*Fv/Fm*) are similar in saline and non-saline soils (about 0.84) in both lines of *Paulownia*, indicating that the primary photochemistry of PSII is not affected by salinity. In contrast to primary photochemistry, the excitation transfer efficiency from antenna pigments to the reaction center of PSII in the light adapted state (*Fv'/Fm'*) in *EE* line is influenced in saline soils. The parameter *Fv'/Fm'* increases with 20% (0.54 in non-saline soil and ≈ 0.65 in saline soils, $p<0.05$) and this increase does not depend on the degree of the soil salinity. In addition, the data reveal increase of the coefficient of the photochemical quenching (q_P) in both lines in saline soils in comparison to the non-saline soil, i.e. increase of the PSII centers that are open (Q_A oxidized). The increase of the parameter q_P is about 62% in *TF* (q_P

- 0.40 in non-saline soil and ≈ 0.65 in saline soils, $p<0.05$) and 37% in the *EE* (q_P - 0.51 in non-saline and ≈ 0.70, $p<0.05$). With the increase of the soil salinity a substantial increase of q_P together with a decrease in non-photochemical quenching coefficient is occurred. In parallel to increase of q_P a rise in the effective photosynthetic electron transport rate (*ETR*) also is registered in plants grown in saline soils. Data reveal that *ETR* in *TF* line increases from 58 % to 78%, depending on the degree of soil salinity, while in *EE* line increases with 56% in both types of the soils.

The rate of Q_A^- reoxidation was measured by following the decay of fluorescence induction by a single saturated flash. The relaxation curves are fitted by two components (fast and slow). The half-time of the fast component in saline soils decreases in *TF* line (from 870 ms in non-saline soil to about 620 ms in saline soils), while in the other studied line (*EE*) increases (from 420 ms to about 550 ms). On the other hand the half-time of the slow component in plants grown in saline soils is higher in comparison to the plants grown in non-saline soil in both studied lines of *Paulownia*. The decrease of the half-time of the fast phase in *TF* line grown in saline soils suggests that salinization results in a stimulation of the electron flow from Q_A to plastoquinone pool. Data in the present study also reveal that the variation in Q_A^- reoxidation kinetics in the studied lines grown in saline and non-saline soils influences on the parameter R_{Fd}, which correlates to the net CO_2 assimilation [4]. In saline soils the parameter R_{Fd} increases in leaves of *TF*, while in the other line (*EE*) it decreases. In addition the decrease of R_{Fd} in *EE* depends on the degree of salinity, but its value remaines relatively high in comparison to the other plant species [4].

The effect of salt stress on PSI reaction centres was studied by measuring the steady-state P_{700} photo-oxidation by far-red light induced absorption changes around 820 nm and the subsequent reduction in the dark after turning off the far-red light. The post-illumination kinetics of P_{700}^+ re-reduction in two lines of *Paulownia* grown in saline and non-saline soils are fitted by two exponents with rate constants k_1 (fast rate constant) and k_2 (slow rate constant). Analysis of the P_{700} photo-oxidation reveals that the salinity decreases k_1 in leaves from both lines, while an increase of k_2 for the plants grown in saline soils is registered only at *TF* lines. On the base of these results it could be suggested that salinity influences PSI in stroma lamellae in both lines, while PSI in grana margin is influenced only in *TF* line.

Conclusions: In summary the results in the present study show that the studied *Paulownia* lines are tolerant to salt stress, as the tolerance is the higher for *TF* in comparison to *EE*.

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References:

1. Ivanova K., Dimitrova V., Georgieva T., Markovska Y., Gen. Plant Physiol., 4, 2014, 163-173.

2. Lichtenthaler H., Methods in Enzymology, 148, 1987, 350–382.
3. Dankov K., Busheva M., Stefanov D., Apostolova E., J. Photochem. Photobiol. B: Biology 96, 2009, 49-56.
4. Lichtenthaler H., Buschmann C., Knapp M., Photosynthetica, 43 (3), 2005, 379-393.

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ДОКЛАДИ/REPORTS

Тематично направление:
ЕКОЛОГИЧНО ЗЕМЕДЕЛИЕ

Topic:
ECOLOGICAL AGRICULTURE

ORGANIC AGRICULTURE – FEATURES, BENEFITS AND NEGATIVE EFFECTS
/A Review/

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Abstract

The benefits from conventional agriculture, which is based on the application of chemical methods of pest management and the use of synthetic fertilizers to improve soil fertility, are obvious. Their application has led to getting more and better crop production. But, from an ecological point of view, the introduction of large amounts of chemicals into the soil, where they fall in the water and plants, brings detrimental environmental outcomes. So that, the organic farming appears as an alternative method, which is based on the use of natural and environmentally friendly methods to control pests in crops. Organic farming integrates biological, mechanical, physical and chemical methods of farm management, without the use of synthetic pesticides and fertilizers, as well as GMOs. This helps for protection the soil characteristics and biodiversity of the ecosystems as a whole. Although the question in terms of yield quantity of the two methods and the costs, associated with them is controversial, the modern environmental science believes, that organic farming is a better agricultural alternative. Last but not of a less importance is the production of agricultural commodities, free of residual chemicals, which is significant for the protection of human health.

Keywords: organic agriculture/farming, pesticides, environment, ecosystem

Introduction

The modern agricultural practice is based on the use of various chemical substances, designed to destroy the pests, weeds and crop diseases, ultimately aiming to get more and better production and yield from the crops. Undoubted benefits of chemical introduction in the agriculture, however can cause a number of negative effects, both for consumers and for the environment, which is the most serious consequence of the application of chemical substances against pests and synthetic fertilizers to enrich the soil. This effect is particularly enhanced when chemical introduction in the agriculture is done improperly and does not comply with the requirements of good agricultural practices. Reduction and disappearance of many useful species, disrupting soil structure and deterioration of soil fertility, the emergence of resistance in pests are some of the most dangerous consequences for the environment in global aspect. The damage of human health, due to the presence of pesticide and other chemical residues in food is also a negative effect, due to the modern agricultural practices, which is based on synthetically produced enhancers.

An alternative to the conventional farming is the so called organic agriculture, whose main purpose is the production of environmentally friendly yield, free of residual substances in it, while conducted with care for the environment and ecosystem protection. Efficiency of agricultural programs for biodiversity conservation and improvement of the environment is a matter of particular importance in recent years. According to USDA (Ministry of the Agriculture of USA), organic agriculture is a management system of ecological production, encouraging and enhancing biodiversity, biological cycles and soil biological activity. It is based on minimal use of non-natural products in agriculture and the application of natural ones, and management practices that restore, maintain or enhance ecological harmony. The main goal of organic agriculture is to optimize the health and productivity of interconnected and interdependent communities of soil, plants, animals and people [1].

Agriculture dates back to about 10 000 years, as pests and plant diseases have always caused losses and have been a major problem for farmers. The conventional agriculture (applying natural remedies and fertilizers) is the original form of agriculture and has been practiced for thousands of years. Synthetic fertilizers appeared for the first time in the 18th century and have become famous because of their low price, powerful impact on the plants and the easy transport. Similar benefits have also the chemical pesticides in the 40s years of the 20th century. But, these new agricultural practices, which were effective in the short term, have serious long-term effect, expressed in compaction and erosion of the soil and also, reducing its overall fertility. Not least in importance is the fact, that these processes are together with a negative effect on human health, due to the toxic chemicals, that enter into the food and human body, as a result [2].

As it is known, conventional modern farming uses chemically produced, water-soluble pesticides and fertilizers, which are purified synthetically. This practice has gained a big popularity, mainly due to the strong and rapid effect, that substances have on diseases and pests, as well as on fruiting. The quantities of chemicals, which are necessary for the treatment of some object or area, are incomparably less than the organic substances and fertilizers, which makes them much easier for transport and application.

Unlike conventional, the organic agriculture is limited in the use of natural pesticides and fertilizers only. Organic farming methods combine the scientific knowledge in ecology and modern technology with traditional agricultural practices, based on natural biological processes. Organic agriculture works in harmony with the nature, not against it. This includes the use of techniques to achieve a good harvest from the crops, without harming the natural environment or the health of people, living and working there.

Key features of organic farming

Biological farming is possible only in ecologically clean regions; it works without the use of chemical pesticides, herbicides and inorganic (synthetic) fertilizers. It has been suggested that this system improves biodiversity in agricultural landscapes. Through application of organic farming, usually there is an increase of species richness, which gives an average of 30% higher species diversity than conventional farming systems. Birds, insects and plants usually show increased species diversity in the system of organic farming [3, 4].

Typical organic farming is a form of agriculture, which includes the following features: application the technique of crop rotation, which is a prerequisite for the effective use of resources from the soil; the so called "green manure" is also used; application of manure composted materials and biological pest control is another feature. The „green manure" is a process, whereby turning the soil is done with it growing on her green mass; the impact of green manure is similar like this of animal manure – enriches the soil with valuable microelements and nitrogen. Organic agriculture applies fertilizers and pesticides, which include herbicides, insecticides and fungicides, if they are considered natural (as bone meal from animals and pyrethrin from flowers) or if they are biopesticides (those, containing biocontrol agents such as various bacteria, viruses, fungi, etc.). But, the application of different methods, including synthetic fertilizers or pesticides, plant growth regulators and hormones also, are excluded or strongly limited; application of antibiotics in livestock is recommended to be avoided; the use of genetically modified organisms and sediments from human sewage is strongly forbidden. The organic farming take advantage of the resources of the space, such as manure for fertilizing or feed, produced in the same farm; plant and animal species that are resistant to diseases and adapted to the local conditions are selected; organic farming is also based on the use of animal breeding practices appropriate to different species [5].

Organic agriculture uses a variety of methods to improve soil structure and fertility, mainly relying on the natural decomposition of organic matter, such as composting of vegetable waste and animal fertilizers and also, their application in the soil. Except by applying the green manure techniques, organic farming works by reducing the soil processing, to absorb nutrients taken from the soil by previous crops. By reducing the processing, the soil is not inverted and the emission of carbon into the atmosphere is reduced. Crop rotation and the green manure help providing nitrogen into the soil by legumes, which fix nitrogen from the atmosphere through symbiosis with the root bacteria. Producers of organic harvest also use manure from the animals, some processed enhancers as ground seeds and various minerals, which are naturally occurring. Biological investigators of the soil and organisms living in the soil, have proved the benefits from

the organic farming. Many bacteria and fungi break down chemicals, plant materials and animal waste into useful nutrients.

The diversity of cultures is another distinctive feature of organic agriculture. Conventional agriculture focuses on the mass production of a kind of culture on an agricultural area, which in practice is called monoculture. The science agroecology has revealed the benefits of polycultures (growing multiple crops in one place), which is often used in organic farming. Planting many different cultures supports a wide variety of beneficial insects, soil microorganisms and other factors which contribute to the overall health of the farm. The diversity of cultures also helps the protection of species from extinction [6].

Organic farming integrates biological, mechanical, physical and chemical methods of weed control, without the use of synthetic herbicides, applying suppression of the weeds instead of their disposal. Mechanical and physical methods for weed control, applied in organic farming can be broadly grouped as: ploughing – turn the soil for incorporation of crop residues and soil improvers, removing weeds and preparation of planting areas; mowing and trimming the tops of weeds; destroying weed thermal or by incineration; mulch – blocking emergence of the weeds with organic materials, plastic films or fabrics. Mulching the soil is also a method for improving its performance and represents the creation of artificial cover (mulch) on the surface of the soil between the plants. Coating is done with plant or different synthetic materials. The purpose of the mulching is to maintain the water supply of the soil by reducing evaporation of soil moisture and improve water absorption ability; also increases the humus content in the soil, when organic materials are used; keeps the soil moist and loose, protects it from severe temperature fluctuations, and prevents weed growth also.

Unlike conventional farming, organic avoids the use of synthetic pesticides, which are considered to damage the environment and human health, as is mentioned above. In organic farming five pesticides are mainly used: bacteria, pyrethrin, rotenone, copper and sulfur. Synthetic pesticides permitted for use in organic farming include insecticidal soaps and horticultural oils for pest management; bordeaux mixture, copper hydroxide, and sodium bicarbonate to the control of fungi. Organic pesticides often complement other strategies to combat pests, such as IPM technology (Integrated Pest Managemen), which applies natural processes for control [7].

Some chemicals of natural origin are authorized for use as herbicides in agriculture. These include certain formulations of acetic acid (vinegar concentrate), gluten from maize meal and essential oils. Some selective bioherbicides, based on fungal pathogens, are also applied [8]. Naturally derived insecticides, permitted for use in organic farming include *Bacillus thuringiensis* (soil bacteria), pyrethrum (an extract of chrysanthemum), spinosad (bacterial metabolite), neem (extract from the same tree) and rotenone (extract of the roots of legumes). Naturally derived fungicides used in organic farming include bacteria *Bacillus subtilis* and *Bacillus pumilus*; fungi *Trichoderma harzianum*. They are effective primarily to diseases affecting the roots. Mixtures of composting contain a mix of beneficial microbes, which can attack or to compete with the some plant pathogens.

For the control of other harmful organisms, including arthropods (mites), nematodes, fungi and bacteria, the organic practice includes the following methods: encouraging

predatory beneficial insects and beneficial microorganisms; a rotation of crops at different places, in order to break the cycle of pests; planting crops and plants that repel pests; usage of covers to protect crops during the periods of migration of pests; use of biological pesticides and herbicides; use of sanitation to remove the habitat of pests; use of insect traps for monitoring and control of insect populations; use of physical barriers [9].

Benefits and negative effects of organic agriculture

A wide range of organisms benefit from organic farming: birds, butterflies, soil microbes, beetles, earthworms, spiders, various types of vegetation, and some kind of mammals. The lack of herbicides and pesticides leads to improved biodiversity and density of population. Many weed species attract beneficial insects that improve soil quality. Soil - inhabiting organisms also benefit due to the increased amount of bacteria, because of the presence of natural fertilizer and reduced intake of pesticides and other chemical substances. Organic farming is beneficial for people also, because of the use of natural products and reduced human intervention in the farming processes (4).

In organic farming soil losses are very limited and the content of soil organic matter is increased. Soil biochemical and environmental performance are also improved. Soil, cultivated through organic farming has a higher water content than conventionally managed, resulting in higher yields of organic farming, compared to the conventional, under conditions of water deficit. Due to the high ability to store carbon in soil, the organic agriculture would constitute a means of improving the CO_2 concentration [10].

Besides the above advantages, due to the implementation of organic farming a cleaner agricultural production is obtained, free from chemical residues, which is crucial for the health of consumers [11].

It is well known, that intensive conventional farming may introduce contaminants into the food chain. It is widely believed that organically produced food satisies the requirements for obtaining agricultural products without chemical residues in it, thereby reducing the damage to the environment and increasing the nutritional value of products. Organically produced crops contain less nitrates, nitrites and pesticide residues, but more dry matter, vitamin C, phenols, essential amino acids and total sugars, compared with conventionally grown plants. Statistically, organic crops contain more minerals and retain their properties in long-term storage [12].

What is the attitude of the organic agriculture to ecosystem services – that are the benefits for human existence that originate from well-functioning ecosystems, mainly the production of oxygen, soil genesis and detoxification of water. Organic agriculture both uses and maintains the ecosystem services. Therefore, it is more resistant than conventional agriculture, which breaks down some ecosystem services. Besides the provision of ecosystem services, organic farming is able to contribute to the global food supply. It offers great potential for the development of cheaper food production, using locally available green technologies, without causing damage to human health and the environment, which is of a great importance for providing food for the growing human population [13, 14].

Regarding the health of consumers, though not fully proven, but it is assumed that the production grown in terms of organic farming is more beneficial to the human body than

conventionally produced fruits and vegetables [15]. Furthermore, due to non-application of synthetic pesticides and other chemicals used in conventional agriculture, they are not expected residues thereof. In this situation, damages to human health, because of acute or chronic effects from the presence of substances can not be caused.

The question regarding the implementation costs of the two types of agriculture – which method is cheaper, remains controversial. And also, it is not possible to say unambiguously, which of the two practices leads to more production, because for now, traditional methods are considered more productive. But, when the best methods of organic practice are used, the difference between the two techniques is about 8-13%, in favor of chemical treatments [16]. According to the authors [12, 17], plants cultivated in organic system have 20% lower yield as a whole, in comparison with conventionally grown crops or, in other words, yields from organic farming are on average 80% of those, produced in conventional.

Besides the positive effect of the organic farming application, it also has some negative consequences: copper sulfate, copper hydroxide and bordeaux mixture may be a problem for the environment. Their frequent use as fungicides may lead to the accumulation of toxic levels of the element copper in the soil. In the European Union, where the replacement of copper-containing fungicides in organic farming is a priority of agricultural policy, researchers are looking for alternative methods for organic production.

Although the view that organic farming has a high energy efficiency (ratio "input/ received" has a positive balance) the average, indicating a low yield, thereby resulting in a reduction in productivity [10]. It is necessary to consider some important problems, such as bacterial and fungal contamination of organic products, and the most important issue: the impact of the consumption of organic food on the health of animals and humans, as this issue has not yet been fully studied [12].

When balancing the benefits of organic and conventional agriculture, a number of factors should be taken into account, because there are no simple criteria by which to determine the better of the two methods in each farm situation. It is necessary to evaluate the costs and benefits of different management options. But finally, to achieve sustainable food security, probably it is necessary to combine different types of techniques, including organic, conventional and hybrid systems, which are able to produce more food at affordable prices [18].

In general, it can be concluded that organic farming is a better alternative to conventional agriculture and provides a number of advantages for the environment by stopping the use of hazardous chemicals and their distribution in the environment and food chain is strongly limitted.

With regard to the future of organic farming, there is a need to explore and study its potential to reduce the impact of farming practices on the environment. The consequences for the socio-economic system of reduced productivity due to organic farming, should also be taken into account and appropriate agricultural policies for the management of agriculture should be developed [10].

Conclusion

Organic farming is a modern system (or group of systems) that is relying more on biological processes rather than chemical, to achieve fertilizing of the soil and pest control. It belongs to a farming system that increases soil fertility through the maximum utilization of local resources, and restricts the use of agrochemicals, GMO and all kinds of synthetic compounds. Organic agriculture counts on the farming practices, based on ecological cycles and seeks to minimize the impact of the food industry on the environment, ensuring the long term sustainability of the soil and also, aims to minimize the use of chemicals.

Of a particular importance is the impact on biodiversity: organic farming systems generally have higher potential to maintain a high biodiversity in terms of flora and fauna, compared with conventional systems of agriculture.

Although the differences between production costs in conventional and organic farming are small, there are significant benefits for human health and the environment, which can be achieved through more widespread adoption of organic farming. Through the application of some regulatory measures from the authorities, such as providing subsidies to the production costs, combined with proportional tax on pesticides and fertilizers tax, provide measures by which it is possible to promote the transition from conventional to organic farming.

References:

1. Gold M. National Agricultural Library, USDA, March, 2014. http://www.nal.usda.gov/afsic/pubs/ofp/ofp.shtml

2. Aubertot J. N., Barbier J. M., Carpentier A., Gril J. J., Guichard L., Lucas P., Savary S., Savini I., Voltz M. (éditeurs). Pesticides, agriculture et environnement. Réduire l'utilisation des pesticides et limiter leurs impacts environnementaux. Expertise scientifique collective, synthèse du rapport, INRA et Cemagref (France), 2005, 64 p.

3. Hole D. G., Perkins A. J., Wilson J. D., Alexander I. H., Grice P. V. Biological Conservation., 122 (1), 2005, 113–130.

4. Bengtsson J., Ahnström J. and Weibull A. J. of Applied Ecology., 42 (2), 2005, 261–269.

5. Directorate General for Agriculture and Rural Development of the European Commission. What is organic farming? http://ec.europa.eu/agriculture/organic/organic-farming/what-is-organic-farming/index_en.htm

6. Pimentel D. P., Hepperly P., Hanson J., Douds D. and Secaев R., BioScience, 55 (7), 2005, 573–582.

7. Smith-Spangler C., Brandeau M. L., Hunter G. E., Bavinger J. C., Pearson M., Eschbach P. J., Sundaram V., Liu H., Schirmer P., Stave C., Olkin I., Bravata D. M. Annals of Internal Medicine., 157 (5), 2012, 348–366.

8. Kremer R. J., Li J., Soil & Tillage Research., 72, 2003, 193–202.

9. Lotter D. Journal of Sustainable Agriculture., 21 (4), 2003, 59.

10. Gomiero T., Pimentel D. and Paoletti M. G. Critical Reviews in Plant Sciences., 30 (1-2), 2011, 95-124.

11. Bourn D., Prescott J. Crit. Rev. Food Sci. Nutr., 42 (1), 2002, 1–34.
12. Rembiałkowska E. Journal of the Science of Food and Agriculture., 87 (15), 2007, 2757–2762.
13. Porter J., Costanza R., Sandhu H., Sigsgaard L., Wratten S. AMBIO: A Journal of the Human Environment., 38 (4), 2009, 186–193.
14. Sandhu H. S., Wratten St. D., Cullen R. Environmental Science & Policy., 13 (1), 2010, 1–7.
15. Brandt K. and Mølgaard J. P. Journal of the Science of Food and Agriculture., 81 (9), 2001, 924–931.
16. Badgley C., Moghtader J., Quintero E., Zakem E., Chappell M. J., Avilés-Vázquez K., Samulon A. and Perfecto I. Renew.Agr. Food Syst., 22, 2007, 86–108.
17. De Ponti T., Rijk B., van Ittersum M. K. Agricultural Systems., 108, 2012, 1–9.
18. Seufert V., Ramankutty N., Foley J. A. Nature, 485, 2012, 229–232; http://serenoregis.org/wp-content/uploads/2012/06/nature11069.pdf

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ДОКЛАДИ/REPORTS
Тематично направление:
ЕКОСИСТЕМНИ ИЗСЛЕДВАНИЯ И УСЛУГИ

Topic:
ECOSYSTEM RESEARCH AND SERVICES

ABIOTIC ENVIRONMENTAL FACTORS AND THEIR RELATIONS TO PRIMARY PRODUCTIVITY OF CARP FISH PONDS

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Abstract

There are about 3000 ha fresh water fish farms for thermophilic fish species in Bulgaria in which common carps, big head carps and grass carps are grown by polyculture technology. The most appropriate water bodies for rearing of carp fishes are the man made reservoirs and earthen fish ponds where the polyculture technology guarantees the best utilization of available food basis.

Aim: The aim of the study was to investigate the effect of organic manure on plankton primary production of fish ponds.

Materials and Methods: The experiment was carried out on seven earthen fish ponds of the Institute for Fishery and Aquaculture in Plovdiv town in the period 2004-2006. The measuring of plankton primary production was carried out by light and dark bottle technique in its oxygen modification.

Results: The application of organic manure caused better development of phytoplankton by increasing intensity of photosynthesis, which is reflected by light conditions suppressing the unfavorable macrophyte development, by statistically significant higher permanganate oxidability, higher concentrations of phosphate ions and better N/P ratio (15/1) in manured than in control fish ponds. The size of primary production in fish breeding gives a good estimation of the result of applied ameliorative measures (i.e. manure) as a first step for achieving the natural fish productivity.

Keywords: primary productivity, carp fish ponds, environmental factors

Introduction

Fishponds are specific open water systems inhabited by communities, whose activities depend on different biotic and abiotic environment factors to which organisms respond by general or specific reactions. The primary production belongs to biological characteristics of aquatic ecosystems and represents their natural food basis.

There are several transitional chains, biological and biochemical processes between it and the final fish production. The primary production is obtained in the course of photosynthesis and is positioned at the bottom of the food chain. Therefore by measuring the speed of photosynthesis we could estimate the primary productivity and get insight into general regularities of biotic transformations of matter and energy in aquatic ecosystems in order to solve important questions related to fish breeding or the sanitary-technical exploitation of waters.

Thus the study of primary production in carp fish ponds and its relations to biotic and abiotic environmental factors as well as the estimation of their strength and influences on it are of great scientific interest and have practical significance.

Materials and Methods

The Institute of Fishery and Aquaculture, Plovdiv is located in the western part of the Upper Thracian valley. The region is characterized by a transitional-continental climate. The study is carried out in the Plovdiv basis during three consecutive years (2004, 2005 and 2006). The ponds are supplied with water from the Maritsa River by means of the "Eni-Ark" irrigation canal.

Seven earthen ponds are involved in the experiment, whose individual size varies between 1.8 and 3.9 da and the mean depth is of about 0.8 m. After Zhang et al. [1] ponds of this size are among the most productive and easy for management. Their bottom is silty but the periphery and the shallowest parts of some of them have a strip of 1-2 m width with increased content of sand.

The size of ponds, their shallowness, vertical and horizontal homogeneity are a part of the preconditions for choosing them for a model object.

About 3000 kg.ha^{-1} mineralized manure was applied to ponds No 6, 12 and 17 once in April each year. Additionally to the natural food, grain forage was given to fish according to a scheme related to their seasonal growth rate. The periodical examination did not reveal any fish diseases.

The applied polyculture technology includes mixed breeding of 30 individuals da^{-1} one-year old bighead carp (T_1), (*Aristhichthys nobilis* Rich.), 50 exemplars da^{-1}of carp (K_1), (*Cyprinus carpio* L.) and 10 exemplars da^{-1} of grass carp (one and two-year) ($A_{1/2}$), (*Ctenopharyngodon idella* Val.) according to Nikolova et al., [2, 3]. The scheme of the experiment is presented on Table 1.

Table 1. Scheme of the experiments in years 2004, 2005 and 2006

Year	Variants of breeding			
	Fertilized ponds, num.da^{-1}		Control ponds, num.da^{-1}	
	Pond No, area in brackets (da)			
2004	6 (3.8)	17 (2.6)	8 (3.8) 15 (3.1)	16 (2.7) 18 (1.8)
2005	12 (3.9)	17 (2.6)	8 (3.8)	16 (2.7)
2006	12 (3.9)	17 (2.6)	8 (3.8)	16 (2.7)

Each fishpond was sampled on one station localized 1-2 m from the shore before the outlet device (savak). The sampling was carried out fortnightly between 8:30 and 11:00 a.m. in the period May-September of th years 2004 and 2006 and in June-September of year of 2005 (Table 2). The final sampling was carried out in the last decade of September. Some of the samples were taken with one to three days difference due to the large number of investigated characteristics.

The collection of water samples was carried out from the 0.3-0.5 m surface layer according to the Bulgarian and European standards [4]. The majority of the samples were processed immediately after sampling. A total of 1120 samples for determination of eight characteristics were analyzed.

The total sun radiation in MJ m^{-2} was recorded by means of a pyranometer type M 80 M. The water column transparency was determined by the Secchi method. For the three years a total of 140 measurements were carried out.

Table 2. Standard methods applied for determination of the physicochemical characteristics of water samples

№	Characteristics	Applied standard
1	Water temperature, °C	BSS 17.1.4.01-77
2	pH	BSS 3424-81, ISO 10523, 1994
3	Dissolved oxygen, $mg.l^{-1}$	BSS EN 25814-2002
4	Oxygen saturation, %	BSS EN 25814-2002
5	Permanganate oxidability, $mg.l^{-1}$	BSS ENISO 8467, 2001
6	Ammonium nitrogen, $mg.l^{-1}$	BSS 3587-79, ISO 5664
7	Nitrate nitrogen, $mg.l^{-1}$	ISO 7890-3, 1998
8	Orthophosphate, $mg.l^{-1}$	BSS ENISO 6878-1:2004

The primary productivity ($g.O_2.m^{-2}.24\ h^{-1}$) was determined by means of light and dark bottle technique in its oxygen modification. The bottles were filled with water from the pond taken by and homogenized in a plastic bucket of a volume of 10 l. Three pairs of two light and two dark bottles were exposed in 0.1, 0.3 and 0.5 m depth layers for a period equal to the second and third of the five equal time intervals in which the day length was separated (in this interval about 55-60% of the total daily production was synthesized according to [5]. The exposure depths depend on the measured Secchi transparency and are in the range 0.25.S - 3.S approximately.

Due to the big productivity of fishponds we have performed the procedures more frequently to shorten the exposure time in order to avoid oversaturation with oxygen of the water in the bottles and its release in gaseous phase as bubbles. For this reason in the majority of cases the exposure time took one hour. In order to avoid the problem with oversaturation of water with oxygen an original author's methodology was developed. The sampled water was moved to an empty plastic bottle twice the volume of the taken water. Then the bottle was pressed by hand till the liquid reached the bottleneck. Then the bottle was closed tightly and vigorously shaken. The elastic bottle walls tried to return to their normal position thus creating underpressure inside the bottle which caused the excessive oxygen to move into the gaseous phase. In this way the oxygen concentration in the water gets lower than the saturation value under the instantaneous atmospheric pressure and no bubbles emerge in the bottles during the exposure. The obtained productivity values are given for 1 m^2.

The diverse characteristics of fishponds presented by big number of measurements offer the possibility to apply statistical methods. The difference between manured and control ponds was tested by means of the Wilcoxon rank paired test with statistical package STATISTICA 7.0 [6, 7, 8].

Results

The seasonal variations of water temperature in all three years follow the seasonal dynamic of sun radiation. In more than 70% of the experiment duration the water

temperature was in the range of 20-26°C, which coincided with the temperature optimum of phytoplankton and reared fish species.

The majority of the recorded sun radiation values were of sufficient intensity for the shallow fishponds, especially bearing in mind that according Hickman [9] the light is limiting the photosynthesis only in deeper waters.

The nutrients were presented by the measurements of the concentrations of inorganic forms of nitrogen and of phosphate phosphorus, which were used to calculate the N/P ratio. The latest was obtained by dividing the sum of the concentrations of inorganic nitrogen forms (nitrate, and ammonium) by the phosphate phosphorus concentration. As shown on Fig. 1 in the majority of sampling dates the value of N/P ratio varies around 16, which is the optimum for phytoplankton. In experimental ponds this ratio is determined mainly by the concentrations of nitrate and phosphates because the concentrations of ammonium and nitrites are very low.

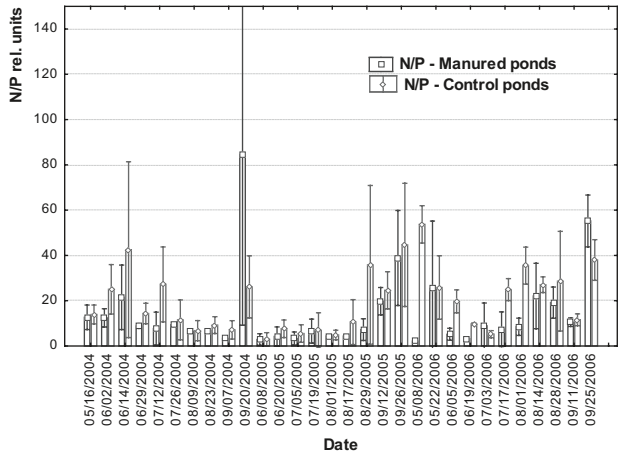

Fig. 1. The N/P ratio in manured fishponds during the experiments in years 2004, 2005 and 2006

The dynamics of the N/P ratio did not show any definite patterns between the months or years during the investigated periods. The values are varying around the optimum and only on restricted number of dates they are higher than 16, which indicate phosphorus limitation due to either by phosphorus depletion or by entering of waters rich in nitrogen. In general the variability of the N/P ratio is higher in the beginning and at the end of the experiment. During the summer months (June, July and August) the values of manured ponds are close to the optimum, while those of the controls are a little higher.

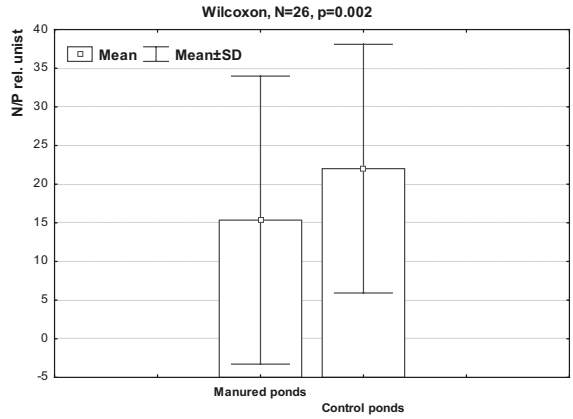

Fig. 2. The arithmetic average values of N/P ratio of manured and control fishponds from all three experiments carried out in years 2004, 2005 and 2006

The basic goal of the applied manuring is to create optimal trophic conditions for phytoplankton development, which is a precodnition for the next levels of the food chain. However sustaining a definite high level of nutrient concentrations during the whole experiment duration is a difficult task. Therefore the achieving of the average N/P ratio of about 16 by the application of organic fertilizer might be considered as a definite success. Such a value favours the development of valuable green algae [10, 11] which are utilized by planktonic grazers in the food chain in fishponds. In opposite, the values of the N/P ratio lower than 16 favor the development of blue-green algae, which is observed in a large number of lakes in the northern hemisphere (USA [12], France [13]) as well as in the southern hemisphere (South Africa [14]). According to Qin and Culver [15] the continuous release of nutrients in the N/P ratio close to 16 can restrict the development of blue-greens. In our experiment the N/P ratio of 22 in the control ponds was statistically different from the fertilized ponds ($P = 0.002$). It seems to be caused by the lower concentrations of phosphate phosphorus in control ponds, where a weak phosphorus limitation is indicated (Fig. 2). The effect of manuring on the other measured abiotic characteritistics of the fishponds is summarized in Table 3.

Table 3. The arithmetic average values of fertilized and control ponds and results of their comparison by Wilcoxon in paired test. The statistically significant differences are indicated by bold font

Characteristics	Arithmetic average Values		Level of significance
	Fertilized ponds	Control ponds	
Secchi transparancy	**0.250 m**	**0.295 m**	**p=0.004**
pH	8.38 mg.l^{-1}	8.29 mg.l^{-1}	p=0.345
Dissolved oxygen	9.56 mg.l^{-1}	9.36 mg.l^{-1}	p=0.909
Permanganate oxydability	**16.8** mg.l^{-1}	**13.9** mg.l^{-1}	**p=0.037**
Ammonium nitrogen	0.127 mg.l^{-1}	0.111 mg.l^{-1}	p=0.548
Nitrate nitrogen	1.2 mg.l^{-1}	1.2 mg.l^{-1}	p=0.122
Phosphate phosphorus	**0.38** mg.l^{-1}	**0.24** mg.l^{-1}	**p=0.003**

The differences between the values of nutrient concentrations and their averages on Table 3 indicate the „bottom-up" effect caused by the application of organic fertilizer. The following statistically significant differences and favorable conditions as a result of the applied manuring are observed:

• More favorable light regime in manured (0.25 m Secchi transperancy) in comparison to control (0.295 m) ponds; this suppreses the development of macrophytes at the expense of better light conditions for the phytoplankton in manured ponds. The growth of macrophytes in fishponds is not desirable and the manuring suppresses its developmet.

• Permanganate oxydability is higher in manured (16.8 mg.l^{-1}) than in control (13.9 mg.l^{-1}) ponds which indicates a higher organic content resulting from the introduced organic fertilizer and subsequent phytoplankton development.

• About 62.5% increase of the concentrations of phosphate ions in manured (0.38 mg.l^{-1}) than in control (0.24 mg.l^{-1}) ponds. Obviously the fertilizing increases the phosphorus concentration, which is the usual limiting element of phytoplankton.

The averages of the other characteristics presented on Table 3 like the concentrations of oxygen, ammonium and pH are also higher in manured fishponds but the differences to control ponds are not statistically significant. The nitrate concentrations, wind speed and hydraulic residence time were also recorded but seemed to have no definite effect on treatment and control ponds.

Conclusions

1. The fertilization of fishponds with 3000 kg.ha^{-1} mineralized manure creates conditions for increased photosynthesis and better development of phytoplankton reflected by the following statistically significant differences between the fertilized and control ponds:

• better underwater light conditions caused by the increased turbidity favoring the phytoplankton and suppressing macrophyte development

• higher permanganate oxidability of fertilized than control fishponds

• by 62.5% higher concentration of phosphate ions of fertilized than control fishponds

• better N/P ratio in fertilized (15/1) than in control (22/1) fishponds, favoring in the first of them the development of green algae, which are more efficiently utilized by the plankton grazer food chain while the ratio is not very low in order to cause development of blue greens.

2. The arithmetic averages of pH and the concentrations of oxygen and ammonium nitrogen are higher in the fertilized fishponds but the differences are not statistically significant.

3. The wind speed and hydraulic residence time were also recorded but seemed to have no definite effect on treatment and control ponds.

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References:

1. Zhang F. L., Y. Zhu X. Y. Zhou. Aquaculture, 60 (2), 1987, 107-116.

2. Nikolova L., Hadjinikolova L., Dochin K., Terziyski D., Atanasova R., Stoeva A., Grozev G., Paskaleva E. Bulg. J. Agric. Sci., 14 (2), 2008a,133-138.

3. Nikolova L., Hadjinikolova L., Dochin K., Terziyski D., Atanasova R., Stoeva A., Grozev G., Paskaleva. E. Bulg. J. Agric. Sci., 14 (2), 2008b, 139-144.

4. Water Framwork Directive 2000/60/EU, http://ec.europa.eu/environment/water/water-framework/index_en.html

5. Vollenweider R. A., Talling J. F., Westlake D. F. A manual on methods for measuring primary production in aquatic environments, Oxford and Edinburgh, Blackwell Sci. Publ., 1969, p. 213.

6. Sokal R. R. and Rohlf F. J. Biometry: the principles and practice of statistics in biological research. W. H. Freeman and Company Publisher, New York, Third edition, 1997, p. 887.

7. Fowler J., Cohen L. and Jarvis P. Practical statistics for field biology Chichester, New York, Brisbane, Singapore, Toronto, 1998, p. 259.

8. McGarigal K., Cushman S. and Stafford S. Multivariate statistics for wildlife and ecology research. Springer Publisher, New York, Berlin, Heidelberg, Barcelona, Hong Kong, London, 2000, p. 283.

9. Hickman M. Oecologia, 6, (3), 1971, 238-253.

10. Левич А. П., Булгаков Н. Г. Потребности планктонных водорослей в субстратных и энергетических ресурсах среды: концепция и измерения. Успехи современной биологии. т. 117, № 1, 1997a, с. 107-121.

11. Левич А. П., Максимов В. Н., Булгаков Н. Г. Теоретическая и экспериментальная экология фитопланктона: управление структурой и функциями сообществ. М., Изд-во НИЛ, 1997b, с. 192.

12. Stockner G., Shortreed S. Limnol. and Oceanogr., 33, 1988, 1348-1361.

13. Michard M., Aleya L., Verneaux J. Archiv fur Hydrobiol., 135 (3), 1996, 337-359.

14. Haarhoff J., Langenegger O., Der Merwe P. J. V. Water S. Afr., 18, 1992, 27-36.
15. Qin J., Culver D. A. Hydrobiologia, 321, (2), 1996, 109-118.

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КРАТКИ СЪОБЩЕНИЯ/SHORT COMMUNICATIONS
Тематично направление:
ЕКОСИСТЕМНИ ИЗСЛЕДВАНИЯ И УСЛУГИ

Topic:
ECOSYSTEM RESEARCH AND SERVICES

SIZE, GROWTH AND DIET OF BLUEFISH (*POMATOMUS SALTATRIX*) IN THE BLACK SEA, BULGARIA

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Introduction: In the last 3 decades, the abundance of the Black Sea predatory species such as bonito (*Sarda sarda*) and bluefish (*Pomatomus saltatrix*) has been severely reduced not only by fishing, but also as a result of pollution. Bluefish is not a substantial target for the Bulgarian commercial fishery, but as a piscivore it is a significant component in the trophic interrelations between the Black Sea's pelagic fishes.

P. saltatrix is a marine, migratory, pelagic species found throughout the world in most temperate coastal regions. It enters the Black Sea in spring for feeding and spawning, and moves back to the Sea of Marmara in winter.

Aim: In the Black Sea region *P. saltatrix* is poorly investigated; therefore, our aim was to explore the bluefish diet, size and growth data, and perform comparisons between our survey (2013-2014) and data from historical studies conducted by Stoyanov [1], Turgan [2], Kolarov [3] and Taranenko [5] (late 1950s, 1960s and 1970s).

Materials and Methods: The samples were collected during the 2013-2014 autumn seasons, from the landings in the Burgas Bay fishing area. A total of 773 individuals were processed for the determination of:

1. Population parameters: total body length (TL, cm), total body weight (W, g), age (otolith reading) and Fulton's condition factor (*cf*, a standard measure for fish health).

2. Stomach content analyses in order to investigate the importance of a given prey type in the bluefish's diet, expressed by an Index of Relative Importance (% IRI) and Frequency of Occurrence (%F) index. Predator feeding intensity was expressed by the Gastro-Somatic index (%Ga.SI).

A comparison between the 2013-2014 and the 1959-1971 periods was implemented, based on the population parameters data.

A linear regression model (R^2=98) was used to adjust the total body length to the fork length (FL, cm) - the preferable measurement in the historical investigations.

Results and Discussion: Young and juvenile individuals aged 0 years (59.9%) and 1 year (33.2%) were mainly represented in the bluefish landings in 2013-2014, while in the 50s, 60s and 70s, sexually mature specimens aged 2 years and older were dominant in the catches, with the complete absence of age group 0.

During the investigated years, the individuals at the age of 1 had similar average body length and weight. The lowest average body weight values were registered in 1967 and 2013 (71g and 71.3 g, respectively), and the highest was recorded in 1970 (169 g). The average body length values ranged from 24 cm FL (1961) to 14.5 cm FL (1962), and the bluefish collected in 1970 and 2014 had the same FL values (18.5 cm).

In 2013-2014 the individuals from the 2nd and the 3rd age classes had significantly lower values of their population parameters. The average values of the body length at the age of 2 were as follows: 24.5 cm FL (2013), 23.1 cm FL (2014) and between 28.7 cm FL and 36.5 cm FL for 1959-1971 period. The average body weight values were: 196 g (2013), 182.3g (2014) and between 408 g and 718 g in 1967-1971. The same significant differences were also recorded for the specimens at the age of 3: 28.8 cm FL (2014); 38.8 - 47.4 cm FL (1967-1971), and average body weight: 315.4 g (2014); 890 - 1000 g (1967-1971). In 2013-2014, the bluefish catches were characterized by the absence of individuals older than 3 years of age.

Compared with the 60s and 70s, in the modern period the specimens from the age groups of 2 and 3 showed a significantly slower growth rate expressed as the average annual weight and length increments. Our results show that the weight and length increments of those age groups were almost three times lower in the current period. Fulton's condition factor also showed considerably lower average values in our investigations than those registered in the 60s and 70s.

In another study concerning *P. saltatrix*'s biology, Kolarov [4] reported a similar situation of slower growth rates for bluefish individuals at the age of 3 and 4. The noted reason for this phenomenon was the insufficient food resources, a fact supported by the complete absence of food in these age groups' stomachs. In 2013, food was found in only 23% of the analyzed bluefish stomachs. The most important food item in *P. saltatrix*'s diet was found to be the horse mackerel, with values of % IRI > 99 and % F = 91.1. In 2014, the percentage of full stomachs was higher (42.5%) and the most preferable food item was the Black Sea sprat, with values of % IRI = 77.2 and %F = 45.8. Other important victims in the bluefish diet were the horse mackerel and the Black Sea anchovy. *Pomatomus saltatrix* feeds mainly on small pelagic fish species whose abundance is a crucial factor for the good state of this piscivore's population.

The Ga.SI shows that the feeding intensity of *P. saltatrix* individuals decreases with the body length increments. The bluefish specimens in the largest size groups mainly had totally empty stomachs. This absence of food and the lower (3 times) growth increment's values in the largest bluefish specimens indicated that in 2013-2014 autumn seasons, the oldest fish of the *P. saltatrix* population were probably starving.

Conclusions: Bluefish reaches sexual maturity during the second year of life [1]. Although in the Bulgarian Black Sea waters the fishing intensity on this fish resource is not significant, the predominance of young individuals in the bluefish landings (93% for 2013-2014) is a serious problem requiring the attention of fisheries scientists and managers.

Compared to the historical data, in the modern period, the older bluefish specimens showed a significantly slower growth rate. This phenomenon could be due to an insufficient food supply, since of the total stomachs examined, 74% were empty and the lowest feeding intensity was registered in the largest size classes [4]. Other factors, including anthropogenic pressures, could also be involved, and more research is needed to ascertain its causes.

The smaller harvest body size and the predominance of immature individuals in 2013-2014 is evidence for the unsustainable *P. saltatrix* exploitation in the Black Sea waters; therefore, effective management regulations should be implemented.

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References :

1. Stoyanov S., Georgiev Z. H., Ivanov L., Nikolov P., Kolarov P., Alexandrova K., Karapetkova M. Fishes of the Black Sea. State Publishing House, Varna, Bulgaria, 1963, p. 106.

2. Turgan G., Rapp P. V. Réun. Comm. Int. Explor. Scient. Mer Mêditerr., 15 (2), 1959, 409-420.

3. Kolarov P. Izv. Inst. Rib, Varna, 4, 1964, 207-220.

4. Kolarov P. Izv. Inst. Rib, Varna,10,1970, 91 – 99.

5. Taranenko N. F.,VNIRO XCIII, 1973, 149-162.

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SUBMARINE LANDSCAPE DIVERSITY OF VARNA COASTAL ZONE (BULGARIAN BLACK SEA): PRESENT-DAY ECOLOGICAL ISSUES AND RECOMMENDATIONS FOR SUSTAINABLE MARINE SPATIAL PLANNING

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Abstract

Having originated in the former USSR, the notion of the submarine landscape as a seabed geo-ecocomplex is a concept rapidly gaining popularity nowadays. Pursuant to these ideas, the landscape comprises a group of inter-linked, hierarchically organized subsystems, implying for the taxonomic subordination of the underwater units.

Aim: To identify, systemize hierarchically and represent graphically the seabed complexes at Varna coastal zone, to evaluate their nature conservation importance and to assess the current ecological issues of the cited sector.

Data and Methods: Several data sources concerning the underwater landscape components (e.g. seabed substrates, seafloor geomorphology, dominant benthic biota etc.) were analyzed in GIS.

Results: The submarine landscape diversity of the study site comprises 43 kinds united in 7 genera, 5 types and 2 classes. The greatest impact upon the seabed is observed at the resorts and port areas of Varna Municipality.

Discussion and Conclusions: The current marine use often runs counter to the conservation value of the benthic landscapes, with the Aladzha bank being a rather alarming example. Recommendations targeted at optimization of the marine spatial planning are accompanied with a map of the suggested marine use types.

Keywords: marine landscape studies, benthic landscape units, marine NATURA 2000, Aladzha bank, ICZM, GIS

Introduction

The scientific concept viewing the landscape as a natural complex is an idea that is widely adopted in some East European countries, former USSR states and Russia in particular. According to this notion, the landscape is a spatially defined part of the geographic sphere, a dynamic geo-ecosystem that is genetically uniform, has a definite resource potential and is influenced to a certain extent by anthropogenic activity [1]. Since the 1990's of the XX century, this theoretic view of what the landscape represents is rapidly gaining popularity in the Nordic countries [e.g. 2, 3]. Furthermore, the scientific

concepts emphasize the spatial delimitation of the landscape complexes and the hierarchic ranking of the landscape units [4].

Despite having emerged initially as a terrestrial interdisciplinary field bridging over geography, ecology, social sciences etc., over the course of the XX century the landscape sciences were subsequently adopted in oceanography as an approach for complex investigation of marine basins, whose primary purpose is to provide detailed maps of the benthic environment in help of the economic sectors involving the use of the marine natural resources [5]. The underwater landscape is again perceived as a natural complex, combining the principal geocomponents and factors of the benthic environment (e.g. loose and solid sublittoral substrates, seafloor geomorphology, hydrodynamic regime, depth zonation and related light availability etc.), with the contiguous group of floristic, epi- and infaunal organisms that inhabit the seabed [3, 5, 6].

Description of the study area

The investigated seabed sector stretches between cape Ekrene on the north and cape Galata on the south, falling entirely within the boundaries of the Varna coastal zone, which occupies the foothills of the Franga and Avren plateaus (Fig. 1). It has a total planimetric area of roughly 6 693.5 ha and an off-the-coastline width varying between 2.4 and 8.3 km, while the maximum depths reach values of approximately 21-23 m. The sublittoral substrates are presented by loose sediments (e.g. varying in grain size sand, sandy silts and silts), additionally diversified by solid lithologic varieties dominated by sandstones and conglomerates. The underwater relief is mostly of accumulative or structural-accumulative type, thereby dominated by corresponding landforms, e.g. wave-formed sandy bars, accumulative and structural-accumulative slopes, structural-accumulative terraces, structural depressions, accumulative platforms etc. The seafloor topography is also characterized by the presence of abrasive and other azonal morphologic features, e.g. plantigrade slopes of structural or landslide-collapse genesis, structural-abrasive benches, rocky banks, geogenic reefs etc. The spatial occurrence and distribution of the macrobenthic biological communities demonstrate a strong relation to the seabed lithologic varieties, grain size of the loose deposits, intensity of the wave-induced sediment transport, hydrodynamic activity and depths [7]. The psammophilous infauna is mostly dominated by clams belonging to the families *Mesodesmatidae (Donacilla cornea), Donacidae (Donax trunculus), Corbulidae (Lenthidium mediterraneum), Veneridae (Chamelea gallina), Tellinidae (Tellina tenuis)*, while the sectors with shelly sand and gravels are populated by species from the *Mytilidae (Gibbomodiola adriatica), Veneridae (Gouldia minima)* etc. In addition, the deeper seabed areas with sandy silts and silts are inhabited by representatives of *Mactridae (Spisula subtruncata), Semelidae (Abra alba), Cardiidae (Parvicardium exiguum)* etc., as well as by thalassinidean shrimps (e.g. *Upogebia pusilla*). The epilithic fauna in the shallow zone is normally dominated by *Mytilidae* mussels *(Mytilus galloprovincialis and Mytilaster lineatus)*, gradually turning into monodominant communities of *Mytilus galloprovincialis* with the increase of depth

[8]. Finally, the dominant macroalgal flora is presented mainly by annual communities of green and red algae, as well as by *Cystoseira* sp. at the rocky seabed areas with clean waters, decreased turbidity and improved light availability [7].

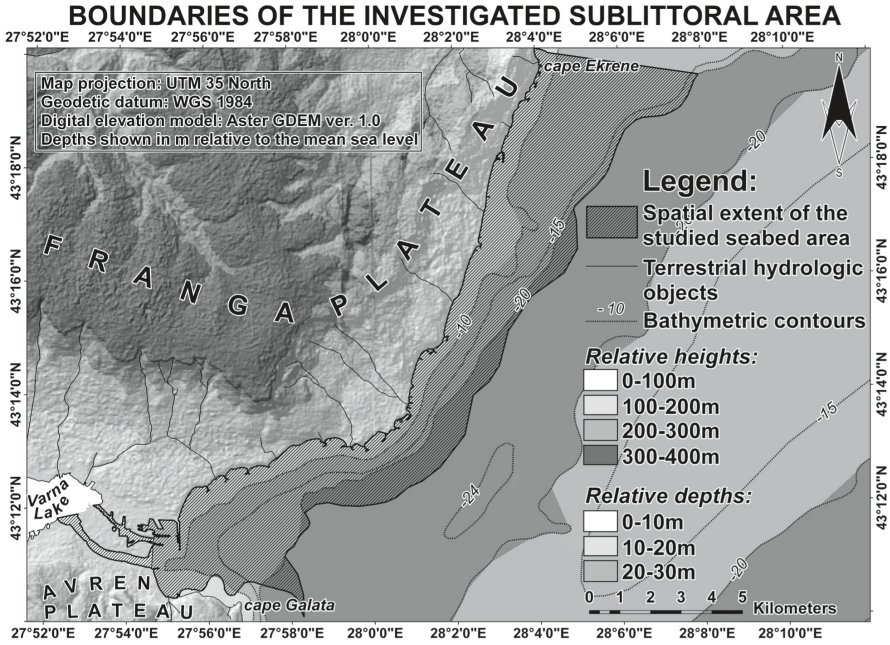

Fig. 1. Location and spatial extent of the investigated seabed sector

Data and Methods

Several graphic and descriptive data sources were used in the process of the GIS-aided submarine spatial analysis for identification and interpretation of the geo-components forming the study area's vertical landscape structure. These include the ESRI shape files on the seabed lithology, bathymetric contours, scanned maps of the seafloor geomorphology etc., as well as limited narrative and point GIS data concerning the dominant macrobenthic communities (Table 1).

Table 1. List of the initial data used in the process of the GIS-aided submarine landscape analysis

Name of the initial data set/source/hard-copy map	Data format	Spatial resolution/ minimum mapping unit/map scale	Data source
Seabed lithology of the Varna coastal zone	ESRI shape file (vector)	1:5,000	Department *"Coastal zone dynamics"* at IO-BAS
Bathymetric contours	ESRI shape file (vector)	2 m	Department *"Coastal zone dynamics"* at IO-BAS
8 GIS layers on the marine use types at the study area	ESRI shape file (vector)	N/A	Department *"Biology and ecology of the sea"* at IO-BAS
Point data on dominant macrobenthic biological communities	ESRI shape file (vector)	N/A	Department *"Biology and ecology of the sea"* at IO-BAS
Boundaries of the „Varna-Beloslav Lake" NATURA 2000 site (Birds Directive)	ESRI shape file (vector)	1:100,000	www.natura2000.moew.government.bg
Boundaries of the "Aladzha bank" NATURA 2000 site (Habitats Directive)	ESRI shape file (vector)	1:5,000	www.natura2000.moew.government.bg
5 scanned seabed morphologic maps of the Varna coastal zone	tiff (raster)	1:10,000	Keremedchiev, 2013 (*personal archive*)
General spatial plan of the Varna Municipality	tiff (raster)	1:25,000	www.varna.bg
Locations of aquaculture farms and fishing nets at the Varna coastal zone	Excel tables	N/A	www.iara.government.bg
Zones prohibited for commercial fishing in 2014 [9]	Pdf document with string data on geographic coordinates	N/A	www.iara.government.bg

All graphic data were integrated, processed and analyzed in Arc GIS 9.3.1 (Fig. 2). The second step of the analysis was the taxonomic grouping of the identified landscape units into the hierarchic categories, applying a four-level classification system developed by the author for the entire central sector of the Bulgarian Black Sea coastal zone [10]. The main aspects of the cited categorization criteria are summarized in Table 2. The final stage of the GIS-aided analysis was an assessment of the current and projected marine use at the Varna coastal zone, alongside with the major ecological issues emanating from its weaknesses, with a stress on the planned spatial development inside the designated marine NATURA 2000 sites. This was

done primarily by reviewing the latest version of the General Spatial Municipal Plan (Table 1). The recommendations for optimization of the marine spatial planning were addressed and visualized as a digital map of the suggested marine use types.

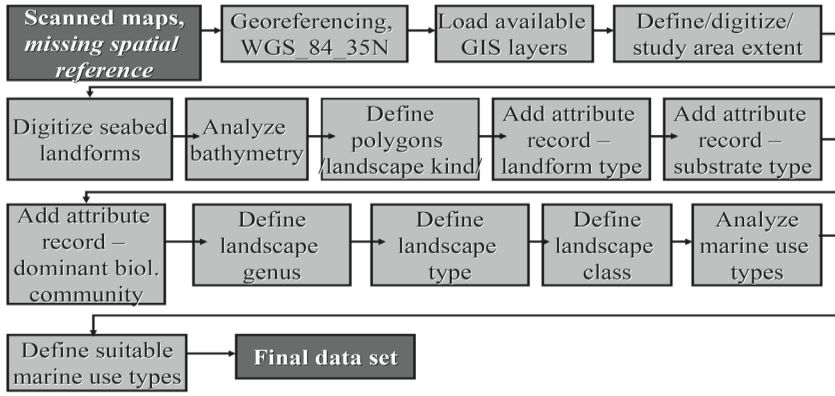

Fig. 2. Generalized flowchart of the procedures carried out in Arc GIS 9.3.1 software environment

Table 2. Main features of the applied landscape hierarchic categorization criteria (Kotsev [10])

Hierarchic category	Conditionally natural submarine landscapes	Anthropogenic submarine landscapes
Landscape class	Macrogeomorphologic criteria (macro-relief of the seabed, e.g. *Landscapes of the submarine coastal slope*)	Introduced for better hierarchic correlation with the classification scheme of the conditionally natural submarine landscapes; one single class, namely *Anthropogenic submarine landscapes*
Landscape type	Intensity of the wave-induced sediment transport and intensity of the geomorphodynamic processes within the submarine coastal slope	Level of alteration of the seabed complex (e.g. *Landscapes with significant to complete transformation of the natural complex*)
Landscape genus	Trends in the geomorphodynamic processes (e.g. abrasion or accumulation) and distribution depths of the landscape units	Trends in the geomorphodynamic processes (e.g. abrasion or accumulation) as a result of the anthropogenic intervention (often irreversible), or used as a category heading for a group of entirely artificial seabed elements
Landscape kind	An underwater complex formed by relatively uniform seabed landforms, sublittoral substrate types and associated dominant macrobenthic biological communities, found at particular depths and constructing altogether the submarine landscape's vertical structure	Indication of the particular anthropogenic landscape kinds that often coincide with given marine use categories, e.g. *port areas, navigable canals, stationary pound nets* etc.

Results

Present-day seabed landscape diversity of the Varna coastal zone. The submarine landscape diversity of the investigated sublittoral region consists of 43 kinds, hierarchically united in 7 genera, 5 types and 2 classes (Fig. 3). The seabed landscape pattern is dominated by accumulative and structural-accumulative complexes, found in the weakly active and inactive (in geomorphodynamic sense) zones, distributed at depths greater than 10 m. The submarine anthropogenic landscape units are concentrated within the relatively shallow near-shore areas contiguous to the recreational, harbor and port zones of the Varna Municipality, as well as at the coastline sections with hydrotechnical structures present, occupying approximately 13.6% of the investigated underwater sector (Table 3).

Table 3. Relative coverage (in % of the studied seabed sector) of the identified submarine landscape genera

Name of the submarine landscape genus	Relative coverage (in % of the study area)
Slope, abrasive-structural & abrasive-landslide-collapse landscapes at depths 0-10 m	2.6
Accumulative (including structural-accumulative) landscapes at depths 0-10 m and rarely reaching 15 m	3
Abrasive (including structural-abrasive) landscapes at depths 5-15 m and rarely reaching 20 m	0.6
Accumulative (including structural-accumulative) landscapes at depths 5-20 m and rarely reaching 30 m	12
Accumulative (including structural-accumulative) landscapes at depths 10-30 m and rarely reaching 42 m	68.2
Accumulative anthropogenic landscapes	5.1
Landscapes formed entirely by anthropogenic activities	8.5

Morphologic seabed features of nature conservation importance. Undoubtedly, the Aladzha bank represents the most important benthic entity when discussing marine nature conservation at the Varna coastal zone. With its area of approximately 522 ha, it is a representative example of the biotope **"Infra- and circalittoral rock overgrown by mussels *Mytilus galloprovincialis* and *Mytilaster lineatus*"** – a variety of 1170 (**Reefs**) NATURA 2000 habitat type. Established as a protected site of the cited European ecological network, the Aladzha bank is important for the geographic coherence and connectivity between the northern and southern reefs in the Bulgarian sector of the Black Sea [11].

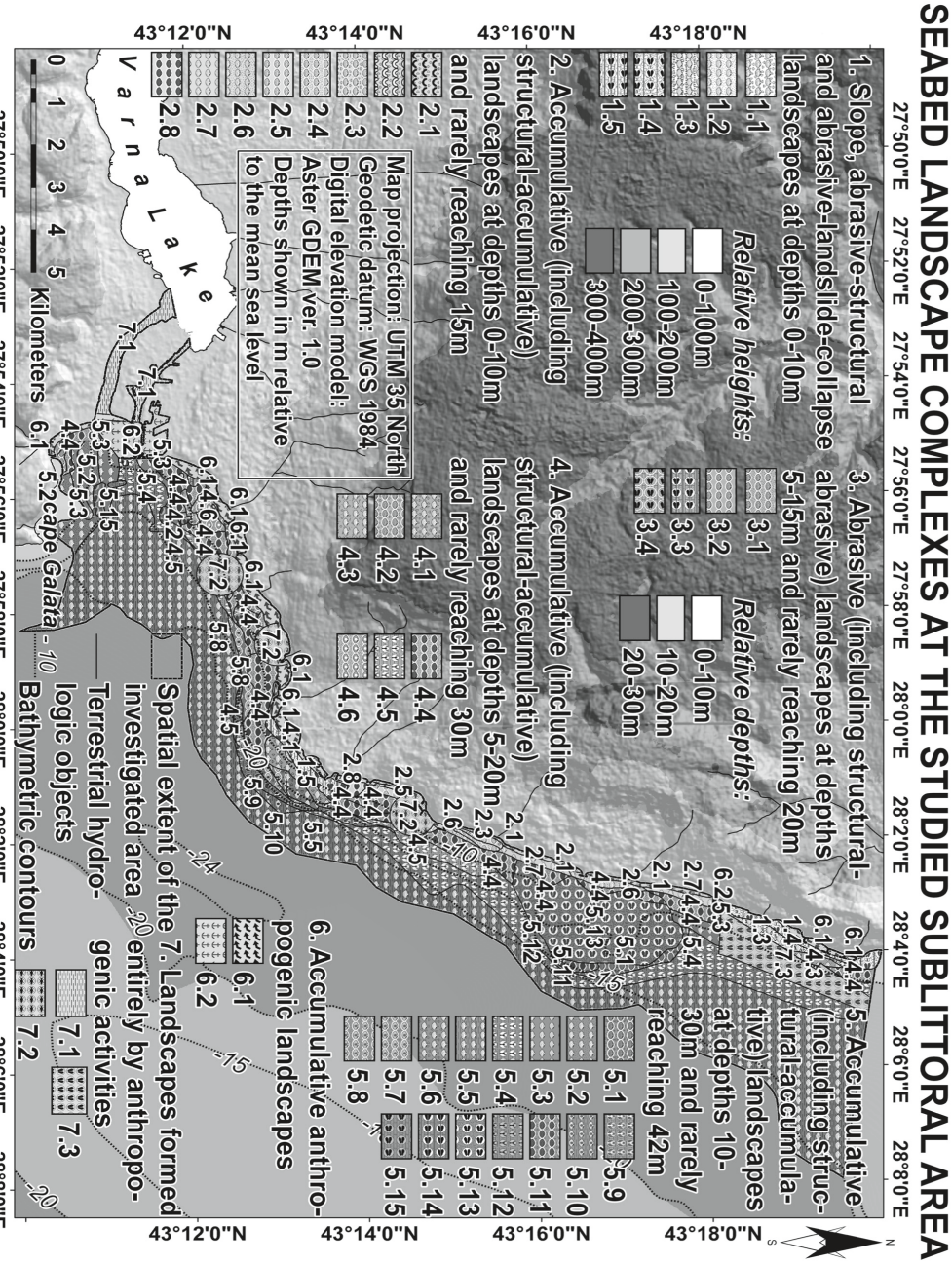

Fig. 3. Map of the contemporary submarine landscapes at theVarna coastal zone

NB: A-B – landscape classes, I-IV - landscape types, 1-7 - landscape genera, 1.1-7.3 - landscape kinds

A. Landscapes of the submarine coastal slope. **I**. Landscapes of the geomorphodynamically active zone. **1. Slope, abrasive-structural & abrasive-landslide-collapse landscapes at depths 0-10m:** 1.1. Abrasive landscapes at structural slopes, with coarse sand and infauna domin. by *Donacidae, Veneridae* etc. clams,

developed at depths of 3-7 m; 1.2. Abrasive landscapes at structural slopes, with medium sand and infauna domin. by *Donacidae, Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 3-7 m; 1.3. Abrasive landscapes at the landslide & landslide-collapse plantigrade slopes, with medium sand and infauna domin. by *Donacidae, Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 2-10 m; 1.4. Abrasive landscapes at the landslide-structural-plantigrade slopes of conglomerates overgrown by *Mytilidae* mussels and *Cystoseira* brown algae, developed at depths of 0-10 m; 1.5. Landscapes of the geogenic reefs and rocky banks of sandstones overgrown by *Mytilidae* mussels and green & red annual algal communities, developed at depths of 0-10 m; **2. Accumulative (incl. structural-accumulative) landscapes at depths of 0-10 m and rarely reaching 15 m:** 2.1. Accumulative forebeach landscapes, with unsorted coarse & medium sand and infauna domin. by *Mesodesmatidae, Donacidae* etc. clams and *Polychaeta* worms, developed at depths 0-7 m; 2.2. Accumulative landscapes at wave-formed bars, with medium sand and infauna domin. by *Mesodesmatidae, Donacidae* etc. clams and *Polychaeta* worms, developed at depths of 0-7 m; 2.3. Accumulative landscapes at wave-formed bars, with coarse sand and infauna domin. by *Donacidae, Veneridae* etc. clams and *Polychaeta* worms, developed at depths of 3-7 m; 2.4. Accumulative landscapes at wave-formed bars, with medium sand and infauna domin. by *Donacidae, Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 3-7 m; 2.5. Accumulative landscapes at wave-formed bars, with fine sand and infauna domin. by *Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 0-10 m; 2.6. Slope-accumulative landscapes, with medium sand and infauna domin. by *Donacidae, Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 3-7 m; 2.7. Slope-accumulative landscapes, with fine sand and infauna domin. by *Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 0-15 m; 2.8. Geostructurally formed accumulative landscapes, with fine sand and infauna domin. by *Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 0-15 m. **II.** Landscapes of the weakly active zone. **3. Abrasive (incl. structural-abrasive) landscapes at depths of 5-15 m and rarely reaching 20 m:** 3.1. Abrasive landscapes at structural terraces, with medium sand and infauna domin. by *Donacidae, Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 5-10 m; 3.2. Abrasive landscapes at structural terraces, with unsorted medium & fine sand and infauna domin. by *Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 5-10 m; 3.3. Landscapes of the geogenic reefs and rocky banks of sandstones overgrown by *Mytilidae* mussels and green & red annual algal communities, developed at depths of 5-10 m; 3.4. Landscapes of the rocky banks of sandstones overgrown by *Mytilidae* mussels, sponges, hydrozoans etc., developed at depths of 10-20 m. **4. Accumulative (incl. structural-accumulative) landscapes at depths of 5-20 m and rarely reaching 30 m:** 4.1. Geostructurally formed slope-accumulative landscapes, with coarse sand and infauna domin. by *Donacidae, Veneridae, Tellinidae* etc. clams, developed at depths of 7-15 m; 4.2. Geostructurally formed slope-accumulative landscapes, with coarse sand and infauna domin. by *Veneridae, Tellinidae* etc. clams and *Polychaeta* worms, developed at depths 8-20m; 4.3. Geostructurally formed slope-accumulative landscapes, with medium (rarely heterogeneous unsorted) sand and infauna domin. by *Donacidae, Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths 5-15 m; 4.4. Geostructurally formed slope-accumulative landscapes, with unsorted medium & fine sand and infauna domin. by *Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 8-15 m; 4.5. Geostructurally formed slope-accumulative landscapes, with sandy silt and burrows of thalassinidean shrimps or infauna domin. by *Veneridae, Tellinidae* etc. clams, developed at depths of 10-20 m; 4.6. Accumulative landscapes at structural terraces, with unsorted medium & fine sand and infauna domin. by *Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 8-12 m. **III.** Landscapes of the inactive deep-water zone. **5. Accumulative (incl. structural-accumulative) landscapes at depths of 10-30 m and rarely reaching 42 m:** 5.1. Slope-accumulative & platform-accumulative landscapes with unsorted

coarse & fine sand and infauna domin. by *Veneridae, Tellinidae* etc. clams and *Polychaeta* worms, developed at depths of 15-20 m; 5.2. Slope-accumulative & platform-accumulative landscapes, with medium sand and infauna domin. by *Donacidae, Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 10-15 m; 5.3. Slope-accumulative & platform-accumulative landscapes, with fine sand and infauna domin. by *Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 10-15 m; 5.4. Slope-accumulative & platform-accumulative landscapes, with sandy silt and burrows of thalassinidean shrimps or infauna domin. by *Veneridae, Tellinidae* etc. clams, developed at depths of 10-20 m; 5.5. Slope-accumulative & platform-accumulative landscapes, with silt and infauna domin. by *Veneridae, Mactridae, Semelidae, Cardiidae* etc. clams and *Polychaeta* worms, developed at depths of 10-30 m; 5.6. Geostructurally formed accumulative landscapes at structural terraces, with coarse sand and infauna domin. by *Veneridae, Tellinidae* etc. clams and *Polychaeta* worms, developed at depths of 12-20 m; 5.7. Geostructurally formed accumulative landscapes at structural slopes & structural depressions, with unsorted coarse & medium sand and infauna domin. by *Veneridae, Tellinidae* etc. clams and *Polychaeta* worms, developed at depths of 12-20 m; 5.8. Geostructurally formed accumulative landscapes at structural slopes & structural depressions, with unsorted medium & fine sand and infauna domin. by *Corbulidae, Veneridae, Tellinidae, Arcidae* etc. clams, developed at depths of 12-15 m; 5.9. Geostructurally formed accumulative landscapes at structural slopes & structural depressions, with sandy silt and burrows of thalassinidean shrimps or infauna domin. by *Veneridae, Tellinidae* etc. clams, developed at depths of 13-20 m; 5.10. Geostructurally formed accumulative landscapes at structural slopes & structural depressions, with silt and infauna domin. by *Veneridae, Mactridae, Semelidae, Cardiidae* etc. clams and *Polychaeta* worms, developed at depths of 20-25 m; 5.11. Landscapes of the structural bars, with coarse sand and infauna domin. by *Veneridae, Tellinidae* etc. clams and *Polychaeta* worms, developed at depths of 12-20 m; 5.12. Landscapes of the structural bars, with sandy silt and burrows of thalassinidean shrimps or infauna domin. by *Veneridae, Tellinidae* etc. clams, developed at depths 12-20m; 5.13. Landscapes of the rocky banks of conglomerates overgrown by *Mytilidae* mussels and *Cystoseira* brown algae, developed at depths 10-15 m; 5.14. Landscapes of the rocky banks of sandstones overgrown by *Mytilidae* mussels and green & red annual algal communities, developed at depths of 10-12 m; 5.15. Landscapes of the geogenic reefs and rocky banks of sandstones overgrown by *Mytilidae* mussels, sponges, hydrozoans etc., developed at depths of 12-20 m. ***B. Anthropogenic submarine landscapes.*** **IV.** Landscapes with significant to full modification of the seabed substrates and the associated macrobenthic biological communities. **6. Accumulative anthropogenic landscapes:** 6.1. Accumulative landscapes in the entry-angle and wave-shadow zones near hydrotechnical structures; 6.2. Port areas. **7. Landscapes with significant to complete transformation of the natural complex:** 7.1. Navigable canals; 7.2. Stationary pound nets; 7.3. Aquaculture installations

Present-day ecological and nature conservation issues at the Varna coastal zone. The study area is well-known for being among the most technogenically transformed sectors along the Bulgarian Black Sea coast, with many negative consequences for the marine environment. Among others, these include:

- Inflow of industrial, chemical and domestic waste, as well as deposition of litter from the land-based resources, leading to a deteriorated state of the marine coastal waters [8];

- Technogenic pressure exerted upon the coastal zone, manifested through erroneously planned, excessive concentration of hydrotechnical structures, which results in sealing, smothering and siltation of the seabed, with main negative consequences being the ongoing physical loss and physical damage of the benthic biotopes [12];

- Direct impacts of recreation upon the marine coastal environment in the summer months, e.g. physical trampling of the soft-bottom mediolittoral (pseudolittoral) habitats at areas with sandy beaches [10];

- Decreased solid runoff from the coastal erosive ravines alongside with the deteriorated nourishment of the accumulative seabed sectors, causing shortage of loose seafloor substrates and eventually leading to seabed conversion at certain sites [13];

- Illegal trawling practicing, resulting in physical damage of the soft-bottom benthic biotopes [14];

- Intensified traffic of marine vessels with related noise and pollution.

Marine spatial planning at the Varna coastal zone - nature conservation vs. current and projected development. The performed GIS-based analysis of the Varna Municipality General Spatial Plan reveals that the current and projected marine use often runs counter to the conservation value of certain seabed sectors, with Aladzha bank being the most disturbing example. Due to the exhausted recreational capacity of the "Golden Sands" maritime resort, the graphic appendices of the cited document foresee the construction of several artificial islands inside the "Aladzha bank" NATURA 2000 site, four of which lying partially or entirely on top of the geogenic reef that is the primary habitat of nature conservation interest and thereby the main reason for designation of the protected area (Fig. 4). Another identified conflict represent the current boundaries of the "Varna-Beloslav Lake" NATURA 2000 site, established in accordance with the Birds Directive. The protected area's spatial extent includes the two navigable canals connecting the Varna Bay with the homonymous lake, as well as a small section of the aquatory adjacent to Port Varna-East (Fig. 5). Results from the regular monitoring campaigns carried out by IO-BAS regarding the quality of the coastal waters confirm that the cited area is in deteriorated ecological state, although a slight improvement was registered in 2013 in comparison to the preceding year [15]. Thus, its inclusion in the European ecological network raises many questions concerning the hardly attainable long-term improvement of the marine environment in Varna Bay and also, to a certain extent, compromises the effectiveness of NATURA 2000 in the Bulgarian sector of the Black Sea.

Recommended marine use at the study area. The recommendations targeted at improvement of the marine spatial planning are based on a complex analysis of the available data sets enlisted in Table 1. Despite being backed by similar feedbacks expressed in relevant articles and scientific project reports, the suggestions addressed are mainly of wishful nature and reflect private opinions. These recommendations are accompanied with a map of the suggested marine use types. The key features of the proposals addressed are designation of the "Aladzha bank" as a protected area in compliance with the Protected Territories Act of Bulgaria, exclusion from NATURA 2000 of the two navigable canals connecting the Varna Bay with the homonymous lake etc. (Fig. 6).

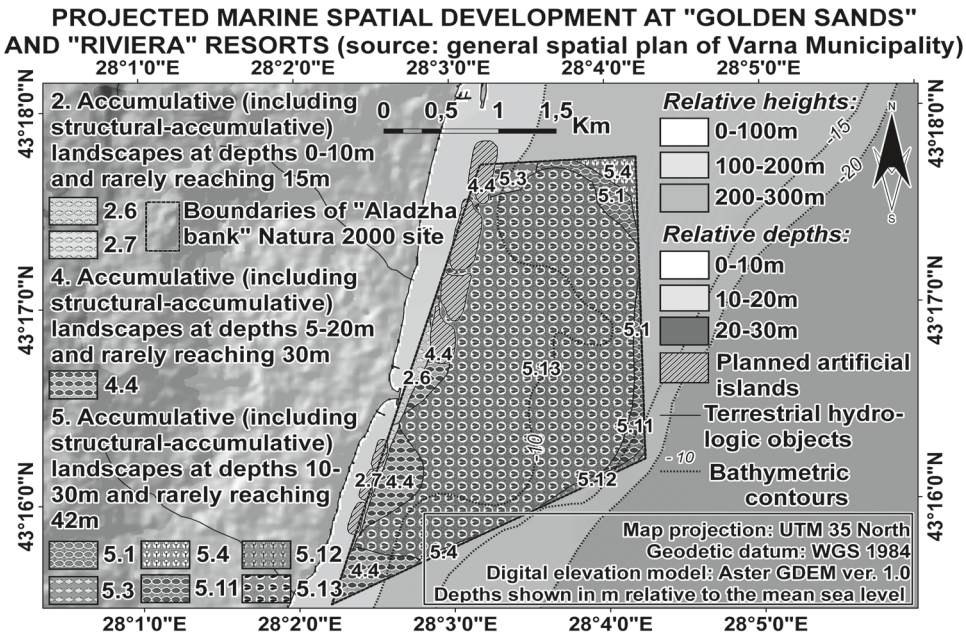

Fig. 4. Boundaries of the "Aladzha bank" NATURA 2000 protected site with the identified seabed landscape complexes and projected marine spatial development

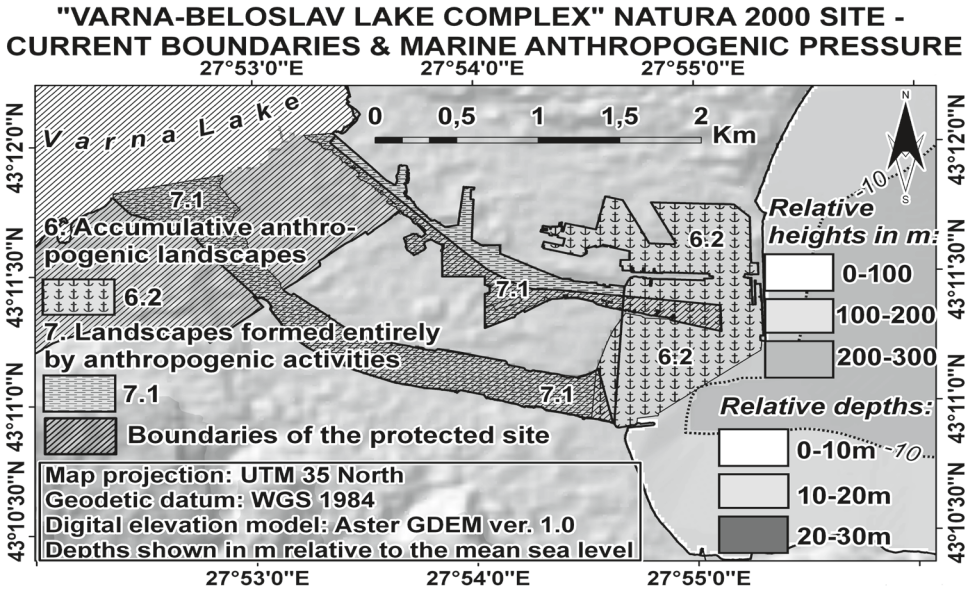

Fig. 5. Boundaries of the "Varna-Beloslav Lake" NATURA 2000 protected site and current marine use

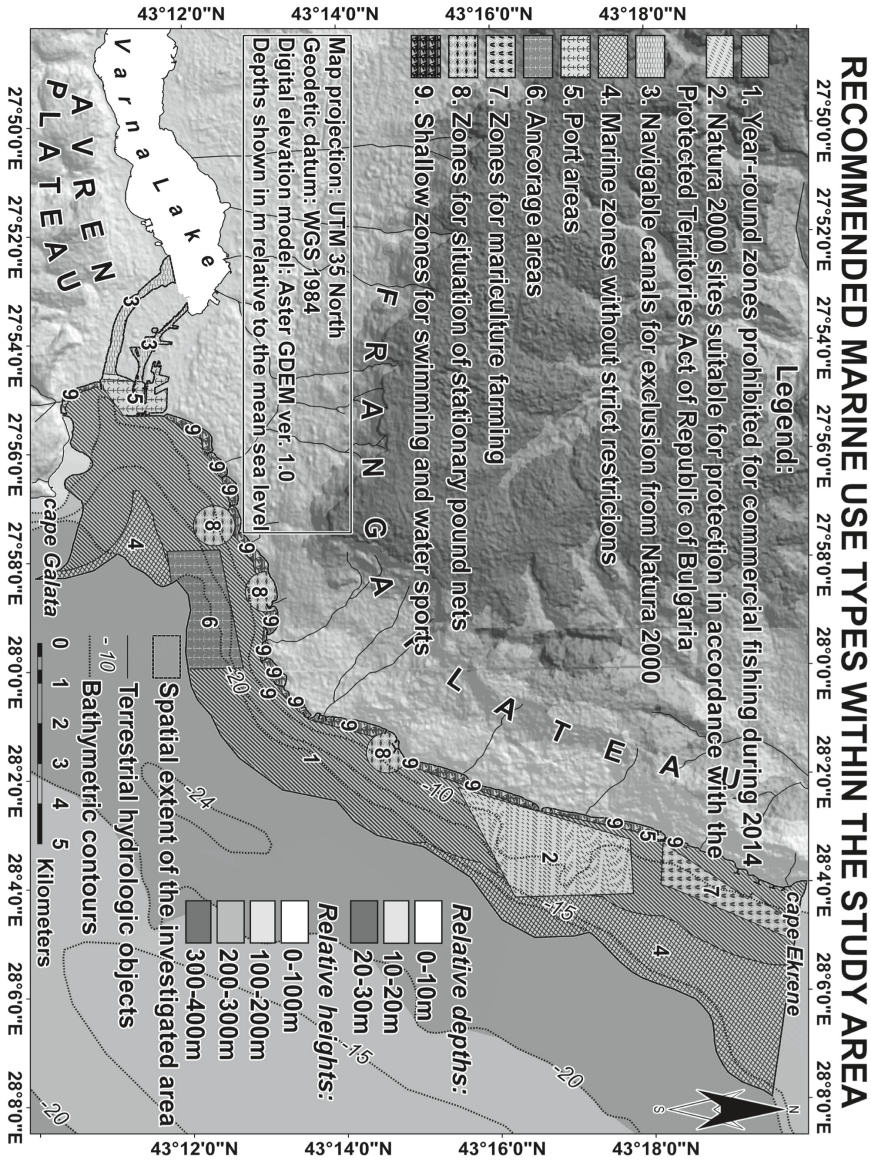

Fig. 6. Map of the suggested marine use types within the spatial extent of Varna coastal zone

Discussion and Conclusions

The results of the study reveal that the submarine landscape pattern of the Varna coastal zone is dominated by accumulative and structural-accumulative complexes, formed typically in the weakly active and inactive in geomorphodynamic sense sectors of the submarine coastal slope, which is in agreement with the geomorphologic setup of

the area. The correct interpretations of the seabed lithologic pattern and landforms are recognized as crucial fundamentals of the submarine landscape mapping [3, 6]. However, while the Nordic researchers traditionally apply geologic and geomorphologic criteria in the broad-scale landscape classifications of the seafloor units, this study incorporates them at the lowest taxonomic levels, thereby adopting a "terrestrial" approach that is widespread among the East European scholars, e.g. in Russia, Ukraine and Bulgaria [1, 4].

As a consequence of the decades of long-term technogenic transformations, the anthropogenic landscape units occupy a significant percentage of the investigated seabed sector. This fact poses further implications about the efficiency of the marine spatial planning as an element of the integrated coastal zone management (ICZM) at the Varna coastal zone, whose current level of consistency with the landscape specifics of the region is highly dissatisfactory. Yet, the General Spatial Plan of the Varna Municipality seldom render an account to the poor ecological state of the marine environment or the nature conservation importance of certain seabed features, with the projected construction inside the "Aladzha bank" NATURA 2000 site being an alarming example. Nevertheless, the suggested marine use targeted at improvement of the ICZM, despite being consistent with the specifics of the submarine landscape pattern, reflects mainly the author's opinion upon the subject.

Due to the poor spatial resolution, certain imperfections characterize the geo-data used, which consequently reflected upon the level of confidence of the results obtained, with GIS layers on the dominant macrobenthic communities being the weakest component of the submarine landscape analysis. Nevertheless, the applied seascape-based approach represents an innovative method for studying the benthic environment.

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References:

1. Velchev A., Penin R., Todorov N., Konteva M. In: Landscape geography of Bulgaria, "Bulvest 2000" publ. house, Sofia, 2011, 31-62 (in Bulgarian)

2. Käyhkö N., Skånes H. Landscape and Urban Planning, 75, 2006, 300-321

3. Mortensen P. B., Buhl-Mortensen L., Dolan M., Dannheim J., Kröger K. Norwegian Journal of Geology, 89, 2009, 163-171

4. Isachenko A. G. In: Landscape science, "Vishaya shkola" publ. house, St. Petersburg, 1991, 8-20 (in Russian)

5. Penno M. V. Annual Journal of "V.I. Vernadsky" Taurida National University, 10 (1) 2014, 166-172 (in Russian)

6. Thorsnes T., Erikstad L., Doland M. J., Bellec V. K. Norwegian Journal of Geology, 89, 2009, 5-16

7. Todorova V., Keremedchiev St., Karamfilov V., Berov D., Dimitrov L., Kotsev I., Dencheva Kr. In: Initial assessment of the current state of the marine environment in compliance with RPEMW, Final project report (V. Todorova, Sn. Moncheva, Eds.), Varna, 2013, 76-164 (in Bulgarian, available at http://bsbd.org/, last accessed on July 22, 2014)

8. Todorova V., Panayotova V. In: Red Data Book of the Republic of Bulgaria, Vol. 3 Natural Habitats (V. Biserkov, Ch. Gussev, Eds.), Sofia, 2011, 56-58 (electronic version available at http://e-ecodb.bas.bg/, last accessed on May 20, 2015)

9. Ordinance № RD 09-112/August 2, 2013 concerning the boundaries of the zones prohibited for commercial fishing in 2014 (in Bulgarian, available at http://iara.government.bg/, last accessed on February 15, 2014)

10. Kotsev I. In: Structure, dynamics and regionalization of the landscape complexes between cape Kaliakra and cape Emine, Bulgarian Black Sea coastal zone. Doctoral thesis, "Aquaprint" Publ. house, Varna, 2014, 108-110 (in Bulgarian)

11. Kotsev I., Keremedchiev St., Todorova V., Trifonova E., Dimitrov L., Andreeva N., Eftimova P. In: Proceedings of the First European SCGIS Conference "Best practices: Application of GIS technologies for conservation of natural and cultural heritage sites", Sofia, Bulgaria, May 21-23, 2012, 31-38

12. Prodanov B., Kotsev I., Keremedchiev St., Todorova V., Dimitrov L. Proceedings of the Second European SCGIS Conference "Conservation of Natural and Cultural Heritage for Sustainable Development: GIS-based Approach", Sofia, Bulgaria, September 24, 2013, 4-13

13. Keremedchiev St., Trifonova E., Valchev N., Andreeva N., Eftimova P., Demireva,D. PLANCOAST – spatial planning of Varna coastal region between cape Ekrene and cape Paletsa. Final project report. Scientific fund of the Institute of Oceanology "Fridtjof Nansen", Varna, 2007, 142pp. (in Bulgarian)

14. Todorova V., Panayotova M., Prodanov B., Keremedchiev St., Kotsev I. In Initial assessment of the state of the marine environment in compliance with RPEMW, Final project report (V. Todorova, Sn. Moncheva, Eds.), Varna, 2013, 257-272 (in Bulgarian, available at http://bsbd.org/, last accessed on July 22, 2014)

15. Assessment report concerning the ecological state of the marine coastal waters. Scientific fund of the Institute of Oceanology "Fridtjof Nansen", Varna, 2014, 370pp. (in Bulgarian)

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ДОКЛАДИ/REPORTS
Тематично направление:

ЕКОЛОГИЯ И ОБРАЗОВАНИЕ

Topic:
ECOLOGY AND EDUCATION

PROJECT-BASED LEARNING ON THE SUBJECT OF "ADVERTISING CAMPAIGN: SEPARATE WASTE COLLECTION"

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Abstract

One of the goals in the field of scientific subjects in high school education is the development of scientific literacy. It is concerned with the comprehension of fundamental scientific terms and hypotheses, as well as with the understanding of the way of interaction between science, mathematics and technology.

Modern teaching practice offers different models of quality teaching and learning. One of them is bound with elaboration of student's projects.

The aim of the following paper is to present a theoretically validated and approbated method of project-based learning in the field of biology and health education in the 10th grade on the subject of "Advertising campaign: Separate waste collection". The order of steps which are followed for the realization of the method, as well as the criteria for evaluation of the final product presented by the students is described in the paper. The method is carried out at the Sofia Professional High-school of Electronics "John Atanassov" with 10th grade students.

In the course of the project-based learning teachers and students switch their roles. The students take on the role of people who actively solve problems in the field of environmental studies and demonstrate personal opinion as they conduct activities which raise the level of public awareness and culture.

Keywords: project-based learning, waste collection, school education

Introduction

Project-based learning (PBL) is a teaching method. Through the process of planning and developing of a certain educational product learners acquire new knowledge and abilities. The technology of PBL is flexible and mobile in respects of organization, duration and content. Educational content is not presented by the teacher. Students seek, extract, structure and present information on their own. Thus they actively comprehend the content through actual context which helps them acquire new knowledge easier. In the process the teacher acts as an advisor and a partner, guiding the learning in meaningful and functional respects, searching for answers to the questions "Why?", "What?" and "How?" [1, 2].

PBL's impact on student's motivation, communication skills and the ability to work in a team is exceptionally valuable [2, 3].

Throughout all stages of the project implementation, students work in groups, discussing and creating collective strategies. This provides circumstances for individual contribution by each student to the attainment of the common goal. PBL holds the potential possibilities for the implementation of permanent feedback, which further motivates the students to study actively. On one hand feedback is given by the teacher to the students, on the other hand it is exchanged mutually between the different members of a workgroup; finally it is shared between the different workgroups at the presentation stage of the project. The most significant advantage of this feedback, except for its permanence, is its objectiveness, and the students become aware of that [1, 2, 4].

The teacher's role is less that of an instructor who transmits information and organizes activities for practice and more that of a guide and a facilitator, it is a critical role, nevertheless. Projects require that teachers get to know their learners' interests. Facilitating project-based learning requires the kind of leadership skills that allow teachers to help a group of learners to move in the direction that they want to go, pointing out the potential pitfalls or making suggestions without getting defensive when students decide they like their own ideas better. It makes a difference if teachers possess a tolerance for ambiguity, some skill in helping learners negotiate conflicts, and enough self-confidence to not give up when a project peters out or refuses to come together [5].

The topic of this particular project relates to separate waste collection. An activity not many people in Bulgaria perform, mostly because of their lack of knowledge on the positive long-term effects it could have on the environment if everyone began doing it. Our aim was to help students, through their own research and work on their projects, realize the advantages of separate waste collection and ultimately share their new knowledge with their school-mates.

Methods

During the course of the project we went through the three stages of PBL organization. At all stages of the project: object planning, planning, implementation, presentation, students work in groups discuss for the accuracy of their knowledge, conclusions and hypotheses, create common strategies, etc. [1, 2]. First we had to go through the stage of initial research and planning [1]. Ecology is included in the Biology syllabus for 9th grade students which meant we had to pick a grade between 9th and 12th, if we wanted the students to have basic knowledge on environmental studies. We also had to pick a suitable school to conduct the project. It was chosen in consideration of the final products that were expected from the students. As we decided that creating an advertisement would require technically proficient students, we chose Sofia Professional High-school of Electronics "John Atanassov". We resolved to carry out the project with 10th graders. Before we could assign the students with the task, we still had to cover several organization matters, such as deciding on the duration of their work on the project, the number of people in the workgroups, the possible types of final products, and the grading criteria.

We chose 26 March 2015 as our first meeting with the students and a starting date for the project. The stipulated date for the presentation of the final products was 20 April 2015. Thus

the time they had to work on the projects coincided with in the Spring break (2 April 2015 – 13 April 2015). The optimal number of people in a workgroup is considered to be two to four. One person is not considered a group, and when there are more than four people in a group, there is a strong possibility some group members will have no contribution to the project at all.

We have had no previous interaction with the students before the starting date of the project, so that we did not know their individual strengths and weaknesses. On that account we entrusted them with the task to divide into groups consisting of two to four people. As a result the majority of the groups were composed of 3 students.

We announced the topic of the project and presented the possible types of final products we have determined. Each group could choose to do a video, an animation, a poster, a brochure, or write an essay, a poem or a song. We told them they were expected to devise an advertisement that would provide information regarding the benefits of separate waste collection and its positive impact on the environment. Their final products were required to be grammatically correct and not to contain obscene words or phrases. The students were given websites with literature on the subject of separate waste collection and were made acquainted with the evaluation criteria. We gave them an e-mail address, where they were able to send their inquiries, if they had any in the process. Eventually we appointed a date to meet with them at the school, several days before the final day of the project, so that they could show us their progress.

Each group was also given detailed instructions in written form containing everything we have already explained to them orally: the start and final dates of the project, the available types of final products to choose from, websites to seek information from, and the evaluation criteria.

The second stage of PBL is the stage of practical implementation [1]. It commenced as soon as we gave the instructions and left the students to themselves. During this stage the groups gather to select and structure the information they've gathered. We have had no control over the course of their projects during the spring break, except for answering any arisen questions via email. 16 April 2015 was the appointed date for the meeting before the final day of the project. The groups brought what they have prepared for three weeks. We gave them final guidelines and pieces of advice. We also recommended the groups to devise an attractive manner to present their product on the final day. For the remaining four days the groups had to put their finishing touches on the projects.

The final stage of PBL is the stage of presentation, discussion, control and evaluation of the research results [1]. On the final day we were given the opportunity to carry out the presentation and evaluation of the projects in the staff room at the school. The students were visibly in high spirits and eager to show their classmates what they had prepared. After all the groups have gathered we expressed our gratitude to the students for taking part in the project. We instructed them that each group would be granted five minutes to present their product, and also that the evaluation would be conducted by our colleagues from the Faculty of Biology, in favor of unbiased judgment. However, the evaluation criteria were not changed and remained as given in the written instructions for each group. Several aspects are graded: the use of plain language and correct terms, the presentational skills of each group, the ingenuity of the approach to the project and its design.

Results

Four of the groups have prepared brochures; one has made an animation; the final group – a PowerPoint presentation. After the presentation of their products they were evaluated and the selection committee awarded the best projects certificates. They agreed unanimously that the animation was the most impressive and original project. It was done by a group of two students and combines genuine ideas, picturesque animations, and appropriate musical setting. The mood after the evaluation of the projects was positive. Some of the students even confessed that the work in groups helped them rediscover the classmates they were working with and become friends with them.

Conclusion

The utilization of project-based learning in the field of environmental studies at school is pivotal in order to draw the student's attention to Ecology and the problems regarding environment pollution. In this way they take up an attitude towards preservation and reasonable utilization of natural resources.

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References:

1. Asenova A., Yotovska K. Key competencies of the biology teacher in the context of e-learning. D. Ubenova Press, Sofia, 2011, p. 159 (in Bulg.)

2. Asenova A., Yotovska K., Evtimova S. International scientific on-line journal Science & Technologies, II (8), 2012, 14-18. http://journal.sustz.com/VolumeII/Number8/Papers/AsyaAsenova.pdf

3. Nikolaeva S. Pedagogy, 5, 2004, 21-32 (in Bulg.).

4. Yotovska K., Asenova A., Popova K. In: Proceedings of the 6-th International Scientific Conference, Global Changes and Regional development,16 – 17 April 2010, Sofia, Bulgaria, "St. Kliment Ohridski" University Press. Sofia, 2011 p. 211-214.

5. Kubiatko M., Vaculova I., Energy Education Science and Technology Part B: Social and Educational Studies, 3 (1), 2011, 65-74.

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FRAMEWORK TO ENSURE AND MAINTAIN THE QUALITY OF NON-FORMAL EDUCATION FOR SUSTAINABLE DEVELOPMENT

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Abstract

In the article is suggested the main criteria for assessing the quality of a training unit for non-formal education for sustainable development, as well as relevant indicators.

During the development process of the framework are implemented the following methods: theoretical analysis and synthesis, pedagogical modeling.

To ensure completeness and objectivity for evaluation of non-formal education quality for sustainable development, the following inter-related areas should be monitored: the expected results upon completion of the training unit, whether the content of the training unit for sustainable development through informal education and evaluation system of learners are effective and relevant to the defined goals; what the achievements of the students in the course for non-formal education for sustainable development are; the resources provided for the effective non-formal learning support and teaching quality; how the system works to ensure and maintain the quality of each training program for non-formal education for sustainable development.

Keywords: quality criteria, non-formal education, sustainable development, framework

Introduction

The idea of the sustainable development was presented in Rio de Janeiro in 1992 for the first time. This concept contains three main elements: combining economic and social development while preserving and improving the quality of the environment.

The classic definition of "sustainable development" is that given by the International Commission for Environment and Developed in the Brundtland Report (1987 г.), and it says: *Sustainable development is development that meets the needs of the present without compromising the ability of future generations to meet their own needs.* Sustainable development is seen as an alternative of the consumer society, as a solution of the crisis in the man's perception of environment and nature resources.

Debates on sustainable development presented in the literature can be classified into several thematic areas: a) conceptual; b) contextual; c) academic; and d) geopolitical [1].

The basic principles of the sustainable development concept are:

• meeting the needs of modern society without depriving future generations of the opportunity to satisfy their needs in the same amount (quantity);

• sparing the use of natural resources and restoration of disturbed ecological balance;

• analyzing the economic impact of any activity related to the environment;

• monitoring of the expenditure for environmental protection from border contamination between current and future generations [2].

Analysis of the sustainable development concept descriptions proved that none of the hundreds of sustainable development definitions found in the literature includ all the aspects of the concept and provide perfect understanding of it. Therefore the appropriate definition that expresses the idea of sustainable development isthe education for sustainable development (ESD) as a lifelong process of personal and social development by which people acquire knowledge and skills and develop attitudes and values. It integrates economic, environmental, social and cultural aspects and builds up a responsible life-style. The ESD upgrades the concept of environmental education and focuses on the following:

1. Expanding the opportunities for the implementation of education for sustainable development through formal and non-formal education.

2. Promotion of European initiatives in the field of education for sustainable development

3. Raising awareness and promotion of integration and inclusion of children with special educational needs and children from ethnic minorities.

The main goal of the ESD is the full use of formal and non-formal education as key opportunities for creating global thinking as a tool for solving global problems of modern society [3, 4]. Based on this conditions, the issue of uniform standards establishment, that ensure and maintain quality in non-formal ESD, becomes more actual.

The result is the preparation of trainers and leaders of cooperatives needs to focus on their professional skills by balancing the existing competition between management, planning, leadership and action, which involves the most direct and practical communication, methodology, motivation, confidence, cooperation, empathy, and has to be extended to those professionals working on inquiry, research, innovation and belonging to the culture of the institution [3].

While the formal education can be quantified and described, this is more difficult in the case of non-formal education, which largely escapes structure and is difficult to assess quantitatively and qualitatively [5]. The challenge of advancing the relationship between education and sustainable development is one of the most valuable lines that guide the training projects, leadership training programs for trainers and training plans of the organizations. One of the most researched contributions is the relationship between the domain of competence of teachers and organization leaders, showing that the updating and training of people is essential to obtain sustainable development [6]. The National education planners should reconsider their policy and practice in order to promote non-formal education as a means of increasing skills and knowledge [7]. There are many publications according to the qualitative evaluation in higher education. The EAATSD scale can be used for testing the anthropocentric and Ecocentric Attitudes Towards Sustainable Development in students of higher education. Based on these results, this scale was found to be revealing of the critical view of paradoxes and challenges inherent in multiple goals of sustainable development as well as useful for testing the anthropocentric and ecocentric attitudes in students of higher education [8].

Within this report is presented a working framework to ensure and maintain the quality of non-formal education for sustainable development, which was elaborated for the project "Platform for Education for Sustainable Development"

To ensure the *completeness and objectivity of the quality evaluation* of the non-formal education for the sustainable development it is necessary to monitor the following inter-related areas:

• the expected *results* upon completion of the training unit, if the learning objectives, formulated for the training unit are relevant to the standards for the non-formal ESD;

• if the content of the training unit for sustainable development through non-formal education and the evaluation system of learners are *effective and relevant* to the defined learning objectives;

• what the *achievements* of students in the training unit of non-formal ESD are;

• what *resources* for effective non-formal learning, for support and for quality of teaching are provided;

• how the *system* works to ensure and maintain the quality of each training unit of non-formal ESD.

Although it is difficult to measure the immediate result of non-formal educational activities, it might be possible to combine the process of evaluation with the implementation of such activities that could give a consistent picture of progress made by the participants. Such method should be based on the ability of the participants of non-formal education to self-evaluation, which itself could be a purpose of non-formal education [7].

The sustainable development must be included as the new competence in which trainers can be prepared to create an environment conducive to entrepreneurship, a culture of constant search and permanent transformation of people climate, cooperative organizations and communities, providing such development as the main line of work for a global, permanent and profound change [6].

Research Methods

Theoretical analysis and synthesis, and pedagogical modeling were used for the research purposes.

Results

Solutions of the non-formal education (for sustainable development) evaluation, the issue of the evaluation object or "what do we evaluate" vary in the different European countries.

Criteria for assessing the quality of non-formal education for sustainable development

The following basic criteria for assessing the quality of non-formal education for sustainable development are proposed:

1. Objectives and expected results.
2. Training content.
3. Evaluation of trainees.
4. Quality of teaching and non-formal learning during the training.
5. Support of trainees and trainee achievements during the training.
6. Training resources.

Within each criteria, different characteristics that could play the role of indicators for building a quality system can be distinguished (Table 1).

Table 1. Criteria for assessing the quality of non-formal education for sustainable development

Criteria	Indicators	Sources of information
1. Objectives and expected results	• formulation of the expected results from the participation in the training unit of non-formal ESD; • correspondence between the objectives of the training unit and the goals of ESD • mechanisms for realization of the set objectives; • correspondence between the results and objectives of the training unit; • ways of inform trainees about the training objectives; • the extent to which trainees are familiar with what is expected of them	Program of the training unit of non-formal ESD; Standards for non-formal ESD; List of the objectives of the non-formal ESD; Knowledge of the trainees about the expected objectives and results, received from their feedback; Form for self assessment of the trainer in the training unit of non-formal ESD; Discussion with trainers and trainees from the training unit; Questionnaires for trainees to study their opinion on the training unit.
2. Training content	• availability of written plans and programs of the educational process, testifying the implementation of non-formal ESD • extent to which the program of the training unit of non-formal ESD contains the necessary (in terms of the training objectives) knowledge, understanding, cognitive skills, specific practical and / or professional skills; • how the training content and the learning process give general skills and key competencies provided in the standards for non-formal ESD; • correspondence of the training content with the latest achievements in the field of education for sustainable development.	Plan and program of the training unit of non-formal ESD; Information from trainees, received from their feedback; Artifacts of students.
3. Evaluation of trainees	• availability of an evaluation strategy; • opportunities of the evaluation system to assess trainees according to their achievements to the expected results; • orientation of the evaluation system methods to all peculiarities and personal qualities of the trainees; • availability of defined criteria to distinguish different achievement levels; • evaluative components and procedures in the training and availability of integrity (internal logic and interconnection) between them; • correspondence of the assessment methods/tools with the planned evaluation strategy; • writing examination, tests, quizzes and computer testing as a component of the evaluation; • external examination (assessment of random papers of trainees from other competent trainers from the organization, conducting the training).	Description of the evaluation methods, tools and criteria in the training program; Procedures for recording and storing the evaluation of trainee achievements; Discussion with trainers, trainees and colleges.

4. Quality of teaching and non-formal learning during the training	• teaching effectiveness in terms of training objectives and content (if the way of teaching meets the training objectives and content); • the extent to which trainers update teaching and increase its effectiveness; • activity of trainees in the learning process; • ways to increase the methodical, scientific and professional competencies of trainers for non-formal ESD; • optimal distribution of work for trainees during the training; • immersion, pace and challenges of teaching; • diversity and effectiveness of the teaching methods, incl. information and communication technologies (Learning), meta-methods, situational, interactive methods; • effectiveness of the teaching in terms of the specific for the training, of the transfer and practical skills.	Program of the training unit of the non-formal ESD; Observation of the real learning process; Reports about the common observation from trainers; Papers and tests from the examination; Information from trainees, received from the feedback; Documents for continuous qualification of the trainers; Results from the trainees evaluation.
5. Support of trainees and trainee achievements during the training	• availability of strategy for learning support (incl. methodical instructions for trainees); • provision of effective learning support of trainees for achievement of the set trainings objectives; • diversity of forms and frequency of the learning support; • dialog between trainees and trainers.	Availability of methodical instructions for the trainees; Results from the trainees progress, that show their advance in the training /for long term training /; Discussion with trainers and trainees.
6. Training resources	• availability of a strategy for the enrichment and improvement of the educational resources for the training unit of non-formal ESD; • maintaining competent and continuously qualifying staff; • relevant technical support for conducting the training unit; • level of use of the resources that facilitate the learning; • quality of information materials for supporting the learning of trainees; • condition of own resources, of material and technical base, incl. for planning and conducting training.	Information about trainers and technical staff; Documents for continuous qualification of the trainers; Observation of the present resources; Inquiries and discussions with students; Observation of the learning process during the training unit; Actual database with information about the participants in the trainings, incl. if certificates are issued.

Discussion

The methodology of evaluation of the training unit of the non-formal ESD includes the summative evaluation - quantitative assessment and formative assessment - verbal (verbal) constructive feedback about the difference between the standard and actual state of the training unit of the non-formal ESD and the needed measures to reach the standard.

Standards for the non-formal ESD

Standards for non-formal ESD are formulated according to several key points:

Definitely **education for sustainable development** (ESD) is education for personal and social development that includes economic, environmental and social (social-political and social-cultural) aspects of development. It integrates global, environmental, health, civic and all other kind of education in a whole. This is the education about the relations in "our common home of life". Education for sustainable development focuses on life itself, in the interaction between nature and culture [3, 9].

The advantages of the use of such tool as framework are the following:

- ♦ criteria and indicators are unified;
- ♦ reporting the status of a particular phenomenon is facilitated;
- ♦ automated processing of data is possible;
- ♦ storage of date becomes possible in order to follow the quality of the process;
- ♦ results of the quality review of all educational units are commensurable and comparable.

Conclusion

The idea of non-formal education for sustainable development is still developing as a broad and comprehensive concept, combining interrelated environmental, economic and social issues. The elaboration of a unified frame to ensure and maintain the quality of non-formal education for sustainable development is essential, especially nowadays, when we talk about the sustainable development of a sustainable society.

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References:

1. Ciegis R., Ramanauskiene J., Martinkus B. Inzinerine Ekonomika-Engineering Economics, 2, 2009, 28-37.

2. Georgiev I., Manolov, M. Ecology and Sustainable Development, University publisher, Sofia, 1999, p. 226.

3. UNECE Strategy for Education for Sustainable Development, 2005.

4. Project of the National Strategy for Sustainable Development of Bulgaria, 2007.

5. Rogers A. Non-Formal Education: Flexible Schooling or Participatory Education. University of Hong Kong/Kluwer, Hong Kong/ Dordrecht, 2004.

6. Rivilla A. M., Domínguez C. M. Iconomic Insights – Trends and Challenges, III (LXVI), (1), 2014, 25 – 34.

7. Dumitrescu C. Non-formal Education Report. Report Committee on Culture and Education. Council of Europe, 1999.
8. Kopnina H. Environ. Dev. Sustain., 15, 2013, 607–623.
9. Programme for Supporting the Education for Sustainable Development in Bulgaria, 2005.

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MACEDONIAN STUDENTS' PERCEPTION OF POLLUTION AND THE ENVIRONMENT THROUGH TEACHING ENVIRONMENTAL TOPICS IN ENGLISH LANGUAGE CLASSES

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Abstract

Aims: Environmental education, considered to be a "blueprint for the future" emerged in the 1970s in recognition of the rapidly escalating deterioration of the environment. In the Macedonian education system, from primary to secondary levels, students are introduced to environmental issues through a range of core subjects. The purpose of this study is to investigate students' perception of environmental topics in the English language syllabus and find out if students feel that these topics help in the improvement of their language skills.

Materials and Methods: The sample for this study comprised of 200 students from a Macedonian secondary school. A two-part questionnaire was designed as an instrument. The mean Score Ranking and ANOVA were the two main methods used to analyze data. A structured interview was used to substantiate the quantitative data.

Results: The findings reveal that gender seems to have limited bearing on students' perceptions of the environment. In addition, students seem to be of the opinion that they gain more content knowledge than English language skills from studying environmental topics.

Conclusion: Many students want to be able to choose the environmental topics to study in their English classes. Teachers should consider giving students the freedom of choice as advocated by the learner-centered approach in CLT.

Keywords: Environmental education, environmental topics, students, English language.

Introduction

While countless themes can spark the interests of language learners (and teachers), one of the most critical issues affecting people globally is the environmental degradation of the planet. Topics such as global warming, deforestation, and water pollution are regularly featured in the news, thereby raising our awareness of the troubled state of Mother Earth. When institutions of elementary education recognize the value of environmental education, language teachers will be in a unique position to promote environmental awareness. Many of today's educators understand the importance of protecting our planet; others, however, might be skeptical about introducing environmental awareness in the classroom because of its political overtones and controversies. Moreover, language professionals generally do not see themselves as science teachers, nor do they, like the general public, always completely understand the environmental issues of the planet [1, 2]. Additionally, many educators wish to remain neutral about environmental topics and withhold their personal opinions from their students. However, Brown [1] argues that one of our goals as teachers should be helping our students "become informed about as many issues as possible that intrinsically affect their lives" [4].

Education about the environment, which is considered to be the "plan for the future" [3], appeared in the 1970s as a result of a rapid, increasing deterioration of the environment. The damage that people inflict on the environment has become a large, international problem and therefore, today, this so-called "plan for the future" is a part of the educational system worldwide [4]. In the Macedonian educational system, starting from primary all through to secondary school, students study issues related to the environment through a range of core subjects. The aim of this paper is to explore the views of students on topics related to the environment through the syllabus of English language. It explores whether students' gender or their ability for mastering has any impact on their understanding of topics related to the environment. So, the research aims to discover if students think that these issues will help them improve their language skills. Testing for this research involved 100 students from one high school in Macedonia. A two-part questionnaire was used for testing. Min Score Ranking and ANOVA were the two main methods used to analyze the data. A specially written interview was used to confirm the data. The results reveal that gender does not have great impact on students' perceptions of the environment. But, the greater the students' ability for mastering, the more positive reflection it has on the attitude they have towards the environment. Students think they acquire sufficient knowledge through the study of topics related to the environment.

The introduction of students of primary and secondary schools to environmental issues through a series of basic items is included in the educational system of Macedonia. Although the studies of ecology and the environment are not considered core subjects in secondary schools, however, topics related to the environment are included in the study of other subjects such as geography, biology, chemistry, and language. The study of language also includes activities related to the environment. Stavreva and Kirova [5] suggest that the most important reason why environmental issues are processed in language classes is the condition in which the environment is.

The environment is the key topic today together with the current trends for language learning. Thus the instructions that students follow are in the direction of how to use language to protect the environment. The program for English Language in Macedonia includes contents related to the environment such as: recycling, World Environment Day, environmental problems - garbage classification and protection of wildlife. These themes provide both learning and developing language skills and moral values. While studying the environment is included in the curriculum, not enough attention has been paid to it by students so they cannot see the need for a friendly attitude towards the environment. It is treated as just another additional academic topic that students have to study without any essential connection with the real world and the things existing outside the classroom. Macedonian students have a long way to go to reach the level of environmental awareness of students in developed countries (for example concerning garbage classification). According to Pillay and North [6] – teachers retain on topics rather than explain skills and the number of topics that need to be processed is great. Ratnawati [7] research and the observations of Pillay and North [6] suggest that learning skills and grammar through processing topics is problematic. Secondary school students know the importance of language for further study, but still they seem uninterested in learning languages. There are many reasons why this is so, but it is probably because these topics required by the curriculum are not interesting enough for many students.

The purpose of this research is to find out the views of students concerning environmental problems imposed by the syllabus for the teaching subject English language. It investigates whether these topics attract secondary school students, i.e. whether there is interest in these topics. Finally, the survey aims to find out whether students believe that these issues help them in their acquisition of language skills.

Questions used to conduct the survey

1. Do the views on the environment of male and female students in Macedonia differ? If yes, in what way are they different?

2. Do students from Macedonia belonging to 3 different knowledge levels have different views on the environment? If yes, what is the difference?

3. Do students from Macedonia have different views on how the study of topics related to the environment help them to improve their language skills? If yes, in what way are they different?

The research through the syllabus for English language aims to prepare students to use their communication skills and ability to perform linguistic functions by using appropriate language forms.

Besides developing the 4 main language skills: listening, speaking, reading and writing and the teaching of grammar, vocabulary and phonetic system, this program

aims to make students citizens with sound knowledge and high moral values that can contribute to a better society and state. According to the syllabus language skills should be built cumulatively and treated with repetition and constant use in order to increase the effect of learning.

The constructive idea for this kind of learning has been developed by Kent [8] who claims that people are not passive recipients but that they actively acquire knowledge. By this he means that students associate the material with the previously acquired knowledge and thereby learn it, which is perceived in their own interpretations.

These topics enable learning and developing language skills and moral values in an integrated manner. According to the Five Teaching Features topics are repeated together with the language skills [6]. When repeating some of the topics designed for every year differently, they are repeated but from different perspectives and different levels of difficulty that suit the intellectual level and maturity of the students. Of great importance for this research are the topics related to the environment in English in high school, as shown in the table:

Table 1. Topic – Environment

Form	Topic
Form 1	Deforestation
Form 2	1. Greenhouse Effect 2. Global Warming 3. Endangered Species

Table 1 shows the topics related to environmental education that are found in textbooks for the subject English language in secondary schools in Macedonia which were processed during the research. These topics were contained in two forms (1 and 2) and they were chosen out of a greater pool of topics in these textbooks for the purpose of this research.

Research procedure

The classes for the study were identified and the researchers fixed a one-period English lesson for the questionnaire survey to be conducted. A 100% return of the questionnaire distributed was obtained. The questionnaire survey was then analyzed. Structured interview questions that would verify the findings were formulated. The respondents were interviewed individually by the researchers over a span of 1 week. Each interview lasted between 5 to 10 minutes.

The raw scores of the respondents were analyzed using SPSS for Windows Version 11.0. Two statistical measures were used in the analysis of data, namely mean scores and analysis of variance (ANOVA). To check the normality of data, homogeneity of variance was undertaken. ANOVA was used to compare the mean differences between groups. In the analysis of data, only the mean scores that were significant were discussed. The

significance level was set at $p<0.05$. For differences in proficiency levels, Post Hoc Tests were used to determine the significance between the groups since more than two groups of subjects were involved in each case. Finally the findings of the interviews were used to verify the quantitative findings where relevant.

Table 2. Comparison of mean scores of the male and female students reveals significant results for question 1, 2 and 3

Question	**m**	**SD**	**M**	**SD**
1. I can play a part in protecting the environment	**3.15**	**0.70**	**4.12**	**0.58**
2. Learning about the environment is a part of education that prepares me for the future	**3.95**	**0.70**	**3.27**	**0.58**
3. My skill in listening in English has improved through learning about the environment	**3.78**	**0.80**	**2.98**	**0.79**

m - mean; SD - Standard Deviation

For question 1, male (m = 3.15) and female (m = 4.12) students show a considerable measure of agreement with the statement: "I can play a part in protecting the environment". However, the mean score of female students is significantly higher than of male students [$p< 0.05$; F (df) = 6.45(1/99)]. This suggests that female students are more concerned about protecting the environment than the male students. There is also a possibility that they see more practical ways in which they could make a difference.

For question 2, while both genders agree that learning about the environment is a part of education that prepares them for the future, male students (m = 3.78) have a significantly higher mean score than female students (m = 2.98) [$p< 0.05$]. This finding suggests that male students regard the environmental topics in the English curriculum as more necessary in preparing them for the future compared to female students.

A comparison of the mean scores of the high (HM), average (AM) and low (LM) proficiency students indicate significant results for question 4, 5, 6 and 7 as shown in Table 2.

Table 3. Comparison of means between high, average and low proficiency students

Question	HM m	HM SD	AM m	AM SD	LD m	LD SD
4. The English lessons teach me skills necessary for taking action against environmental abuse.	2.95	0.80	2.09	0.76	2.16	0.75
5. My skill in reading has improved through learning about the environment.	2.84	0.54	2.63	0.42	3.25	0.51
6. After learning environmental topics in English I have no difficulty in listening to spoken English.	2.82	0.75	2.29	0.79	2.83	0.53
7. After learning environmental topics in English I can write better compositions.	2.64	0.76	1.95	1.15	2.14	0.75

m - Mean; SD - Standard Deviation; HM - High mastery; AM - Average mastery; LM - Low mastery.

The data for question 4 show a significant difference between the mean scores of HM and LM students [$p<0.05$]. More HM ($m = 2.95$) students seem to agree with the statement while more LM ($m = 2.16$) students disagree. This is an indication that generally HP students feel that they learn more skills in the English class to act against environmental abuse than LM students with the AM students.

For question 5, the majority of HM, AM and LM students agree with the statement with a significant difference between the mean scores of AM and LM students [$p<0.05$]. The mean score of LM ($m = 3.25$) students is notably higher than that of AM ($m = 2.63$) students. This finding suggests that the majority of students believe that their reading skills have improved, with LM students being the most positive in their responses.

The data for question 6 indicates a significant difference between the mean scores of the HM and AM students [$p<0.05$]. HM ($m = 2.82$) and LM ($m = 2.83$) students agree with the statement while the AP ($m = 2.29$) students disagree. This means that, of the three groups, the AP students find the environmental topics the least helpful in improving their spoken English and HM and LM students think otherwise.

The data for question 7 indicates a significant difference in the mean scores between the HM and AM students' responses [$p<0.05$]. HM ($m = 2.64$) students agree with the statement while AM ($m = 1.95$) and LM ($m = 2.14$) students disagree.

Discussion

The questionnaire data reveal that both male and female students seem to share similar perceptions of the environment. They indicate a positive attitude towards the environment. Regardless of the gender, all students are concerned about the environment and say they take steps not to damage it and want to conserve it for the future generations. They also indicate a desire for the opportunity to choose the topics they like.

The data also suggest that the female students are more concerned about protecting the environment than the males. This could perhaps be due to the nurturing instinct in females which makes them want to care and protect more than males. The interviews provide evidence to verify this interpretation.

These findings reflect evidence from social, psychological and educational research that many of the world's children are genuinely fearful about the changing world and are preparing to face future challenges. Similarly, the paper shows that students are aware of the environmental crisis and feel that environmental education is imperative to curtail this problem [1].

Generally, students feel that the use of environmental topics to teach English has helped to develop and improve their language skills. But this improvement is only secondary, as they seem to have learned more content knowledge and ideas about the environment through these topics than language skills. This is in line with the findings of [6, 9]. They found that teachers were teaching the topics instead of the language skills.

Vocabulary is important in a language learning program and findings show that respondents believe they have improved in this area too. This is mainly due to the opportunities present in the English lessons for the introduction of useful vocabulary by the teacher. But more importantly, it could be due to the opportunities students had to practice the meaningful use of new vocabulary.

HP students seem to be better in discussing environmental issues and have more ideas to share about the environment compared. They report picking up skills that help them deal with environment concerns such as listening for ideas and writing. AP students have greater confidence in discussing environmental issues too. Hence both appear to have picked up the content for discussion rather than the skills.

Conclusion

Language teachers have many opportunities to heighten students' awareness about the environmental problems plaguing the planet while remaining committed to improving students' English skills. In addition to supporting meaningful language instruction, such theme-based instruction encourages students to think critically, provides them with real world knowledge, increases motivation, and promotes learner autonomy. Given teachers' busy schedules, some may feel hesitant about developing and incorporating environmental education into their curricula.

Students learn more, as through sharing responsibility for creating their classroom environment they become more skillful learners. Teachers also get a chance to better understand their students as well as adapt their teaching to specific groups of students.

Many students want to be able to choose the environmental topics to study in their English classes. This could imply that the currently assigned topics are uninteresting. A number of classroom practices and suggestions may also be considered to bring variety and interest to learning English. Though the inclusion of environmental education in the English language classroom may seem difficult, it is worth trying if we actually look forward to the welfare of both our environment which is becoming an eminent

threat to humanity and our young generations who are going to live in the world of tomorrow.

References:

1. Brown H. D. The Language Teacher. 15 (8), 1991, 4–5.
2. Cotton D. R. E. Educational Research. 48 (2), 2006, 223–41.
3. Ryan F., and Ray S. The Environment Book, Activities and Ideas for Environmental Education. South Melbourne: Macmillan, 1991. p. 252-256
4. Jacobs G. M. and Goatly A. ELT Journal 54 (3), 2000, 256-264.
5. Stavreva Veselinovska S., Kirova S. Integracija nekih nastavnih sadržina ekološkog vaspitanja sa učenjem engleskog kao stranog jezika, Zbornik radova TIO 2007, 2007, 283-286
6. Pillay H. and North S. The English Teacher, 26, 1997, 1–23.
7. Ratnawati M. A. The English Teacher, 25, 1996, 4–10.
8. Kent O. Journal of Jewish Education 72 (3), 2006, 205-33
9. Jacobs G. M. Integrating environmental education in second language instruction. Occasional Paper No. 46. Southeast Asian Ministers of Education Organization, Singapore, Regional Language Centre. 1993, p 68.

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ДОКЛАДИ/REPORTS

Тематично направление:

ДРУГИ КОРЕСПОНДИРАЩИ НАПРАВЛЕНИЯ

Topic:
OTHER RELATED TOPICS
ДОКЛАДИ/REPORTS

ENVIRONMENTAL HEALTH INVESTIGATION OF SANDPITS AT PUBLIC PLAYGROUNDS IN SOFIA CITY

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Abstract:

Aim: The purpose of this investigation is to evaluate the environmental contamination and the hygiene of natural sand from sandpits at public playgrounds of Sofia city, followed by relevant environmental health conclusions.

Materials and Methods: 20 representative samples of sand were collected from 20 public playgrounds in Sofia city. The samples were analyzed for chemical, microbial and parasitic contamination.

Results: The sand appeared to be clean from harmful chemicals and parasites, though the indicative quantifications of total coliforms, *E. coli, Enterococcus* and *Cl. perfingens* reveal external fecal contamination and potential risk for the presence of enteric pathogens in the sand.

Conclusion: The results of this study indicate the need for introduction of more regular replacement of the sand and efficient disinfection procedures for the public sandpits of Sofia city.

Keywords: sandpits, playgrounds, environmental health, contamination

Introduction

In Bulgaria, Ordinance No. 1 of 12 Jan. 2009 on the Terms and Conditions for the Structure and Safety of Playgrounds (SG No. 10/2009) [1] set the new requirements for children playgrounds. Subsequently, many municipalities, including the Sofia Greater Municipality started the construction or rehabilitation of a number of such facilities, which are currently available in the public parks and areas of Sofia city, with extremely high functional load. With a contribution to ensuring the health safety of those facilities,

NCPHA organized the first complex sanitary study on quality of playgrounds' sand in Sofia city, in terms of heavy metals and asbestos content, sanitary-bacteriological and parasitological indices.

Materials and Methods

Sand was sampled at 20 playgrounds in Sofia city, where 8 were situated in the Triaditza municipality, 3 in Krasno Selo, 3 in Sredetz, 4 in Oboriste and 2 in Slatina. The spatial distribution of the sampling points was selected randomly, mostly at the central area of the city, in order to assure both unbiased sampling and presence of intensively used playgrounds.

The methodology was based on the available literature references - pointing out the bacteriological and the parasitological contamination of the sand as of major health concern – potential presence of pathogenic bacteria and parasitic worms (*Toxocara* spp, *Trichuris* spp, *Toxascaris leonine, Ancylostomatidae, Ascaris lumbricoides*) [2, 3, 4, 5, 6, 7]. For each sand sample taken from 0-10 cm depth, a protocol was provided, followed by a common laboratory protocol with the analytical results from all samples.

The determination of heavy metals content involved a wide spectrum of accredited methods, including mineralization and atomic absorption spectroscopy. The analytical procedure is validated using a certified reference material for the soil. Defined were the procedure parameters - limit of detection, recovery and measurement uncertainty. Asbestos was determined through ISO 22262-1:2012: Air quality – Bulk materials - Part 1: Sampling and qualitative determination of asbestos in commercial bulk materials and MDHS-77 Asbestos in bulk materials: identification by polarized light microscopy HSE Books 1994. The parasitological indices were investigated in NCIPD through a validated method II/19/1995. The bacteriological parameters included *Salmonella* sp., coliforms, *E. coli, Enterococcus, Cl. perfringens,* determined through titrimetric methods.

Results and Discussion

Table 1 presents the mean contents of seven heavy metals of all investigated sands and the 95% confidence intervals (CI) and other descriptive parameters.

Table 1. Mean contents of heavy metals in the sands. ***N is the number of samples; x – the mean value; SD – the standard deviation; SE – the standard error; CI – the confidence interval; LPC - limit permissible content in mg/kg of dry mass [8]; P presents the P-value difference between the mean and the LPC, one-tailed test of significance (with alternative hypothesis: x <LPC); ** - the limit of detection***

Statistical Index	pH	Lead	Cadmium	Arsenic	Copper	Zinc	Chromium	Nickel
N	20	20	20	20	20	20	20	20
x	7.51	22.87	<0,20**	1.78	7.72	22.87	4.60	2.03
SD	0.37	5.29	-	1.66	1.78	5.29	1.74	1.03
SE	0.08	1.18	-	0.37	0.40	1.18	0.39	0.23
95% CI	7.34-7.69	20.39-25.34	-	1.00-2.56	6.88-8.55	20.39-25.34	3.79-5.41	1.55-2.51
Min	6.65	15.00	-	0.57	5.25	15.00	1.75	0.90
Max	8.05	35.80	-	8.62	11.00	35.80	8.50	4.50
LPC (mg/kg)	-	*200*	*8*	*25*	*300*	*400*	*200*	*100*
P	-	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*

The data obtained reveals the main characteristics of the variables and their distributions, the P-values of significance and determined the level of environmental health risk.

Obvious are the low heavy metals content in all sands, and the relatively narrow CIs. Conclusion can be derived that the heavy metals presence in the investigated sands is significantly below the LPC, with small variations caused predominantly by the initial differences in metal contents from the sand sources, and not in connection to later pollution during the exploitation of the sandpits.

Regarding asbestos, the investigated sand samples are not contaminated by this kind of dangerous to health fibers, and there is no health risk to the general population.

Determined is the contamination of the sand samples with organic non-asbestos fibers. The presence of this type of fibers in the sand before delivery to the sandpit is unlikely, because of the current regulatory requirements for washing and disinfection of sand before use. In this case, it can be assumed that the presence of many non-asbestos fibers is an indirect indicator of a secondary contamination of the sand during its exploitation and stresses the need for more frequent complete replacement of the sand in the playgrounds.

Regarding the laboratory analyses in NCIPD for parasitic contamination of the sand, the results do not prove increased health risk to the population when using of the studied children's playgrounds. Intestinal protozoa and helminths are not found in all 20 samples. Only in one sand sample are determined larvae of free-living nematodes, which are not pathogenic to humans.

The results of the bacteriological investigation for the presence of sanitary-indicative microorganisms are figured in Table 2.

Table 2. Bacteriological investigation of the sand samples

Sample	Coliforms (titer/g)	*E. coli* (titer/g)	*Enterococcus* (titer/g)	*Clostridium perfringens* (titer/g)	*Salmonella* spp. (in 20 g)
1.	0.001	0.001	0.001	0.001	not isolated
2.	0.0001	0.0001	0.0001	0.001	not isolated
3.	0.00001	> 1	0.01	0.01	not isolated
4.	0.001	0.001	0.01	0.001	not isolated
5.	0.001	0.001	0.01	0.01	not isolated
6.	0.1	0.1	0.01	0.1	not isolated
7.	0.001	0.001	0.1	0.01	not isolated
8.	0.01	0.01	0.1	0.01	not isolated
9.	0.01	> 1	0.1	0.1	not isolated
10.	0.001	> 1	0.01	0.001	not isolated
11.	> 1	> 1	0.01	0.01	not isolated
12.	0.01	0.1	0.01	0.01	not isolated
13.	0.1	0.1	0.1	> 1	not isolated
14.	0.1	0.1	0.01	0.01	not isolated
15.	0.001	> 1	0.001	0.01	not isolated
16.	0.0001	> 1	0.01	0.1	not isolated
17.	0.0001	0.0001	0.001	0.001	not isolated
18.	> 1	> 1	0.001	0.01	not isolated
19.	0.001	> 1	0.01	0.001	not isolated
20.	0.0001	1	0.0001	0.01	not isolated

The indicative quantifications of total coliforms and *E. coli, Enterococcus* and *Cl. perfingens* prove their persistence in different proportions in most of the samples, thus indicating external fecal contamination and potential risk for the presence of enteric pathogens in the sand. Pathogenic *Salmonella* spp. in 20 g is not isolated.

Concerning coliforms and *Cl. perfringens,* the results are compared with the criteria for sanitary clean environment (sand/soil), presented in Tables 3 and 4. Used are qualitative scales for the presence of coliforms and *Cl. perfringens* - in three rank groups as follows:

• samples meeting the requirements for clean sand (titer ≥ 1.0 for coliforms; titer ≥ 0.01 for *Cl. perfringens*);

• samples corresponding to contaminated sand (titer from 0.9 to 0.01 for coliforms; titer 0.009 – 0.0001 for *Cl. perfringens*);

• samples corresponding to strongly contaminated sand (titre ≤ 0.009 for coliforms; titre ≤ 0.00009 for *Cl. perfringens*).

This way of presenting the results corresponds to the criteria for determining the sanitary condition of soils (in titer) according to the national reference document [9].

Table 3. Coliform presence in the sand samples

Sample	Total number of samples N	Results from the bacteriological investigation, presented as a percentage of samples with:			Requirement for sanitary clean sand (Coliforms titer)[9]
		Titer ≥ 1.0	Titer 0.9-0.01	Titer ≤ 0.009	
Coliforms	20 (100%)	10.0%	30%	60.0%	> 1.0

Table 4. *Cl. perfringens* presence in the sand samples

Sample	Total number of samples N	Results from the bacteriological investigation, presented as a percentage of samples with:			Requirement for sanitary clean sand (*Cl. perfringens* titer)[9]
		Titer ≥ 0.01	Titer 0.009-0.0001	Titer ≤ 0.00009	
Cl. perfringens	20 (100%)	70.0%	30.0%	0.0%	> 0.01

Conclusion

1. In all analyzed sand samples from Sofia city, the heavy metal contents are far below the relevant LPCs, thus not being an essential environmental health concern. Heavy metals exposure from sandpits is not a cause for an increased public health risk, both for children and adults, visiting the investigated playgrounds.

2. Regarding asbestos, the sand samples are not contaminated with these hazardous to health fibers. The presence of many non-asbestos fibers is an indirect indicator of a secondary (exploitational) pollution of the sand and reflects the need of its more frequent replacement.

3. Concerning potential contamination with intestinal protozoa and helminths, the results of the parasitological study do not prove their presence in the sand.

4. In most of the sand samples persist sanitary-indicative microorganisms in quantities, exceeding the average values for clean soil (sand). The microbiological indicators are with major hygienic significance in order to assure sand safety at children playgrounds.

5. The results of the microbiological testing prove, that the municipal authorities should organize more frequent replacement of the sand, and improve the quality of its periodic disinfection.

References:

1. Council of Ministers, Sofia. Ordinance No. 1 on the Terms and Conditions for the Structure and Safety of Playgrounds. − State Gazette, 10/2009.

2. Bartosik M., Rzymowska J. Annales Universitatis Mariae Curie-Sklodowska, Lublin-Poland, XXIII (1), 2010, 61-66.

3. Gyorkos T. W., Kokoskin-Nelson E., Dick MacLean J., Soto J. Can. J. Infect. Dis., 5 (1), 1994, 17-20.

4. Carabin H., Gyorkos T. W., Kokoskin E., Payment P., Joseph L. and Soto J. Can. J. Infect. Dis., 9 (3), 1998, 149-156.

5. Hayward C., Knight R., Rossum N., Wickham B., Struwig M. C., Venter E. C., Joubert G. The South African Journal of Epidemiology and Infection., 21(4), 2006, 173-177.

6. Badura A., Luxner J., Feierl G., Reinthaler F. F., Zarfel G., Galler H., Pregartner G., Riedl R., Grisold A. J. Environmental Pollution, 194, 2014, 24-30.

7. Fernandes M. C., Takai S., Leite D. S., Pinto J. P., Brandão P. E., Santarém V. A., Listoni F. J., Da Silva A. V., Ribeiro M. G. Braz. J. Microbiol., 44 (2), 2013, 485-491, http://dx.doi.org/10.1590/S1517-83822013005000044

8. Council of Ministers, Sofia. Ordinance No. 3 on the Limit Permissible Content of Harmful Substances in Soils. − State Gazette, 71/2008.

9. Ministry of Health, Sofia. Soil. Methods for bacteriological examination., Proceedings of the methods for mandatory laboratory tests on sanitary-epidemiological control, 1985, 76-83.

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A CLASSIFICATION SYSTEM FOR EVALUATION OF ECOLOGICAL STATUS OF COASTAL MARINE WATERS IN RESPECT OF ZOOPLANKTON BIOLOGICAL QUALITY ELEMENT

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Abstract

Goal: The main aim of the study was to implement the WFD classification system for zooplankton and to develop a classification system for the ecological state assessment of coastal marine waters.

Materials and Methods: An ecological classification system was defined based on the candidate indicators: mesozooplankton biomass, *Noctiluca scintillans* biomass, *Mnemiopsis leidyi* biomass and Shannon diversity index. The initial assessment of the ecological status of the coastal waters of Bulgaria is based on the data collected during the sampling campaigns in 2012 (May-November).

Results: Among the candidate indicators mesozooplankton biomass index prevailed in the evaluation of the final ecological state of the coastal marine waters.

Conclusions Water bodies situated to the north BG2BS000C001, BG2BS000C002, BG2BS000C003 and BG2BS000C006, BG2BS000C007 to the south met moderate conditions while all other sites reflected a poor state.

Key words: indicators, zooplankton, coastal waters, Water Framework Directive

Introduction

The EU Water Framework Directive environmental objectives are established on the basis of ecological status assessment, focused on species groups as phytoplankton, macrophytes, marine angiosperms and macrozoobenthos, however zooplankton was not considered, despite the fact that it is a key and integrated component of pelagic food webs [1]. Zooplankton is mentioned in the WFD CIS Monitoring guidance [2] and in the Bulgarian legislation, Ordinance № 1/11.04.2011 (Article 56) as a 'supportive/interpretative parameter''. The importance of zooplankton as an indicator of ecological conditions stems from its position in the food web, sandwiched between the top-down regulators (fish or jellyfish) and bottom-up factors (phytoplankton), thus providing information about the relative significance of top-down and bottom-up controls and their impact on water clarity [1].

The main aim of the study was to apply the WFD approach to zooplankton and develop a classification system for the ecological state assessment of coastal marine waters. The first step in the assessment of the ecological status was selecting reliable zooplankton indicators followed by reference conditions establishment and determination of relevant class boundaries.

Materials and Methods

Study area and sampling

Preliminary investigation was carried out in 2012 within the frame of Agreement D-33-4/08.05.2012 between the Ministry of Environment and Waters and Institute of Oceanology-BAS for the Black Sea monitoring accomplishment regarding to Article 171, Paragraph 2, Item 3 of the Water Act. Twenty stations from Krapets (North) to Veleka (South) located on the one mile strip along the Bulgarian Black Sea coast were monitored in May, June, July, August, September-October and November, 2012.

Zooplankton samples were collected by a vertical plankton Juday net, 0.1 m^2 mouth opening area, 150 μm mesh size, from 2 meters above the bottom to the surface. Total of 120 zooplankton samples were collected during the cruise. Species quantity was determined by the Dimov's method [3]. Biomass was estimated using species individual weight by Petipa [4].

Approach - indicators and analysis

The list of selected potential zooplankton metrics (indicators) tested during the National Monitoring Programme campaign in 2012 included: a) Mesozooplankton biomass [$mg.m^{-3}$], b) *Noctiluca scintillans* biomass [$mg.m^{-3}$], c) *Mnemiopsis leidyi* biomass [$g.m^{-3}$], d) Shannon-Weaver index [$ind.bit^{-1}$].

The European WFD requires a definition of reference conditions, i.e. pristine conditions, for all surface waters. Reference conditions are equated with the "high ecological status" of the classification system and are meant to represent the structure and functioning of biological communities in the absence of or under minimal anthropogenic disturbances. Key approaches for deriving ecological type-specific reference conditions, namely historical evidence and professional judgment are outlined in the study. Next, biological, respectively zooplankton attributes of ecological quality (metrics) were selected with respect to their sensitivity to particular types of environmental stress. Finally, reference conditions for the selected metrics and the appropriate strata (thresholds) were specified. A long- term zooplankton data (1966-2007) from c. Galata and Varna Bay (Western Black Sea) as the best available historical data set analysed and the percentile approach for thresholds identification was applied [5]. Even though this series was not considered to be the monthly series data, these biological series could be the best and useful series available as indicators of the system's alteration over a long-term period since 1967. Due to the high seasonal variability of zooplankton community quantity a classification tool was developed on seasonal bases. The values estimated from 1967 to 1972 correspond to the reference conditions (for the definition of "High" status the 90 percentile was calculated) and those from the period 1980 - 1993 of "Bad" status. The same period was the base for a definition of the "bad values" of *N. scintillans* biomass indicator using the 95 percentile of data from the intensive anthropogenic eutrophication phase. The identified Ecological Quality Ratio (EQR) for each indicator expresses the relationship between the observed and reference condition values and lies between 0 and 1.

For the overall classification of the ecological status in respect of zooplankton biological quality element the "one-out-all-out" principle and in minor cases an expert judgment have been applied, resulting in the worst value of all stations and all indicators per coastal water body.

The metrics and WFD classification system are presented in Table 1.

Table 1. Metrics and classification system for the coastal marine waters (according to WFD) a) mesozooplankton biomass [mg.m^{-3}], b) *Noctiluca scintillans* biomass [mg.m^{-3}], c) *Mnemiopsis leidyi* biomass [g.m^{-3}], d) Shannon-Weaver index [ind.bit^{-1}]

a)

Season/state	High	Good	Moderate	Poor	Bad
Spring	400-300	300-150	150-70	70-10	<10(>400)
EQR	1.00-0.70	0.69-0.40	0.39-0.20	0.19-0.10	0.00
Summer	900-600	600-350	350-200	200-40	<40(>900)
EQR	1.00-0.70	0.69-0.40	0.39-0.20	0.19-0.10	0.00
Autumn	350-250	250-150	150-70	70-10	<10(>350)
EQR	1.00-0.70	0.69-0.40	0.39-0.20	0.19-0.10	0.00

b)

State	High	Good	Moderate	Poor	Bad
N. scintillans	<50	50-250	250-500	500-2500	>2500
EQR	1.00-0.90	0.89-0.80	0.79-0.30	0.29-0.10	0.00

c)

State	High	Good	Moderate	Poor	Bad
M.leidyi	0	1-4	4-20	20-50	>50
EQR	1.00-0.90	0.89-0.70	0.69-0.20	0.19-0.10	0.00

d)

State	High	Good	Moderate	Poor	Bad
H'	>3.5	3.5-2.5	2.5-1.5	1.5-1	<1
EQR	1.00-0.80	0.79-0.60	0.59-0.20	0.19-0.10	0.00

Results

Description of indicators and ecological state

The initial assessment of the ecological status of the coastal waters of Bulgaria is based on the data collected during the sampling campaigns in 2012 (May-November).

The mesozooplankton biomass indicator reflects the composition of the zooplankton community and includes information about the major key groups - Copepoda, Cladocera, Meroplankton and the species O*ikopleura dioica and Parasagitta setosa* as well. This zooplankton metric exhibits strong variability in time and space under the influence of natural

and anthropogenic factors. Mesozooplankton is indirectly exposed to the eutrophication process (the amount of food composition and size) and catches of commercially exploited fish (through changes in the pelagic food chain), while the direct impact is shaped by climate change and predation of fish and gelatinous plankton. The Black Sea ecosystem evolution passed through several phases such as: a) pre-eutrophication - up to the early 70s, representing an oligotrophic regime of the ecosystem, b) eutrophication period with two phases of early and intensive eutrophication (from the 70s to the early 90s) which coincided with the invasion of *M. leidyi*, overfishing and loss of biological diversity, and c) the post-eutrophication period after 1995 which corresponded to reduction in the anthropogenic nutrient and pollutantload, *B. ovata* introduction and *M. leidyi/B. ovata* interaction [6, 7, 8].

Historical data of zooplankton biomass were not normally distributed in respect of seasons (normtest, PASTECS, $p<0.05$). Long-term time series analyses showed that the zooplankton biomass shifted as a term of the anomalies at the studied stations in distinct periods. As indication of anomalies (deviation from the relevant data), starting with a positive phase at the beginning of the time-series (1967 - 1972), zooplankton biomass decreased steadily and was consistently lower than the average value during much of the 1970s and 1980s (Table 2).

Table 2. Basic statistic information of mesozooplankton biomass in distinguished periods and seasons at c. Galata station

Season	*summer*	*summer*	*spring*	*spring*	*autumn*	*autumn*
periods	**1967-1972**	**1980-1993**	**1967-1972**	**1980-1993**	**1966-1972**	**1980-1987**
n	**29**	**43**	**25**	**53**	**28**	**22**
Average	499.78	161.59	123.07	80.58	120.98	94.56
Standard Error	85.07	30.42	22.57	21.36	19.09	31.55
Median	364.52	87.44	93.00	41.93	87.17	41.95
Standard Deviation	458.10	199.46	112.85	155.52	100.99	147.98
Minimum	50.48	12.20	15.09	2.31	11.63	4.41
Maximum	2141.08	1058.83	454.62	1084.68	404.92	641.10
Confidence interval (95.0%)	174.25	61.38	46.58	42.87	39.16	65.61

Mesozooplankton biomass presented high variability especially in summer during the monitoring campaigns in May-November, 2012. The results suggested a wide range of ecological states between poor to good in the spring, poor-to moderate in the summer and moderate to very good in the autumn. Thus, the lowest values were recorded in the summer which defined the season as critical for the pelagic ecosystem. Also in summer a high dispersion in the biomass distribution was observed (Fig. 1).

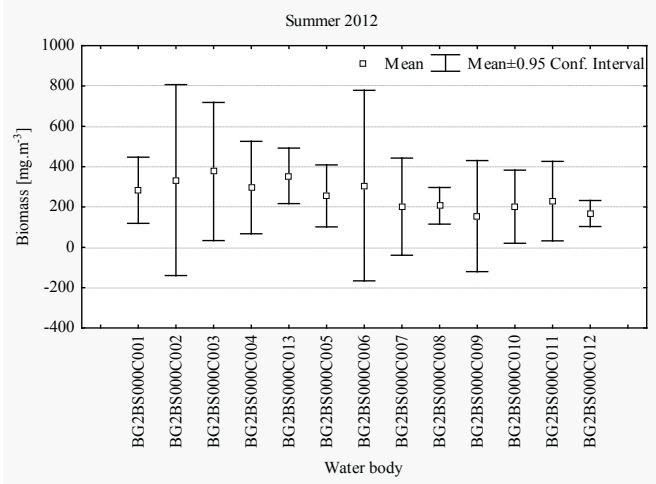

Fig. 1. Mesozooplankton biomass (average values ± 95% confidential interval) in summer 2012

The mesozooplankton biomass indirectly reflects the *Noctiluca scintillans* and *Mnemiopsis leidyi* contribution to the plankton community. *N. scintillans* biomass could be used as a good indicator of water quality and eutrophication. Its density is usually higher in coastal areas where maximum phyto- and zooplankton were registered. In 2012, the categorization of the ecological state of the coastal waters was defined as poor (between 500-1300 $mg.m^{-3}$) in 75% of the cases and as good – only in 20% (Table 3).

Based on the calculated critical biomass of ctenophore *M. leidyi* that does not affect mesozooplankton abundance, 4 $g.m^{-3}$ was identified [9] as a threshold for good ecological status while the absence of the species - as a very good status. *M. leidyi* biomass varied in a wide range during the past 10 years (0.1 to 136 $g.m^{-3}$±34), the same as most zooplankton metrics. Monitoring data of 2012 supported sharp fluctuations of the *Mnemiopsis* biomass throughout the whole study period with the average- 12 $g.m^{-3}$ ±34.5 and maximum – 209$g.m^{-3}$. The ecological state of water bodies according to the *M. leidyi* biomass indicator demonstrated lower quality at the stations located on the north in comparison with those on the south part (Table 3).

The Shannon - Weaver diversity index is one of the most used in the assessment of pollution in an ecosystem. The ability to quantify diversity in this way is an important tool trying to understand community structure. The classification was made according to the maximum number of species found in the reference period, not taking the seasons into account. According to the proposed classification, index H' in 2012 reflected the state between moderate and good with an exception at c. Kaliakra (poor status < 1.5 ind.bit-1). Although the index is not presented seasonally, the variations were higher in spring and summer while in autumn in 50% of the cases the values corresponded mainly to a good ecological status (Table. 3).

The lowest values were in May, which probably correlated with the *N. scintillans* prevalence in the mesozooplankton complex.

Discussion

Coastal water bodies are amongst the most intensively used marine water areas and are an integral part of the marine environment, and as such they are also covered by the MSFD. With the implementation of the WFD member states had to classify the ecological status of surface waters regarding a series of biological quality elements excluding zooplankton. Further, the National Monitoring Programme of Bulgaria - 2012 required the assessment of the coastal Black Sea ecosystem in broader aspects including fish and zooplankton as complementary biological quality elements. Preliminary results of the study together with relevant data from numerous zooplankton studies, suggest the zooplankton metrics as reliable indicators of water quality and ecological state.

High mesozooplankton biomass suggests higher trophic environment [10], increasing the concentrations of the plankton fauna are an indirect indicator of the food ability in the water column respectively eutrophic conditions. On the other hand mesozooplankton biomass reduction indicates the enhanced predator pressure in the food chain (jellyfish, ctenophores and small pelagic fish). The development of *M. leidyi* reflected directly (predation) while *N. scintillans* indirectly on mesozooplankton biomass, since both negatively correlated with the biomass of plankton fauna.

The wide feeding spectrum (phytoplankton, zooplankton and detritus) of the species and development in high bloom concentrations especially in spring, usually after the mass development of phytoplankton, determine its ecological importance for the pelagic ecosystem [11]. Eutrophication leads to an increase in the number and productivity of phytoplankton, which provokes *N. scintillans* development simultaneously with favorable hydrological factors-temperature rise and calm weather [12]. *Noctiluca* is recognized as a "dead-end" in the food web due to the species regarded as an end user in the food chain. In addition, the increased species biomass implies a reduced quantity and quality of the fish food supply. Very often, the biomass of *N. scintillans* corresponds to more than 90% of the zooplankton during bloom periods. *N. scintillans* blooms subsequently overcome zooplankton biomass by feeding on their eggs and competing for food resources [13].

As a key factor for the mesozooplankton community, *M. leidyi* becomes a reliable indicator of the pelagic ecosystem dynamics and relationship with other trophic groups to determine the functioning of the pelagic food web. Gelatinous predator, which consumes less than 10% of the zooplankton biomass per day, can not reduce mesozooplankton abundance and biomass [14]. However, the higher consumption rates (more than 20% of zooplankton biomass per day) result in a sharp reduction of the prey abundance [15].

Diversity indices provide important information about rarity and commonness of species in a community and are mainly an approach to biological quality determination.

Table 3. Ecological state of water bodies (May-November 2012) according to proposed zooplankton indicators

Water body	Station	Mesozooplankton biomass			*N.scintillans* biomass	*M.leidyi* biomass	H'	Ecological state
		Spring	Summer	Autumn				
BG2BS000C001	Krapets	166.66±34.24	330.27±217.79	525.70	718±491.16	70.04±121.1	2.93 ±0.55	Moderate
BG2BS000C002	Shabla	148.21±122.43	333.45±190.62	538.07	121.3±65.39	2.16±3,5	3.01±0.37	Moderate
BG2BS000C003	Rusalka	212.96±203.054	375.97±137.88	503.21	241.8±136.66	6.11±10.5	2.66±0.35	Moderate
BG2BS000C004	Kaliakra	49.39±28.88	349.75±194.61	161.20	536.8±108.30	5.39±9.3	1.89±0.9	Poor
	Balchik	100.78±109.8	242.91±269.72	549.54	585.4±90.83	0.71±1.2	2.91±0.92	
BG2BS000C013	Albena	57.55±44.16	315.88±152.47	415.34	1077.4±1017.37	0.01	2.85±0.98	Poor
	Galata	39.73±0.87	393.06±123.48	499.79	600.1±542.10	1.88±3.2	3.05±0.79	
BG2BS000C005	Varna Bay-North	36.51±5.53	249.80±171.88	362.43	1300±1023.41	2.78±4.7	2.41±0.84	Poor
	Varna Bay-South	79.92±30.63	260.47±154.59	296.82	499.6±83.5	5.99±10.2	2.23±1.03	
BG2BS000C006	Kamchia	64.71±9.55	306.14±190.18	241.29	695.9±260.21	2.34±3.5	2.78±0.73	Moderate
BG2BS000C007	Dvoinica	48.64±11.25	201.67±96.98	129.10	843±611.64	0.034±0.1	2.73±0.58	Moderate
BG2BS000C008	Nesebar	142.34±113.25	176.29±6.50	182.20	551,8±668.12	0.00	3.02±0.88	Poor
	Rosenets	120.90±20,87	273.25±202.31	503.34	1220.2±1337.03	7.11±11.9	2.54±0.79	
	Sarafovo	44.17±20.58	167.99±67.07	224.30	414.6±346.63	6.51±11	2.46±0.94	
BG2BS000C009	Koketrais	106.27±56.35	154.94±110.87	181.74	125.9±109.95	1.89±3.2	2.88±0.85	Poor
BG2BS000C010	Bourgas 2	48.86±4.28	169.28±191.81	82.17	613.3±453.35	0.00	2.25±1.02	Poor
	Maslen nos	51.90±22.84	233.71±186.552	115.26	319.9±199.41	0.23±0.3	2.23±0.7	
BG2BS000C011	Sozopol	39.05±3.38	229.18±79.34	222.85	47.1±57.17	0.021±0.02	2.87±0.79	Poor
BG2BS000C012	Varvara	36.81±2.96	196.56±43.19	152.41	440.8±441.76	0.031±0.1	2.59±0.7	Poor
	Veleka	53.13±3.26	138.75±71.37	122.36	591±560.31	0.02±0.023	2.27±0.73	

Mesozooplankton biomass index prevailed in the evaluation of the final ecological state of the coastal marine waters. According to various indices, cases of moderate and poor conditions were the most common (~ 40%), as good or high were 12% and 8%, respectively (Table 3). Water bodies situated to the north BG2BS000C001, BG2BS000C002 and BG2BS000C003 (Krapets, Shabla and Rusalka) and BG2BS000C006 and BG2BS000C007 (Kamchia and Dvoinitsa) to the south met moderate conditions while all other sites reflected poor state (Fig. 2).

Conclusions

Long-term data analyses together with new data support the high indicative ability of zooplankton to reflect the state of the ecosystem and water quality. The results point to:

• Tested zooplankton indicators have a high value as invaluable tool in assessment of the trophic state and water quality of water bodies.

• Preliminary distinction into five ecological classes (as required in WFD) was done for various zooplankton indicators but further development is needed with focus on validation of candidate indicators.

• Suggestions for new indicators and development of an integrated zooplankton indicator for integration of different metrics.

Fig. 2. Map of the water bodies ecological state along the Bulgarian coast according to the biological quality element - zooplankton in the period May-November 2012

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References:

1. Jeppesen E., Nõges P., Davidson T. A., Haberman J., Nõges T., Blank K., Lauridsen T. L., Søndergaard M., Sayer C., Laugaste R., Johansson L. S., Bjerring R., Amsinck S. L., Hydrobiologia, 676, 2011, 279–297.

2. CIS. Monitoring under the Water Framework Directive. Common Implementation Strategy for the Water Framework Directive (2000/60/EC). Guidance Document No 7. Working Group 2.7 – Monitoring. European Communities, Luxembourg, 2003, p. 153.

3. Dimov I., Rep. BAS, 12 (5), 1959, 427-429. (in Russian).

4. Petipa T. S., Tr. Sevast. Biol. St. 9, 1959, 39-57.

5. Heiskanen A-S., Gromisz S., Jaanus A., Kauppila P., Purina I., Sagert S., Wasmund N. JRC, 2005, EUR 21582/EN/1, http://publications.jrc.ec.europa.eu/repository/handle/JRC32862

6. Moncheva S., Doncheva V., Kamburska L. In: Proceedings of IX International conference on "Harmful Algal Blooms", Hobart, Tasmania (G. M. Hallegraff et al., Eds.), UNESCO-IOC, Paris, 2001, 177-182.

7. Yunev O. A., Carstensen J., Moncheva S., Khaliulin A., Ærtebjerg G., Nixon S. Estuarine, Coastal and Shelf Science, 74, 2007, 63-76.

8. Mee L. D. Scientific American, 295 (5), 2006, 54–61.

9. Vinogradov M. E., Shushkina E. A., Anokhina L. L., et al., Oceanology 40 (1) 2000, 46–49.

10. HELCOM. Development of a set of core indicators: Interim report of the HELCOM CORESET project. PART B: Descriptions of the indicators. Balt. Sea Environ. Proc. No. 129 B. 2012, p. 219.

11. Dela-Cruz J., Middleton J. H., Suthers I. M. Limnol. Oceanogr., 48 (2), 2003, 656–674.

12. Adnan Al-Azri, Khalid Al-Hashmi, Joaquim Goes, Helga Gomes, Ahmed I. Rushdi, Harub Al-Habsi, Salem Al-Khusaibi, Raya Al-Kindi, Nasser Al-Azri,. International Journal of Oceans and Oceanography, 2 (1), 2007, 51–60.

13. Nakamura Y. Japan. J. Plankton Res., 20, 1998, 2213–2222.

14. Finenko G., Anninsky A., Abolmasova G., Romanova Z., Bat I., Kideys A. In: BSC: Trophic relationship and food supply of heterotrophic animals in the pelagic ecosystem of the Black Sea (B. Ozturk, A. Kideys, G. Finenko, G. Shulman, L. Bat, Eds.) Black Sea Commission Publications, Istanbul, Turkey, 2009, p. 308.

15. Shiganova T., Musaeva E., Araskievich E., Kamburska L., Stefanova K., Michneva V., Polishchuk L., Timofte F., Ustun F., Oguz T., Khalvashi M. In: BSC, State of the Environment of the Black Sea (2001-2006/7) (T. Oguz, Ed.) Publications of the Commission on the Protection of the Black Sea Against Pollution (BSC), Istanbul, Turkey, 2008, p. 448.

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