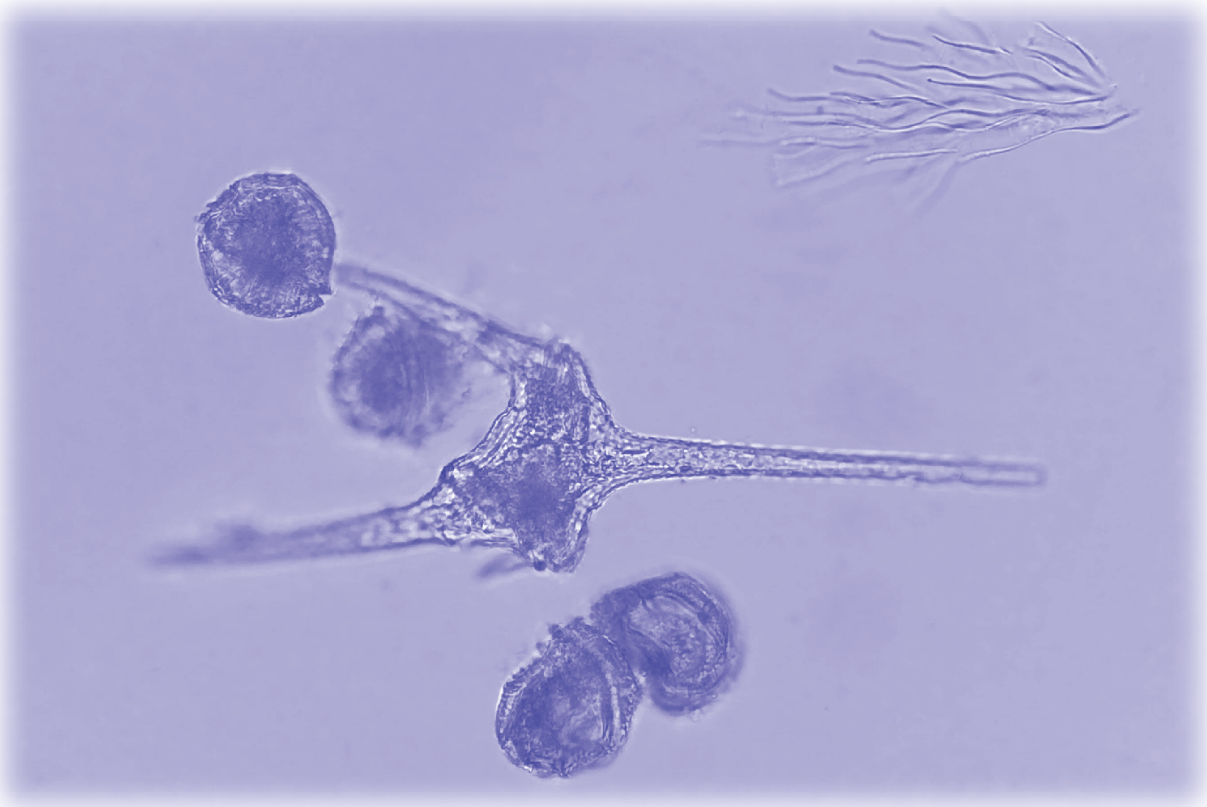
STUDIA UNIVERSITATIS
BABEŞ-BOLYAI



BIOLOGIA

1/2017

STUDIA
UNIVERSITATIS BABEŞ-BOLYAI
BIOLOGIA

1 / 2017
January – June

YEAR **Volume 62 (LXII) 2017**
MONTH **JUNE**
ISSUE **1**

PUBLISHED ONLINE: 2017-06-20
PUBLISHED PRINT: 2017-06-30
ISSUE DOI:10.24193/subbbiol.2017.1

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA

1

STUDIA UBB EDITORIAL OFFICE: B.P. Hasdeu no. 51, 400371 Cluj-Napoca, Romania, Phone + 40 264 405352, www.studia.ubbcluj.ro

SUMAR – CONTENTS – SOMMAIRE – INHALT

All authors are responsible for submitting manuscripts in comprehensible US or UK English and ensuring scientific accuracy.

Original pictures on front cover: The algal species: *Ceratium hirundinella*, *Peridinium cinctum* and *Dinobryon divergens* from Lake Iezerul Ighiel (Transylvania, Romania) (40x magnification) © Anca-Mihaela Ciorca

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXII, 1, 2017 (p. 5-7)

=== IN MEMORIAM ===

Professor Emeritus Constantin Crăciun

(15 September 1937 – 25 November 2016)

CONSTANTIN CRĂCIUN was born on September 15, 1937, in Pîrscoveni (Olt County, former Romanați County), Romania. Teodor Crăciun, the father of Constantin, graduated Philosophy and Theology at the University of Bucharest and became a devoted priest. He married Eva Cunescu, the daughter of a Moldavian boyar. Tragically, Eva Crăciun passed away while all her four children were very young (Constantin himself being only 3-years-old). Following this suffering moment, Teodor Crăciun moved with his family to Corabia where he was appointed as bishop. Constantin went on a deprived childhood and teenage, graduating the secondary school (Corabia), and the Technical High School for Topography (Bucharest, 1957). Constantin was afterwards appointed a job in Transylvania (Bihor County). At that time, he could not enroll in academic studies because of his politically 'unhealthy' ascendance, by the communist standards. Soon, however, Constantin was formally adopted by the family that hosted him in Batar village (Bihor County). It was a crucial moment in Constantin's fate, as he was now 'eligible' to become a student at the Faculty of Biology (Babeş-Bolyai University of Cluj, 1961-1966). During his undergraduate studies in Biology, Constantin Crăciun was tutored by Professors Eugen A. Pora, Emil Pop, Oreste Marcu, Victor Pop, Ștefan Kiss and others that left their marks on the progress of biological sciences in Cluj-Napoca and Romania. As a student, Constantin was remarked by Professor V. Gh. Radu for his hardworking, sense of responsibility and keenness to take on technical and science challenges. Prof. V. Gh. Radu foresaw

Constantin as a promising researcher and therefore, provided him a position at the Institute of Biological Research in Cluj-Napoca (1966-1978). The Romanian Nobel laureate Professor George E. Palade granted Constantin Crăciun a fellowship for attending a specialization at the Medical School of Yale University, but he was again denied to follow his dreams by the communist policy that restricted the travels in foreign countries. Following a specialization in electron microscopy at the University of Bucharest, Constantin Crăciun together with Professor V. Gh. Radu established the Laboratory of Electron Microscopy of the Babeș-Bolyai University starting in 1971. Since 2000, this Laboratory became the Electron Microscopy Center, which was continuously managed by Constantin Crăciun until his most regretted decease in 2016.

Professor Dr. C. Crăciun by the transmission electron microscope (TEM) unit at the Electron Microscopy Center (Babeș-Bolyai University of Cluj-Napoca) ***(Source: Photography Archive of Electron Microscopy Center - Babeș-Bolyai University of Cluj-Napoca)***.

Constantin Crăciun (or simply Costică as known by his family, close friends and colleagues) was appointed as Professor at the Department of Animal Physiology (Faculty of Biology and Geology, Babeș-Bolyai University) as well as associated

Professor at the Faculty of Biology ("Vasile Goldiş" West University, Arad) and the Faculty of Medicine (University of Oradea). The teaching tasks included lectures and laboratories of Cytology, Electron Microscopy and Advanced Cell Structure and Ultrastructure for undergraduate, Master and PhD students. Prof. Crăciun was honored as *Professor Emeritus* for his tremendous contributions to the research development and international visibility of Babeş-Bolyai University. The scientific expertise, active engagement in strengthening and broadening numerous collaborations in addition to development of the state-of-the-art research infrastructure and his unconditioned devotement to science, justified numerous prizes that Prof. Crăciun was awarded. Prof. C. Crăciun was awarded as *Doctor Honoris Causa* of two Universities ("Iuliu Haţieganu" University of Medicine, Cluj-Napoca, and "Vasile Goldiş" West University, Arad) and became a Honorary member of the Romanian Academy of Medical Sciences.

Professor Crăciun deeply dedicated his scientific interest to understanding the structure and function of cell components by employing sophisticated, although highly-accurate electron microscopy techniques. Ultrastructural imaging of a large variety of microbial, plant or animal cell samples either in their native state or impacted by natural compounds (e.g. alcohols, toxins) or xenobiotics (e.g. therapeutics) was brought up to the level of perfection by the skillful hands, sharp eyes combined with a prepared mind and enthusiastic heart of Constantin Crăciun. Numerous scientific papers (more than 500 in total including around 100 papers in international peer-review journals or volumes), books (28 in total including 4 books printed by international publishers), edited volumes (26), patents (9) and research grants (17 as a director) were authored during his entire prodigious career.

Besides his professional qualities as a motivated, focused and highly demanding researcher, Constantin Crăciun was much esteemed and beloved by colleagues and students for his warm and friendly appearance, restless, dynamic and optimistic character, openness to help and collaboration in every aspect of biology. In the same time, Professor Crăciun was known as an equilibrated and easy-going character by balancing the academic duties with outdoor hobbies such as gardening, fishing and hunting. He was a true model both as a human being and as a professional for many generations of students, some of which later became his collleagues and collaborators.

Acknowledgments. The preparation of this material was possible thanks to the contribution of Veronica Crăciun and Adrian Radu Crăciun. The kind support of Lect. Dr. Lucian Barbu, Assoc. Prof. Corina Roşioru, Prof. Nicolae Dragoş, Prof. Corneliu Tarba, and Acad. Octavian Popescu is gratefully acknowledged.

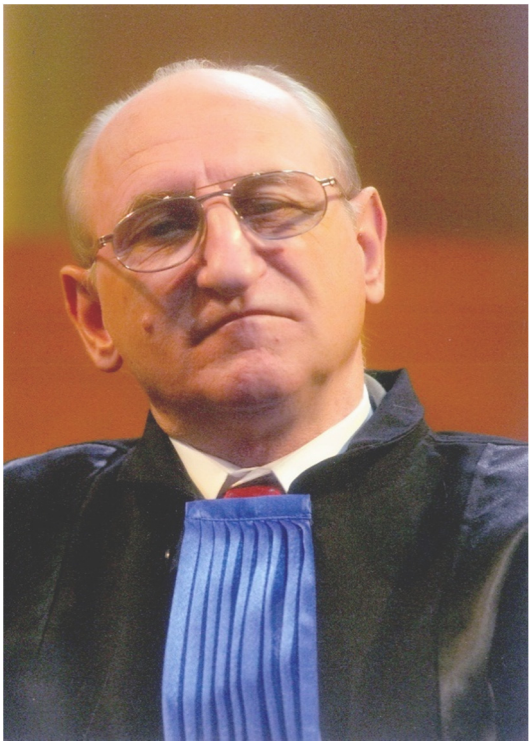
Horia L. BANCIU

Faculty of Biology and Geology
Babeş-Bolyai University, Cluj-Napoca

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXII, 1, 2017 (p. 9-11)

=== IN MEMORIAM ===

Professor Gavril Ardelean
(5 May 1942 – 3 October 2016)



GAVRIL ARDELEAN was born on May 5, 1942 in Seini, Maramureş County, Romania. He graduated the elementary school in Seini (1956) and "Gh. Şincai" High School in Baia Mare (1960). During the next years, Gavril Ardelean attended the Faculty of Biology, Geography and Geology (Babeş-Bolyai University) in Cluj, graduating in 1965 with a major in Biology and Geography. Until 1990, he taught Biology and Geography in several high schools in Vişeu de Sus, Tăşnad, and Satu Mare.

In 1979, Gavril Ardelean came back to *Alma Mater Napocensis* as a doctoral student, under the prestigious supervision of Academician Eugen A. Pora, who cherished him as an eminent disciple.

As Professor Pora passed away in October 1981, Gavril continued his research under the supervision of Professor Dumitru I. Roşca. The experimental part of his thesis was performed in the Biochemistry facilities of the Faculty of Biology, with the scientific help and kind assistance of Professor Ioan V. Deaciuc.

In those years, Gavril Ardelean used to come to Cluj as often as possible, to spend weeks or months working with dedication and perseverance on his doctoral thesis. At that time, he was already married and had two children, Doru and Raluca.

Unfortunately, he had to go through a tremendous tragedy, as Raluca passed away at the age of seven, after a ruthless suffering. Gavril found consolation in his work, both as a teacher and as a doctoral student, showing discretion in his deep distress.

He successfully defended his doctoral thesis *Mechanisms preventing fatigue* in January 1985.

Gavril Ardelean was appointed, by public contests, lecturer (1993), associate professor (1994) and full professor (1996) at the Northern University in Baia Mare, Faculty of Sciences. Since then, his scientific, teaching and organizing efforts were entirely dedicated to build a strong academic life in the Northern part of our country. In 1997, he founded the Satu Mare, and later the Baia Mare branches of the Western University "Vasile Goldiş" in Arad. He served as vice-provost of the Western University "Vasile Goldiş" in Arad (between 2007 and 2012), and as director of the two founded branches, until he had to live us and move away into a better world.

As a son of *Alma Mater Napocensis*, Gavril Ardelean (Puiu, for his friends and family) never neglected the collaboration with the staff of the Faculty of Biology and of the Biological Research Institute in Cluj. Although his start in the scientific activity was in the field of Animal Physiology, afterwards he successfully explored a new research avenue, addressing fauna biodiversity of the North-Western area of the country, species preservation and ecological reconstruction. His diligent endeavor of several decades materialized in almost 200 scientific papers, numerous textbooks, books (as author), and treatises (as coordinator).

Equally important, Gavril was an outstanding organizer and manager; he conducted seven research grants (most of them cross-border projects, in collaboration with researchers from Hungary and Ukraine) and participated as an expert in others. He understood how crucial are team work and communication among scientists; therefore, he spent a lot of energy in organizing 19 national and international scientific symposia. Gavril was always on the move: teaching, writing, organizing, putting things together – these were his reasons to be.

As a result of his prodigious activity, Gavril Ardelean was distinguished with the "Grigore Antipa" award of the Romanian Academy (2002) and received the distinction "Honorary Member in Commander Grade" from the Romanian Military Order. In the 5th of May, this year, Gavril would have been 75 years old. At the homagial scientific meeting organized on this occasion by the University he served so many years, he was awarded post-mortem with the highest academic distinction – *Doctor Honoris Causa* of the Western University "Vasile Goldiş".

Besides his dedication for science, Gavril Ardelean was a good Professor, who knew how to pass on to his students not only knowledge, but also – and far more important – passion, motivation, the curiosity to investigate. Gavril was also a reliable friend, always available when someone needed help in professional or personal problems. As long as we will keep him a good memory, he will remain present in the University and in our hearts.

Corina L. ROȘIORU

Faculty of Biology and Geology
Babeș-Bolyai University, Cluj-Napoca

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXII, 1, 2017 (p. 13-15)

=== IN MEMORIAM ===

Professor Iosif Viehmann

(1 September 1925 – 6 August 2016)

IOSIF VIEHMANN (Pepi, for family and friends) came to this world the first of September 1925, as the son of Iosif Viehmann, violonist at the Romanian Opera in Cluj, and Elisabeta Mureșan.

In 1940, after the loss of a part of Transylvania by the Vienna Dictum, Iosif (16 years old) and his brother Eugen (11 years old) fled to Timișoara with their mother. Here, the young Iosif Viehmann graduated the National College "C. D. Loga" in 1944.

After returning to Cluj, Iosif enrolled in the Faculty of Natural Sciences of the "Victor Babeș" University and graduated in 1950 with a thesis on the genesis of stalagmites.

There is a Romanian saying: "The man blesses the place". This is certainly true for Pepi, throughout his life. The university assistant position was refused to the newly licensed Iosif Viehmann, because of his declared sympathy for the Liberal Party and for the Americans, his monarchist feelings, and for having close relatives in Germany (his father and brother). Therefore, he ended as a highschool teacher in Năsăud (Pedagogical School, 1952-1956), where he succeeded to inspire his pupils the love for studying, for nature, culture, and... for caves. He founded, with their help, a Laboratory for Natural Sciences, organized school trips and spelelological explorations. In 1956, he was finally nominated scientific researcher at the "Emil Racoviță" Speleological Institute in Cluj, where he worked for the rest of his long and successfull career.

He never accepted the enrollment in the Communist Party. As a consequence, he was banned from matriculating in any doctoral program, and could finally defend his PhD thesis "*Carst in the Apuseni and Rodnei Mountains: a comparative study with geomorphological and stratigraphic considerations*" only in 1991, at the age of 66.

Nevertheless, his scientific activity was marked by numerous discoveries that gained international recognition: the "permanent drop" in Pojarul Poliței Cave, the ancient human footprint in Vârtop Cave, the thermoindicatory stalagmites in the Glacier Cave of Scărișoara, the cave bug *Drimeotis kovacsi viehmani* in Ienișțea Cave were premieres in Romanian speleology. As a researcher, he developed the Geze-Viehmanm theory on the genesis of eccentric monocrystals (crystalictites) in Pojarul Poliței Cave, explored and mapped the "record" caves Tăușoare (Rodnei Mountains) and Cetățile Ponorului (Bihor Mountains), and actively participated in the arrangement of Bears Cave (Bihor Mountains) for tourism use. As a legacy of his mentor, Professor Emil Racoviță, Iosif Viehmann actively participated in monitoring the seasonal evolution of ice in Scărișoara Glacier Cave. He also attended internships and scientific visits in the Yugoslavian karst, Eisriesenwelt ice cave (Austria), Bergen University (Norway), Nahal Soreq (Israel). He published more than 230 scientific papers and was cited by speleologists all over the world.

One of my professors told me once that "Pepi will never be a true scientist, because he is not serious enough". Indeed, Pepi was never frowning, because he loved young people, he was very close to students and, as an Associate Professor in the Faculty of Biology and Geology, did his best to educate them.

For many years, he teached an optional class in Biospeleology, attended by huge numbers of students. He founded and conducted the Speleological Student Club "Emil Racoviță"; here, every Tuesday evening, new speleological trips were planned, and Pepi was presenting a cave, a scientific meeting, an ecological theme, together with the geography, the culture and the inhabitants of that specific area. Students, friends, even strangers often attended these special evenings. Many of us learned to take performant pictures, and "got infected" with cave exploring and protecting, by Pepi.

At the age of 85, Pepi was still organizing and conducting cave applications for his students. When asked about his tremendous vitality, he used to say that he feels young because he was fortunate to do all his life only what he liked – speleology and jazz.

His interest for jazz started in his teenage, when he conducted a jazz sextet in which he played as a drummer. Later on, he teached, for many years, the class of History of Jazz in the Students' Culture House and in the Music Academy.

He published several books for the use of his students: *The History of Jazz Music, General Speleology, Ecology, Environment Protection*.

For his achievements, has been distinguished with the title of Laureate of the Belgian Speleology Federation (1958), the "Emil Racoviță" award of the Romanian Academy (1963), the award "Lions International of the Invaluable Services" (2003), Senior of the City (2009), Honorary Citizen of Cluj (2004 and 2014).

Iosif Viehmann was a distinguished scientist, a man of culture who promoted local and national values, but, above all, he was a good person. He was deeply attached to the peasants of the hamlet of Scărișoara and of Gârda village, helping them whenever they needed in issues concerning health and education of their children.

Iosif Viehmann (Pepi) was a prominent personality in the scientific, cultural and academic environment of our faculty. Now, our auditoriums are more silent without him.

Corina L. ROȘIORU

Faculty of Biology and Geology
Babeș-Bolyai University, Cluj-Napoca

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXII, 1, 2017 (p. 17-28)
doi:10.24193/subbbiol.2017.1.01

Nitrate, nitrite and microbial denitrification in drinking water from Ozun village (Covasna County, Romania) and the association between changes during water storage

Orsolya-Csilla Ráduly[1] and Anca Farkas[1, 2] ✉

SUMMARY. Severe groundwater contamination affects drinking water quality in many vulnerable zones across Romania. Ingestion of high levels of nitrate and nitrite can lead to the emergence of compounds with major toxicological effects. The present study was aimed to assess nitrate and nitrite concentrations in parallel with the activity of denitrifying bacteria, in different groundwater sources from Ozun village, Covasna County. Natural denitrification potential based on local microbiota was investigated during water storage for one month. Results indicate that tap water supplied from the public system complies with drinking water criteria, but most domestic wells are not safe. Nitrate concentrations exceeded the maximum limit allowed for drinking water in most of the samples collected from dug wells, while nitrite ions occurred within the mandatory limit. Denitrifying bacteria were detected in all groundwater samples, with the exception of one well. Regrowth of denitrifying bacteria was observed during water storage, but significant reduction of nitrate, nitrite or their sum of ratio did not occurred as a general rule. The association between percentage changes in bacterial counts, nitrate and nitrite concentrations was not statistically significant. In conclusion, enhancing the bioremediation potential of local microbiota by groundwater storage at household level is not an efficient strategy for nitrate/ nitrite removal.

Keywords: denitrifying bacteria, groundwater, nitrate, nitrite, water storage.

Introduction

Water, together with air, nutrients, light and climate represent important natural resources, sustainability of life on Earth becoming questionable as a result of their continuous deterioration and depletion. Over the last decades, local and global consequences of

[1] *Babeş-Bolyai University, 1 M. Kogălniceanu Street, 400084/Cluj-Napoca, Romania*
[2] *Laboratory of Plant Biology, Genetics, Microbiology and Biotechnology, Center of Systems Biology, Biodiversity and Bioresources, Babeş-Bolyai University, Cluj-Napoca, Romania*
✉ ***Corresponding author: Anca Farkas**, Babeş-Bolyai University, 1 M.Kogălniceanu Street, 400084 Cluj-Napoca, Romania,*
E-mail: anca.farkas@ubbcluj.ro

environmental degradation and pollution became more obvious (Hill, 2010), impacting the ecological balance and the human health (Myers *et al.*, 2013; Suk *et al.*, 2016). Pollution occurs as a result of accidental or deliberate discharge of harmful substances (fertilizers, pesticides, organic contaminants, metal compounds, etc.), after which the environment suffers significant changes and the biogeochemical cycling is altered. The most common contaminants affecting water and soil are nitrates, phosphates and organic matters. These substances are harmless and even necessary at certain levels, but are harmful at higher concentrations (Butiuc-Keul, 2014).

Many thresholds for human and ecosystem health have been exceeded due to pollution with nitrogen compounds (Erisman *et al.*, 2013).Especially in agriculture, soil fertilization is required for the high productivity of crops. Excessive application of chemical fertilizers, natural compost, or mixtures, often leads to over-fertilization, soil becoming saturated in reactive nitrogen species (Fields, 2004). The soil and vegetation cannot retain and use them and therefore, depending on their solubility, such contaminants end up leaching into the vadose zone and groundwater (Dahan *et al.*, 2014). During the nitrogen cycle, nitrogen compounds are affected by the ammonifying, nitrifying and denitrifying bacteria. Ammonium ions ($NH4^+$) resulting from mineralization of organic matter are oxidized to nitrite (NO_2^-) and then to nitrate (NO_3^-) by nitrogen-fixing bacteria in a process known as nitrification. Denitrifying bacteria, also known as nitrate reducers, belong to the category of chemotrophic microorganisms that oxidize hydrogen to release energy, having the ability to reproduce the natural circuit of nitrogen. Denitrification occurs by reduction of inorganic nitrogen compounds, nitrate being reduced to nitrite and then to nitrous oxide (N_2O) and to molecular nitrogen (N_2), under anaerobic conditions (Muntean, 2009).

Although nitrate concentrations have slightly decreased over the past decades in some European rivers, overall, nitrate levels in groundwater have remained constant (Grizetti *et al*., 2011). Across Romania, many aquifers are highly contaminated, in different regions of the country, from the karst systems into the mountains (Epure and Borda, 2014) to the seaside (Vulpaşu and Racovițeanu, 2016). Nitrate and nitrite have been frequently detected in drinking water drawn from underground sources (Burcă *et al.*, 2015; Mureşan *et al.,* 2011; Pele *et al.*, 2010; Roşu *et al.,* 2014; Török-Oance *et al*., 2013; Vasilache *et al.*, 2012), in concentrations exceeding the maximum limit of 50 mg/L for nitrate and 0.5 mg/L for nitrite, respectively (Council Directive 98/83/EC; Law 458/2002).

Nitrate removal methods include biological denitrification. The possibility of enhancing natural denitrification is currently receiving attention in relation to the problem of nitrate in groundwater (WHO, 2011).

The purpose of this study is to investigate the well water quality in Ozun village, Covasna County, an area vulnerable to nitrate contamination. Also, given the population habits to store drinking water in households, an experiment was conducted in order to assess the changes of water properties over one month. Fresh water samples

from private wells and from the public network were analyzed for nitrate, nitrite and denitrifying bacteria. After 30 days of storage, chemical and microbiological analysis was repeated.

Materials and methods

Ozun is the largest commune of Romania, located in the southern part of the Covasna County, Romania (Fig. 1). Seven villages belong to Ozun commune: Ozun, Sântionlunca, Lisnău, Bicfalău, Lunca Ozunului, Măgheruș, Lisnău-Vale. The commune has a population of 4599 inhabitants (Ráduly, 2011).

Figure 1. Location of the study area. Image taken from GoogleEarth

The main source of drinking water is groundwater. The old centralized system, supplied through 3 drilling boreholes of medium depth (50-80 m), is currently under expansion. Measurements on the quality of drinking water are carried out regularly by the Water Management System Sfântu Gheorghe. Previous data indicate an excess of iron, manganese and nitrate. As a result, the area is included in the nitrate vulnerable zone. The households not connected to the public network are using private hand-dug wells. Well water quality is not regularly monitored to ensure compliance with drinking water standards. Domestic wells are highly exposed to nitrate and other types of pollution, mainly due to leakage from manure storage, mineral fertilizers, sewage and detergents from private homes, household waste deposits and those from authorized and unauthorized industrial activities (Ráduly, 2011).

Location of sampling points

For the investigations and experiments performed in the present study, a number of 10 samples have been collected from domestic wells and from the public system (Fig. 2), in April 2015:

- Eight samples were collected from private wells with daily use for drinking, household needs and irrigation (W1-W8);
- Two samples were collected from the public water supply, one from the reservoir before treatment (R) and one from the tap (T).

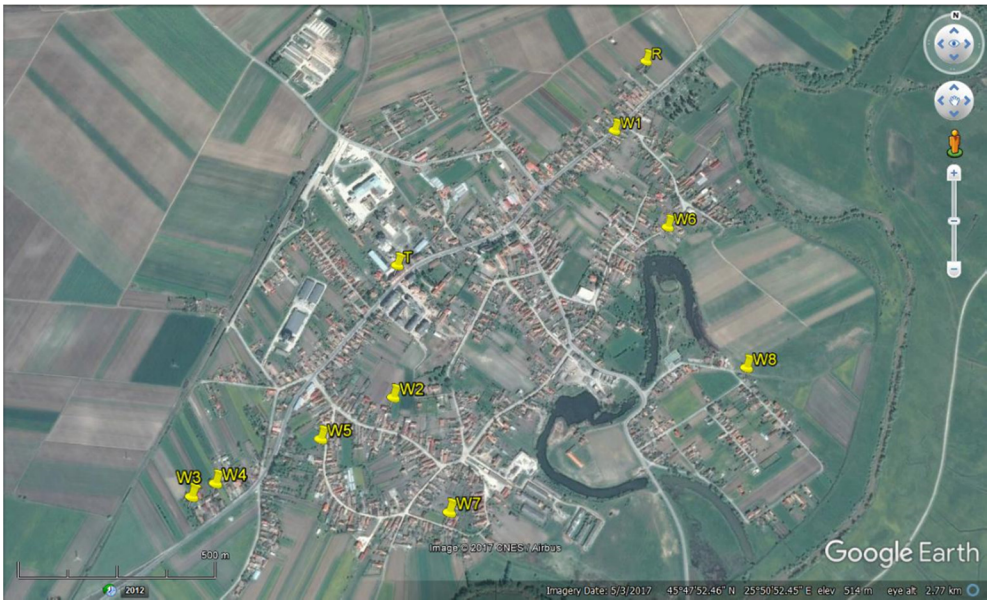


Figure 2. Location of sampling points across Ozun village. W1 - W8: domestic wells; R: reservoir of the public drinking water system; T: tap water from public network. Image taken from GoogleEarth

Water storage and analysis

Samples were analysed for nitrate, nitrite and denitrifying bacteria within 12 hours after collection (day 1). Then, considering the common practices of household water storage, the samples were kept in closed polypropylene bottles for one month at room temperature, and the analysis was repeated (day 30). All the procedures for sampling, sample processing and execution of microbiological analysis were carried out in sterile conditions. The analyzes were performed in laboratories of Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania.

Determination of nitrate and nitrite

The measurement of nitrate was carried out using the spectrophotometric method, according to the international standard (SR ISO 7890-1/1986). It is based on the formation of yellow 4-nitro-2,6-dimethylphenol, when nitrate reacts with 2,6-dimethylphenol, under acidic conditions. Nitrite levels were measured by spectrophotometric reading, a pink diazonium salt resulting from nitrite reaction with Griess reagent, sulphanilic acid (4-aminobenzosulfoacid) and 1-naphtylamine (SR EN ISO 26777/2002).

Assessment of microbial denitrification

The most probable number of denitrifying bacteria was estimated by the method of multiple tubes (Farkas, 2015). Each water sample and at least three subsequent dilutions were inoculated in Allen broth, a culture medium rich in nitrate (KNO_3 1g/L, KH_2PO_4 0.4 g/L, K_2HPO_4 0.5g/L, $CaCl_2$ 0.2 g/L, $MgSO_4x\ 7H_2O$ 0.25 g/L, $FeCl_3$ 0.001 g/L). The inoculated tubes were incubated at 25 °C for 14 days. Denitrification was evident at the end of the incubation period through the accumulation of gas (nitrogen or nitrous oxide) in the Durham tubes.

Risk assessment of nitrate and nitrite in drinking water

Because of the possibility of the cumulative hazard due to simultaneous occurrence of nitrate and nitrite in drinking water, the sum of the ratios (R) of the concentration (C) of each to its guideline value (GV) was assessed for each sampling point, in freshwater and stored water:

$$\frac{C_{nitrate}}{GV_{nitrate}} + \frac{C_{nitrite}}{GV_{nitrite}} \leq 1$$

The GV of 50 for nitrate and GV of 3 for nitrite were considered (Council Directive 98/83/EC; Law 458/2002). Based on epidemiological evidence for methaemoglobinaemia in infants, the sum of ratios should not exceed 1 (WHO, 2011).

Statistical analysis

In exploratory analyses, the results were statistically assessed using the analysis of variance (Kruskal-Wallis non-parametric test) to find out any significant differences between the parameter levels in fresh water and also after 30 days of storage. The change in nitrite or nitrate concentration was compared with the variation of denitrifying bacteria after storage, by using the association between percentage changes in a regression framework. Statistical analyses were performed using the Real Statistics Resource Pack software for Microsoft Excel (Zaiontz 2015), with a significance level of $p < 0.05$.

Results and discussion

Occurrence of nitrate and nitrite in drinking water

At day 1, nitrate concentrations varied between 9.94 and 150.16 mg/L, exceeding the maximum limit allowed (50 mg/L) in 6 groundwater samples. Only four samples, including two wells and the public drinking water system were found to correspond with drinking water criteria. After one month of storage, recorded nitrate levels ranged between 2.40 and 151.81 mg/L. The changes were not uniform, in some of the samples (W1 and W2) a nitrate raise have been recorded, while in other samples (W3-W8, R and T) a nitrate reduction have been observed (Fig.3).

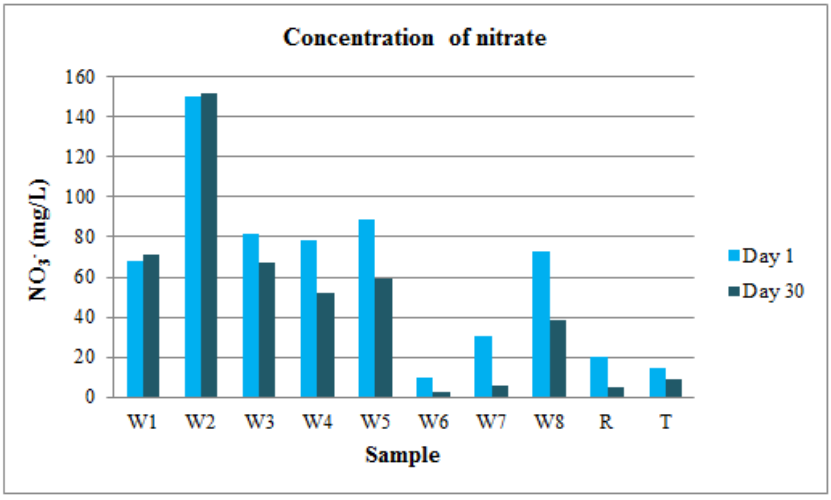


Figure 3. Nitrate concentrations in groundwater and tap water. W1-W8=well water, R=reservoir, T=tap water from the public network

A percentage of 88% groundwater samples from dug wells, in Ozun village, contained high levels of nitrate (above 10 mg/L), 75% being non-compliant with drinking water standards. Other studies investigating drinking water from domestic wells across Romania also warn on nitrate contamination. In Cluj County, 46,4% of wells and springs were found contain more than 10 mg NO_3^-/L (Mureşan *et al*., 2011), while all groundwater samples from the most urbanized village near Cluj-Napoca exceeded 10 mg NO_3^-/L, 56% of the wells being non-compliant with drinking water criteria (Roşu *et al*., 2014). Unfortunately, in Romania there are still many areas in which these values are significantly exceeded (Fleşeriu and Oroian, 2010). Extreme values, over 400 mgNO_3^-/L were found in Matca, Galaţi County, Săhăteni, Buzău County (Pele *et al*., 2010) and Ştefăneşti, Botoşani County (Vasilache *et al*., 2012),

while more than 500 mgNO_3^-/L have been detected in Brăneşti, Ilfov County (Pele *et al*., 2010; Tociu *et al*., 2016). Factors such as the complex biogeochemical cycle of nitrogen in soils, nitrate leaching from the agricultural system and dispersion in the groundwater contribute to extended nitrate contamination (Marinov and Marinov, 2014; Wick *et al*., 2012). Severe contamination leads to nitrate concentration in shallow groundwater.

At day 1, nitrite was detected in concentrations up to 0.309 mg/L (Fig. 4), the maximum limit allowed for drinking purposes (0.5 mg/L) being not exceeded. After 30 days, nitrite levels were observed to increase in three water samples (W1, W2 and W6), while in five samples a nitrite reduction have been noticed (W3, W4, W5, R and T). In samples W7 and W8 nitrite has not been detected neither at the time of water collection, nor after one month of storage.

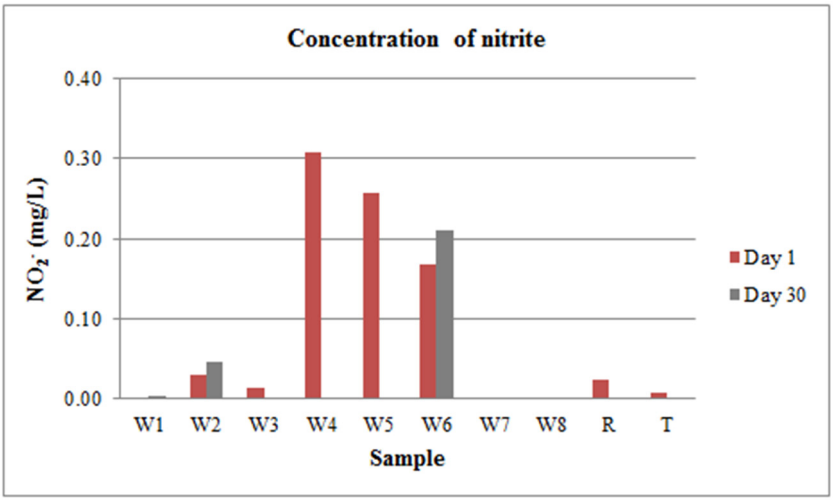


Figure 4. Nitrite concentrations in groundwater and tap water. W1-W8=well water, R=reservoir, T =tap water from the public network

Both the groundwater from private wells and drinking water from the public system of Ozun village contained low nitrite levels. Previous studies investigating drinking water from domestic wells across Romania frequently indicate compliance with drinking water limit (Mureşan *et al*., 2011; Roşu *et al*., 2014; Tociu *et al*., 2016), but extreme nitrite values, over 1.1 mg NO_2^-/L were found in Matca, Galaţi County (Pele *et al*., 2010), 1.4 mg NO_2^-/L in Gătaia, Timiş County (Török-Oance et al., 2013), 1.5 mg NO_2^-/L in Sadoveni, Botoşani County (Vasilache *et al.*, 2012) and over 1.6 mg NO_2^-/L in Feleacu, Cluj County (Burcă *et al.*, 2015).

Activity of denitrifying bacteria

In all fresh water samples, bacteria able of denitrification were present excepting one well (W7), where a maximum of 3.1 cells/mL (W1) have been reached. A general increase in the number of denitrifying bacteria was achieved during water storage, but not for W1. At day 30, the highest values were obtained for wells W5 (92 cells/mL), W8 (54 cells/mL) and for the public reservoir (35 cells/mL) (Fig. 5).

Previous studies proved that in drinking water systems denitrifying bacteria are generally present in low number. Denitrification occurs mostly in biofilms, the ecophysiological group of denitrifying organisms being usually overwhelmed by the ammonifyiers (Farkas *et al.*, 2013). Biological nitrate reduction occurs in the presence of organic material, under anaerobic conditions, leading to the production of nitrite which is then broken down further to harmless elemental nitrogen (WHO, 2011).

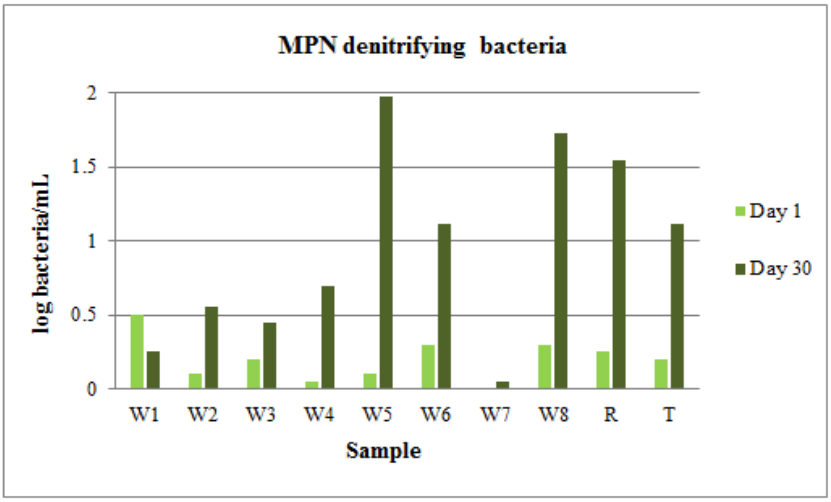


Figure 5. Changes in concentration of denitrifying bacteria in groundwater and tap water. MPN=most probable number, W1-W8=wells, R=reservoir source for public network, T=tap

Nitrate/nitrite risk for human health and water quality changes during storage

The sum of ratios for nitrate and nitrite, signifying the cumulative hazard due to their simultaneous occurrence in drinking water, was greater than 1 in six out of eight wells, reaching the maximum value of 3.01 in W2. After one month of storage, a slight decrease in the sum of ratios for nitrite and nitrate was observed in most of the samples, except for W1 and W2. At day 30, in five groundwater samples the ratio was greater than 1, with a maximum value of 3.05 in W2 (Table 1). Both samples collected from the public system were compliant with drinking water guidelines that recommend a ratio below 1 (WHO, 2011). Comparing to domestic wells, a lower ratio

was obtained for the groundwater sampled from the deep reservoir supplying the public network. Well depth, type, age and condition are important variables influencing the water quality. Nitrate contamination is often associated with shallow wells (Fewtrell, 2004; Tociu *et al*., 2016), but drilling at higher depths does not guarantee the higher quality water in terms of nitrate concentration (Vulpaşu and Racovițeanu, 2016). In such cases, groundwater nitrate may rather occur from geological sources, than as a consequence of anthropic activities. Nitrogen release form bedrock has a potentially significant impact on localized nitrogen cycles (Holloway and Dahlagren, 2002).

Therefore, water from dug wells should be used with caution for human consumption, especially by vulnerable categories such as infants, toddlers and pregnant women. It is well known that nitrate itself and in small quantities is relatively harmless, but its reaction products, e.g. nitrite or nitrosamines, can affect human health (Kross, 2002). Ingestion of water contaminated with nitrate and nitrite leads to the disease called methaemoglobinaemia (Fewtrell, 2004). This intoxication is a serious hazard for Romanian infants, especially in areas where drinking water is used from polluted wells (Zeman *et al*., 2002; Iacob *et al.*, 2012). The association between high groundwater nitrate levels and elevated methaemoglobin levels in infants fed with drinking water–diluted formulas has been demonstrated by epidemiological studies. However, more recent investigations suggest other sources of nitrogenous substance exposures in infants, including protein-based formulas and foods, or the production of nitrate precursors by bacterial action in the infant gut in response to inflammation and infection (Richard *et al*., 2014). Other health effects associated with drinking water nitrate and nitrite are cancer, inflammatory bowel disease, adverse reproductive outcomes, thyroid hypertrophy, diabetes, increased blood pressure and acute respiratory tract infections (Ward *et al.*, 2005).

Table 1.

Nitrite, nitrite, denitrifying bacteria and R in fresh water and stored water.
R = sum of ratios for nitrate and nitrite.

Sample	Nitrate (mg/L)		Nitrite (mg/L)		R		Denitrifying bacteria (cells/mL)	
	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30
W1	68.23	71.18	0.000	0.004	3.1	1.4	1.36	1.42
W2	150.16	151.81	0.030	0.047	0.4	3.4	3.01	3.05
W3	81.32	67.56	0.014	0.000	0.7	2.2	1.63	1.35
W4	78.72	51.65	0.309	0.000	0.2	4.9	1.68	1.03
W5	89.06	59.43	0.258	0.000	0.4	92.0	1.87	1.19
W6	9.94	2.40	0.168	0.210	1.7	13.0	0.25	0.12
W7	30.77	5.68	0.000	0.000	0.0	0.2	0.62	0.11
W8	72.50	38.22	0.000	0.000	1.3	54.0	1.45	0.76
R	19.92	4.80	0.024	0.000	1.0	35.0	0.41	0.09
T	14.79	8.81	0.007	0.000	0.8	13.0	0.30	0.18
Kruskal-Wallis test	p = 0.15		p = 0.08		p = 0.15		p = 0.003	

The multiplication of denitrifying bacteria was observed during water storage, but nitrate and nitrite reduction did not occurred as a general rule. In some samples, an increase of nitrate and/or nitrite content occurred at day 30, comparing to day 1. The difference between the number of denitrifying bacteria at day 1 and at day 30 was statistically significant (p-value = 0.003), while no significant differences occurred in nitrate, nitrite concentrations and their sum of ratios within one month of storage (Table 1). No significant associations were observed in the percentage changes of denitrifying bacteria and nitrate (r = 0.01; p-value = 0.97) or nitrite (r = 0.39; p-value = 27), neither between percentage changes of nitrate and nitrite (r = 0.28; p-value = 0.44). Therefore, the complex microbial activity of heterogenic consortia present individually in each well hinders generic predictions on drinking water quality during storage. Despite the enhancing denitrification due to microbial regrowth, groundwater storage at household level is not efficient for nitrate/nitrite removal. Optimized bioremediation strategies are needed in order to ensure drinking water safety.

Conclusions

According to analyses carried out in this study, water from the public network of Ozun village complies with drinking water regulations, but domestic wells are not safe. Nitrate concentrations exceeded the maximum limit allowed for drinking water in most of the samples collected from dug wells, while nitrite ions occurred within the mandatory limit. Denitrifying bacteria were detected in all groundwater samples, with the exception of one well.

The multiplication of denitrifying bacteria was observed during water storage, but a significant reduction of nitrate, nitrite or their sum of ratio did not occurred as a general rule. In conclusion, enhancing the bioremediation potential of local microbiota by groundwater storage at household level is not an efficient strategy for nitrate/nitrite removal.

REFERENCES

Burcă, S., Indolean, C., Măicăneanu, A. (2015) Groundwater quality in shallow wells from Feleacu village, Cluj County, Romania, *Studia Universitatis Babeș-Bolyai, Chemia* **60**(3): 247-255

Butiuc-Keul, A. (2014) *Biotehnologie Generală*, Presa Universitară Clujeană, Cluj-Napoca, pp. 276 [in Romanian]

Council Directive 98/83/EC (1998) on the quality of water intended for human consumption, Official Journal of the European Communities, L330/32

Dahan, O., Babad, A., Lazarovitch, N., Russak, E. E., Kurtzman, D. (2014) Nitrate leaching from intensive organic farms to groundwater. *Hydrology and Earth System Sciences* **18**: 333–341

Epure, L., Borda, D. R. (2014) Groundwater contamination and the relationship between water chemistry and biotic components in a karst system (Bihor Mountains, Romania), *Travaux de l´Institut de Spéologie Emil Racovita* **53**: 69-84

Erisman, J. W., Galloway, J. N., Seitzinger, S., Bleeker, A., Dise, N. B., Petrescu, A. M. R., Leach, A. M., de Vries, W. (2013) Consequences of human modification of the global nitrogen cycle, *Philosophical Transactions of the Royal Society B* 20130116

Farkas, A. (2015) *Apa potabilă și biofilmul*, Presa Universitară Clujeană, Cluj-Napoca, pp. 198 [in Romanian]

Farkas, A., Drăgan-Bularda, M., Muntean, V., Ciatarâș, D., Țigan Ș. (2013) Microbial activity in drinking water-associated biofilms, *Central European Journal of Biology* **8**(2): 201-214

Fewtrell, L. (2004) Drinking water nitrate, methemoglobinemia, and global burden of disease: a discussion. *Environ Health Perspectives* **112**(14): 1371–1374

Fields, S. (2004) Global nitrogen: cycling out of control. *Environmental Health Perspectives* **112**(10): A556–A563

Fleşeriu, A., Oroian, I. (2010) Water pollution with nitrates from agricultural sources. *ProEnvironment* **3**: 110 – 112

Grizzetti, B., Bouraoui, F., Billen, G., van Grinsven, H., Cardoso, A. C., Thieu, V., Garnier, J., Curtis, C., Howarth, R. W., Johnes, P. (2011) *Nitrogen as a threat to European water quality.* In: The European nitrogen assessment, Sutton, M. A., Howard, C. M., Erisman, J. W., Billen, G., Bleeker, A., Grennfelt, P., van Grinsven, H., Grizzetti, B., Cambridge University Press, Cambridge, New York, pp. 379-404

Hill, M. K. (2010) *Understanding environmental pollution.* Cambridge University Press, New York, pp. 585

Holloway, J. M., Dahlgren, R. A. (2002) Nitrogen in rock: Occurrences and biogeochemical implications, *Global Biogeochemical Cycles* **16**(4): 65-1–65-17

Iacob, O., Tudor, A., Neamțu, A., Cristea, A. (2012) *Apa de fântână: contaminarea cu nitrați și methemoglobinemia*, Carol Davila, București, pp. 31 [in Romanian]

Kross, B. C. (2002) Nitrate toxicity and drinking water standards – a review, *The Journal of Preventive Medicine* **10**(1): 3-10

Law 458 (2002): Legea nr. 458 din 8 iulie 2002 privind calitatea apei potabile, Monitorul Oficial, 552 [in Romanian]

Marinov, I., Marinov, A. M. (2014) A coupled mathematical model to predict the influence of nitrogen fertilization on crop, soil and groundwater quality, *Water Resources Management* **28**(15): 5231–5246

Muntean, V. (2009) *Microbiologie generală*, Presa Universitară Clujeană, Cluj-Napoca, pp. 332 [in Romanian]

Mureșan, C., Farkas, A., Chakirou, C. (2011) Studiu asupra surselor proprii de apă – fântâni și izvoare – din județul Cluj, Romaqua **73**: 38-42 [in Romanian]

Myers, S. S., Gaffikin, L., Golden, C. D., Ostfeld, R. S., Redford, K. H., Ricketts, T. H., Turner, W. R., Osofsky, S. A. (2013) Human health impacts of ecosystem alteration. *Proceedings of the National Academy of Sciences* **110**(47): 18753–18760

Pele, M., Vasile, G., Artimon, M. (2010) Study regarding nitrogen pollutants in well waters from Romania. *Scientific Papers, UASVM Bucharest*, **53**: 145-151

Ráduly, I. (2011) *Strategia de dezvoltare locală a comunei Ozun*, Alma Mater, Cluj-Napoca, pp. 126 [in Romanian]

Richard, A. M., Diaz, J. H., Kaye, A. D. (2014) Reexamining the risks of drinking water nitrates on public health. *The Ochsner Journal* **14**: 392–398

Roşu, C., Roba, C., Piştea, I., Neş, M, Ozunu, A. (2014) Groundwater quality and its suitability for drinking and agricultural use in rural area from Cluj County (Floreşti Village), *Scientific Papers Series Management, Economic Engineering in Agriculture and Rural Development* **14**(2): 247-252

SR EN ISO 26777 (2002) Calitatea apei. Determinarea conținutului de nitriți. Metoda prin spectrometrie de absorbție moleculară. Asociația de Standardizare din România [in Romanian]

SR ISO 7890-1 (1986) Calitatea apei. Determinarea conținutului de nitrați. Asociația de Standardizare din România [in Romanian]

Suk, W. A., Ahanchian, H., Asante, K. A., Carpenter, D. O., Diaz-Barriga, F., Ha, E. H., Huo, X., King, M., Ruchirawat, M., da Silva, E. R., Sly, L., Sly, P. D., Stein, R. T., van den Berg, M., Zar, H., Landrigan, P. J. (2016) Environmental pollution: an underrecognized threat to children's health, especially in low- and middle-income countries, *Environmental Health Perspectives* **124**(3): 41-45

Tociu, C., Marcu, E., Ciobotaru, I. E., Maria, C. (2016) Risk assessment of population exposure to nitrates/nitrites in groundwater: a case study approach. *Journal of Environmental Research and Protection* **13**(3): 39-45

Török-Oance, R., Tulhină, D., Lazăr, M. (2013) Preliminary study on groundwater pollution with nitrites and nitrates in Gătaia (Timiș County), *Annals of West University of Timişoara, Biology* **16**(2): 89-96

Vasilache, V., Filote, C., Cretu, M. A., Sandu, I., Coisin, V., Vasilache, T., Maxim, C. (2012) Monitoring of groundwater quality in some vulnerable areas in Botoşani County for nitrates and nitrites based pollutants, *Environmental Engineering and Management Journal* **11**(2): 471-479

Vulpaşu, E., Racoviţeanu, G. (2016) Evaluation of the groundwater quality in Constanta County, seaside area. *Energy Procedia* **85**: 612 – 619

Ward, M. H., deKok, T. M., Levallois, P., Brender, J., Gulis, G., Nolan, B. T., VanDerslice, J. (2005) Workgroup report: Drinking water nitrate and health. Recent findings and research needs. *Environmental Health Perspectives* **113**(11): 1607-1614

WHO (2011) *Guidelines for drinking-water quality*, 4th edition, World Health Organization, Geneva, pp. 541

Wick, K., Heumesser, C., Schmid, E. (2012) Groundwater nitrate contamination: Factors and indicators, *Journal of Environmental Management* **111**: 178-186

Zaiontz, C. (2015) Real statistics using Excel. www.real-statistics.com.

Zeman, C. L., Kross, B., Vlad, M. (2002) A nested case-control study of methemoglobinemia risk factors in childrenof Transylvania, Romania, *Environmental Health Perspectives* **110**(8): 817-822

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXII, 1, 2017 (p. 29-42)
doi:10.24193/subbb.2017.1.02

SPION size dependent effects on normal and cancer cells

Sergiu Gabriel Macavei[1,2], Maria Suciu[2,3,✉], Izabell Crăciunescu[2,3], Lucian Barbu-Tudoran[2,3], Septimiu Cassian Tripon[2, 3], Cristian Leoştean[2] and Radu Bălan[1]

SUMMARY. Iron oxide nanoparticles have become widely used today in medical applications. In this study, we report a hyperthermia treatment with 10 and 100 nm naked and polyethylene glycol(PEG)-coated Super Paramagnetic Iron Oxide Nanoparticles (SPIONs) to normal and tumor cells in culture. Cells' responses to nanoparticles were analyzed by cell viability assays (MTT and LDH) and transmission electron microscopy. Results indicate that even if 10 nm SPIONs have good magnetization saturation, the hyperthermia treatment is not effective due to the fact that cells do not endocytose them. 100 nm SPIONs are better engulfed by cells, and their hyperthermia effect is slightly increased.

Keywords: hyperthermia, melanoma cells, SPION

Introduction

Nanoparticles are present in our every-day life, whether we like or not, whether we know it or not. We breathe micro and nanoparticles evacuated from cars exhaust pipes, we put them on our skin deliberately as sunscreens or cosmetics, or in our foods, we even have nanoparticles on our walls (Karjalainen *et al*., 2014; Smijs and Pavel, 2011; Das *et al*., 2011; Kaiser *et al*., 2013). We also use them in medicine for magnetic resonance imaging, as cytostatic enhancer or drug delivery system (Blasiak *et al*., 2013; Duan *et al*., 2016).

[1] *Department of Mechatronics and Machine Dynamics, Faculty of Mechanical Engineering, Technical University, 103-105 Muncii Bvd., 400641, Cluj-Napoca, Romania*
[2] *National Institute of Research and Development for Isotopic and Molecular Technologies, 67-103 Donath Str., 400293, Cluj-Napoca, Romania*
[3] *Faculty of Biology and Geology, Babes-Bolyai University, 5-7 Clinicilor Str., 400006, Cluj-Napoca, Romania*
✉ ***Corresponding author**: **Maria Suciu**, 5-7 Clinicilor Str., 400006, Cluj-Napoca*
E-mail: maria.suciu5@gmail.com

Super Paramagnetic Iron Oxide Nanoparticles (SPIONs) are currently used in medicine for magnetic resonance imaging and other types of imaging or as treatment for iron deficiency (Revia and Zhang, 2016). SPIONs are magnetite or maghemite nanoparticles that can be produced at desired dimensions and shapes, and can be covered with biocompatible materials and drugs or markers for various applications. SPIONs have the great advantage that they are biodegradable in the human body and a plus is that they have special magnetic properties. One of the many potential uses in medicine for SPIONs is for hyperthermia treatment. For this, SPIONs are placed in an alternating magnetic field which will determine SPIONs to produce heat that can be used for medical purposes (Bañobre-López *et al*., 2013; Giustini *et al*., 2010).

In this study, we explored the effect of size and PEG coverage on hyperthermia treatment *in vitro* on normal and cancer cells. Our results give proof to the fact that besides the magnetic properties of a nanoparticle, one should also consider the nanoparticle's dimension and surface coverage for the end effect of hyperthermia treatment on cells. These aspects are important for designing the most effective nanoparticles for hyperthermia treatments against cancer.

Materials and methods

Nanoparticles synthesis. For the 10 nm SPIONs synthesis we used the co-precipitation method of ferric and ferrous salts under the presence of argon gas (Turcu *et al.*, 2015). Our recipe was: 0.1 M $FeCl_3$ and 0.05 M $FeCl_2$ were dissolved into 200 mL of distilled water and stirred for 60 minutes. At 700 °C and under vigorous mixing we added 200 ml of 25 % NH_3 also under argon gas, and then left to precipitate for 2 h at pH of 12. After cooling to room temperature, the precipitates were magnetically separated, washed extensively with distilled water until neutral pH was reached. At the end, Fe_3O_4 magnetic nanoparticles were washed in acetone and dried at 60-70 °C. These resulted in 10 nm naked SPIONs.

SPIONs were then covered with PEG 2000 which produced the 10 nm SPION-PEG, considered to be biocompatible (Kostiv *et al*., 2017; Silva *et al*., 2016). For PEG coated SPIONs the recipe was as follows: 10 wt. % PEG and 1 wt. % of magnetic nanoparticles were mixed at room temperature, under vigorous magnetic stirring overnight in distilled water. Then SPIONs were magnetically separated and washed with distilled water and re-dispersed in water.

For the synthesis of 100 nm clusters of SPIONs, we used the oil in water mini-emulsion method (Crăciunescu *et al.*, 2017). Our recipe was as such: 0.5 wt% toluene based ferrofluid (Fe_3O_4) was added to an aqueous solution containing surfactant (sodium lauryl sulphate). This led to the formation of micelles dispersed in toluene.

This two-phase mixture was then homogenized using an ultrasonic finger for 2 minutes and the organic phase was evaporated under magnetic stirring (500 rpm), at 100^0 C in an oil bath. 100 nm SPIONs clusters were then washed with a methanol-water mixture to remove excess of reactants and re-dispersed in distilled water. These clusters represent the 100 nm naked SPIONs. From these we obtained the 100 nm PEG-coated SPIONs as described above.

TEM analysis of SPIONs. 10 and 100 nm SPIONs dispersed in water were placed on carbon coated 300 mesh copper grids. Multiple images were taken on a Hitachi STEM HD-2700 transmission electron microscope (TEM) at 200kV acceleration voltage.

VSM analysis of SPIONs. Room-temperature magnetic behavior of 10 and 100 nm SPIONs was recorded using a vibrating sample magnetometer (VSM) produced by "Cryogenic Ltd."

Cell culture procedures. For the hyperthermia study, we used normal human keratinocyte cells (HaCaT cell line), which was a gift from dr. Alina Sesarman, ICEI-BNS Cluj-Napoca, and human melanoma cells (A375 cell line) from ATCC. Keratinocytes were first cultured on plastic 25 cm^2 dishes in DMEM supplemented with 10% fetal calf serum, 1% penicillin-streptomycin and 1% L-glutamine. Melanoma cells were cultured according to the producer recommendations in 4.5 g/l glucose DMEM supplemented with 10% fetal calf serum, 1% penicillin-streptomycin and 1% L-glutamine. Cells were grown in a humidified incubator at 37 ºC and in a 5% CO_2 atmosphere. All cells were used when spread to 80% confluency, at which point they were trypsinized from the culture plate and seeded onto 96 wells plates for MTT and LDH analyses and on glass slides for TEM analysis.

A volume of 10 µl naked or PEG-coated SPIONs was added to cell media, at concentrations between 0.1 and 500 µg/ml. Their effects were tested at 24 hours of contact with the cells.

Hyperthermia treatment. For the hyperthermia induction, we used a Resistor-Inductor-Capacitor (RLC) circuit powered by a sinusoidal signal of an Arbitrary Waveform Generator type WW2571A and a custom-made power wide band amplifier having a frequency range of 100kHz-100MHz and a power range of RF 1-200W. Cells plated in 96 wells plate were incubated with the 10 and 100 nm SPIONs for 24 hours to ensure endocytosis. Plates were then placed in the alternating magnetic field for 20 minutes at 100 Oe and 0.75 MHz (for the 100 nm SPIONs) or 3 MHz (for the 10 nm SPIONs), and then returned to the incubator for another 24 hours. Before treatment, cell medium was replaced with fresh one to remove non-endocytosed

nanoparticles. The magnetic field for each type of nanoparticle was calculated according to their magnetization saturation (obtained by VSM) to the maximum power indicated by the Atkinson-Brezovich limit for human applications (Atkinson *et al*., 1984).

MTT method. Cells were seeded in 96 wells plate, at 12 x 10^3 cells/well density, and left to reach exponential phase for 24 hours. 10 or 100 nm SPION's were added to the culture media, and 24 hours later the mitochondrial activity was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) method. The MTT compound was added to each well to the final concentration of 0.5 mg/ml and left with the cells for 1.5 hours in the incubator. Afterwards, the media were removed and formazan crystals were lysed with acidified iso-propanol. The formazan absorbance was read at 550 nm (with background read at 630 nm) using BioTek Synergy HT plate reader and Gen5 Plate Reader Program (Riss *et al.*, 2016). Each concentration was tested six times and each plate contained untreated cells as positive control and negative controls (cells treated with Tween 20 2%). Data refers to mean ± standard error from at least three independent experiments. Comparison between control group and treated groups was performed with student's t-test and values of $p<0.05$ were considered significant; all calculations were performed in Microsoft Excel.

LDH method. For this method, we used 50 µl of culture medium from the MTT plates, which were transferred to a new 96 wells plate. To this we added 50 µl 50 mM lithium lactate solution, 50 µl 200 mM tris solution at pH 8, and 50 µl NAD solution. LDH reaction was read at 490 nm (with background at 690 nm) using BioTek Synergy HT plate reader and Gen5 Plate Reader Program (Chan *et al*., 2013). Data refers to mean ± standard error from at least three independent experiments. Comparison between control group and treated groups was performed with student's t-test and values of $p<0.05$ were considered significant; all calculations were performed in Microsoft Excel.

TEM analysis of cells and nanoparticles uptake. Cells were plated on 6 mm glass coverslips in a 12 wells plate. 100 µg/ml of 10 and 100 nm naked and PEG-coated SPIONs were added to cells and part of these glass coverslips were subjected to hyperthermia. 24 hours after the applied treatment, cells were fixed with 2.7% glutaraldehyde and post-fixed with 1% osmium tetroxide, dehydrated in ethanol, embedded in Epon resin and polymerized at 60 ºC. Samples were trimmed and ultrathin 50 nm sections were obtained using a Diatome diamond knife on Leica UC6 ultramicrotome. Sections were recovered on a carbon coated 200 mesh copper grids, and were left unstained and analyzed in a Jeol JEM 1010 TEM with MegaView II CCD Camera.

Results and discussion

In this study, we tested 10 and 100 nm SPIONs covered or un-covered with PEG in skin cell cultures. We used normal keratinocytes cells (HaCaT) and malignant melanoma (A375), since skin cells are daily subjected to all types of micro and nanoparticles from the surrounding environment. This fact is important because it gives us a better understanding of how the resistant cells might respond to the nanoparticle and hyperthermia treatments.

The TEM analysis of 10 and 100 nm SPIONs revealed the way nanoparticles look like at their nano sizes. In Fig. 1A, 10 nm SPIONs are imaged closely together due to physical forces that draw nanoparticles close when dried on the grid. Nanoparticles have around 8 to 12 nm and are well dispersed in water. No clumps were seen during analysis. The 100 nm SPIONs (Fig. 1B) are clusters of well defined spherical forms, having 70 to 150 nm diameters. In their structure, 10 nm magnetic nanoparticles can be identified. The 100 nm SPION clusters remained stable in their spherical form throughout the entire experimental procedures.

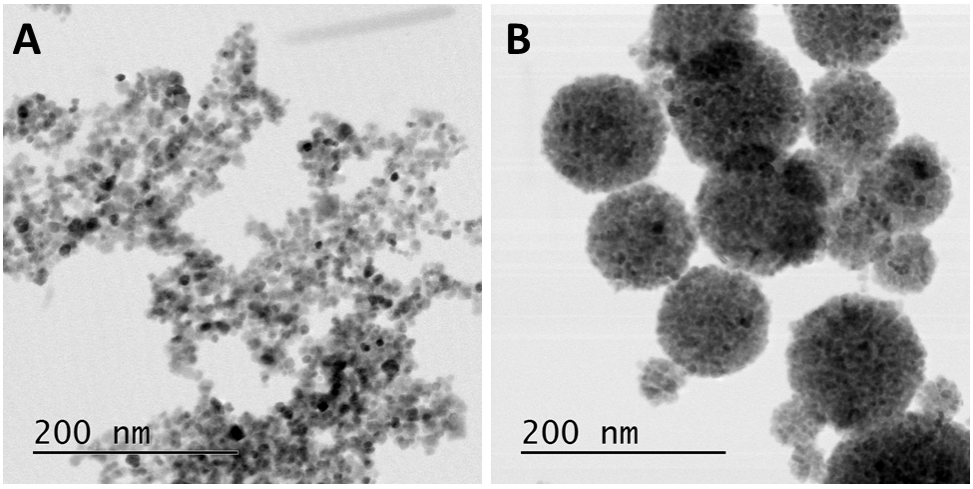


Figure 1. TEM micrographs of SPIONs. A - 10 nm SPIONs, B - 100 nm SPIONs.

The magnetization curves at room temperature of the SPION samples is shown in Fig. 2. As expected, the magnetization shows only a very small hysteresis loop, which is consistent with superparamagnetic behavior. The saturation magnetization (Ms) and coercitive field (Hc) values are 74.3 emu/g, 26 Oe for 10 nm SPIONs and 61.8 emu/g, 27 Oe for 100 nm SPIONs. The exchange field remains at low levels (<1 Oe). The lower value of Ms for the cluster structured 100 nm SPIONs is attributed to interparticle dipole-dipole interactions due to close packing.

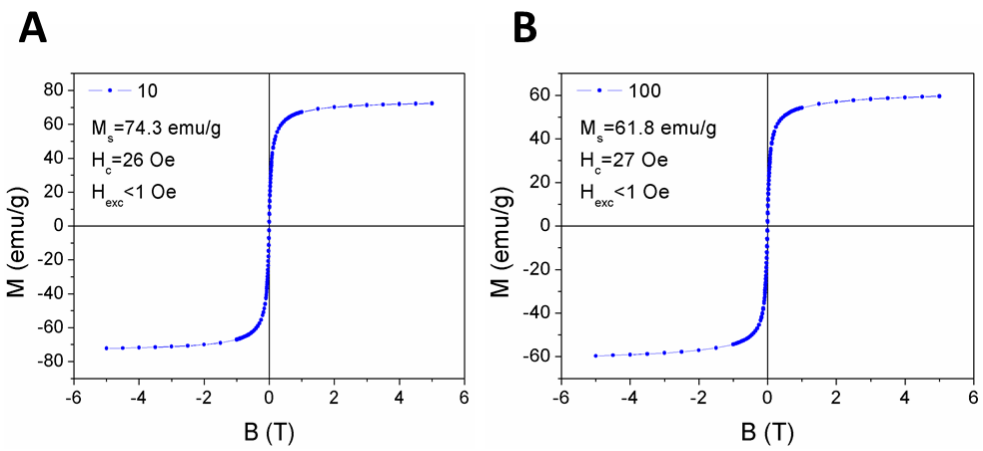


Figure 2. The magnetization curves versus applied magnetic field at room temperature of SPIONs. A - 10 nm SPIONs, B - 100 nm SPIONs.

The MTT analyses revealed that normal keratinocytes were not affected by the 10 nm naked SPIONs, as their mitochondrial activity was above 80% at all tested concentrations (0.1-500 µg/mL) and they were not affected even by the hyperthermia treatment (Fig. 3A, columns). On the contrary, the 10 nm PEG SPIONs, which are supposed to be biocompatible, reduced the mitochondrial activity of keratinocytes down to 50% (at the 10 µg/mL concentration). This reduction in the mitochondrial activity was not concentration dependent and gave different results in different separate MTT analyses. This, we believe, is due to the fact that when placed in contact with the cell culture medium, the nanoparticles clump together, are not well dispersed and therefore, are not uniformly endocytosed, giving different results. But what remains constant, though, is the fact that the 10 nm PEG SPIONs do reduce the cells' viability. When placed in a magnetic field, the mitochondrial activity remained reduced, although slightly higher than the cells not treated by hyperthermia (Fig. 3B, columns). This increase in the mitochondrial activity of the hyperthermia treated cells could be the result of 24 hours' time of recuperation from the contact with the SPIONs. Because, if the 10 nm naked SPIONs did not produce enough heating damage when subjected to magnetization, it is our belief that the PEG coating could not have raised the heating.

When placed in contact with the 100 nm naked SPIONs (Fig. 3C, columns), normal keratinocytes had a concentration dependent decrease in mitochondrial activity, remaining in the non-toxic percentage (100-80%) at 0.1 and up to 100 µg/mL, and dropping to 70% mitochondrial activity at the 500 µg/mL concentration. When placed

in the magnetic field, all concentration points dropped to 50-60% mitochondrial activity. The 100 nm PEG SPIONs (Fig. 3D, columns) gave a slightly reduced mitochondrial activity (80%) at the 500 µg/mL concentration, and non-toxic values for the other tested concentrations (0.1-100 µg/mL), even though somewhat variable (with large standard deviations). The hyperthermia treatment resulted in reduced mitochondrial activity to all tested concentrations, compared to same groups not treated by hyperthermia, with best results at 50 and 100 µg/mL concentrations (40-50% mitochondrial activity).

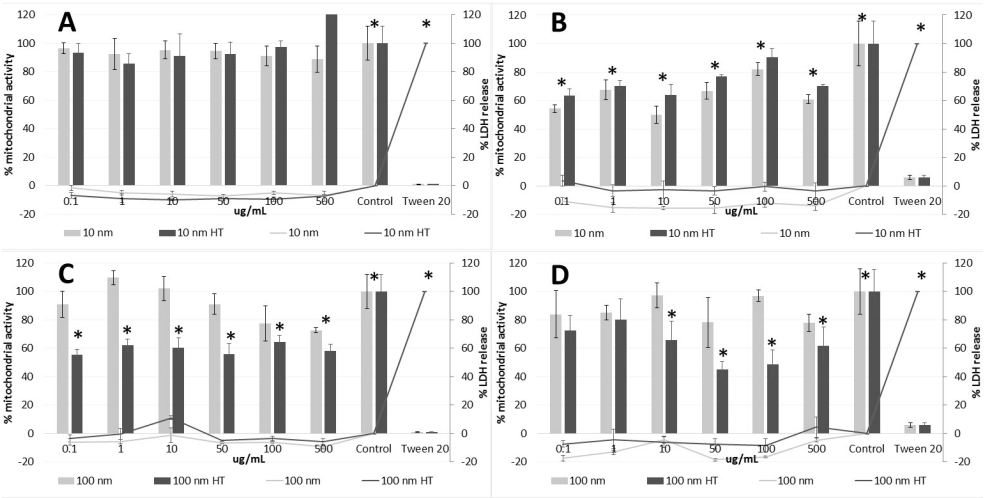


Figure 3. MTT and LDH analyses of normal keratinocytes treated with 10 and 100 nm SPIONs with and without hyperthermia. (A) 10 nm naked SPIONs; (B) 10 nm PEG SPIONs; (C) 100 nm naked SPIONs; (D) 100 nm PEG SPIONs. Columns represent mitochondrial activity values, and lines - LDH release percent values. Asterisks indicate statistical relevance between control and tested groups, $p<0.05$.

The human melanoma cells responded with a drop in their mitochondrial activity when put in contact with 10 nm naked SPIONs for 24 hours (Fig. 4A, columns). Values were between 60 and 70% mitochondrial activity in a concentration dependent manner. After the hyperthermia treatment, melanoma cells had a reduced mitochondrial activity (60-80%) starting from 10 to 500 µg/mL concentrations. The 10 nm PEG SPIONs gave a slightly reduced mitochondrial activity in the presence and absence of the magnetic field, in a concentration dependent manner, dropping below 80% from 10 µg/mL and reaching 60% at 500 µg/mL (Fig. 4B, columns).

Melanoma cells treated with 100 nm naked SPIONs had a reduced mitochondrial activity, down to 60-80%, independent of the administered concentrations. When cells were put in the alternating magnetic field, some were at the same mitochondrial level as without hyperthermia (0.1; 50 and 100 μg/mL), but some had higher mitochondrial activities (1; 10 and 500 μg/mL), with no evident pattern (Fig. 4C, columns). The 100 nm PEG-coated SPION gave a dose response mitochondrial activity, starting from non-toxic values (100-90%) concentrations (0.1-10 μg/mL) and ending at (80-70%) at 50-500 μg/mL. The cells treated by hyperthermia had a reduced mitochondrial activity (60-80%) at all concentrations (Fig. 4D, columns).

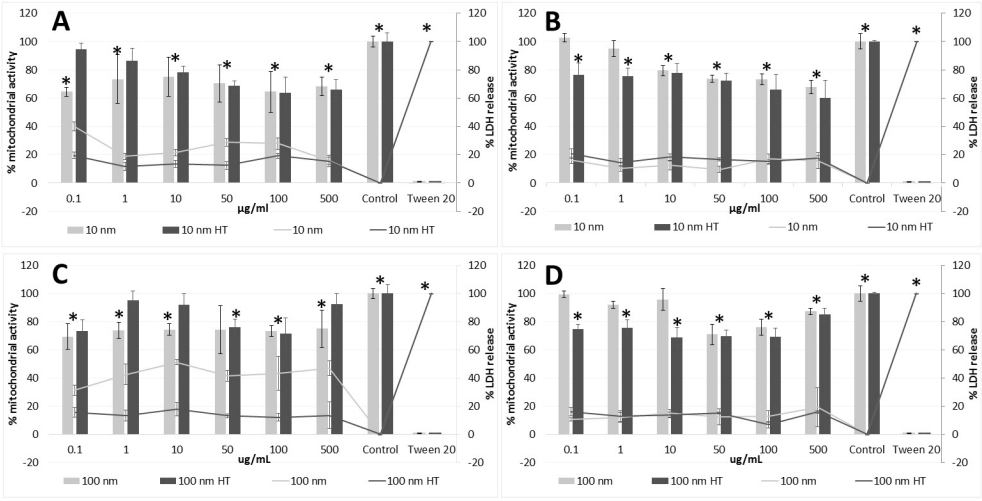


Figure 4. MTT and LDH analyses of melanoma cells treated with 10 and 100 nm SPIONs, with and without hyperthermia. (A) 10 nm naked SPIONs; (B) 10 nm PEG SPIONs; (C) 100 nm naked SPIONs; (D) 100 nm PEG SPIONs. Columns represent mitochondrial activity values, and lines - LDH release percent values. Asterisks indicate statistical relevance between control and treated groups, $p<0.05$.

LDH analyses showed no release for keratinocytes at any of the tested concentrations or conditions (with or without alternating magnetic field) under the action of 10 and 100 nm SPIONs, coated or uncoated with PEG. All values were around the untreated control values of release. Therefore, we can assume that these nanoparticles do not affect keratinocytes plasma membranes (Fig. 3, lines).

For the melanoma cells, LDH release had elevated values compared to keratinocytes. 10 nm naked nanoparticles produced a 20 to 40% LDH release at 24 hours of contact with cells, with the highest spike at 0.1 μg/mL and the lowest

value at 500 μg/mL concentration. After the hyperthermia treatment, the LDH release was lower (20%) and maintained constant throughout the concentrations (Fig. 4A, lines). In the case of PEG-coated 10 nm SPION, the LDH was 20% higher than the untreated cells' release, and remained constant at all concentrations, even under the effect of the magnetic field (Fig. 4B, lines).

The 100 nm naked SPIONs triggered the highest LDH release, ranging from 30 to 50% release (30% at 0.1 μg/mL and 50% at 10 and 500 μg/mL). This effect was not seen when put in the magnetic field, although it remained elevated to 20% at all concentrations (Fig. 4C, lines). The 100 nm PEG-coated SPIONs, again, produced an elevated LDH, but only just to 10-20%, throughout the entire concentrations and independent of the hyperthermia treatment (Fig. 4D, lines).

TEM analysis of cells treated with nanoparticles revealed the ultrastructural effects of SPIONs on normal and tumor cells. Both 10 nm and 100 nm SPIONs could be found in normal keratinocytes after 24 hours of exposure, some free in the cytoplasm, some contained in endosomes, but none of them reached the nuclei. Large groups of SPIONs could still be identified outside the cells, but attached to the cells' membranes. Endocytosed SPIONs were grouped in small clumps in which individual particles could be identified, meaning that the nanoparticles were not yet transformed to hemosiderin. Some evident derangement could be seen in keratinocytes exposed to 10 nm SPIONs for 24 hours (Fig. 5A): the cytoplasm was homogenous with constant, normal granulations, but in some places, there could be seen lysis areas of the cytoplasm, not surrounded by a membrane; mitochondria were scarce and swollen. Keratinocytes exposed to 100 nm SPIONs (Fig. 5B) had electron-dense cytoplasm packed with vesicles, and nuclei with homogenous chromatin. The strongly electron-dense cytoplasm and homogenous nuclei suggest protein synthesis problems, and the high number of cytoplasmic vesicles suggests an overdrive of cell functions (Panariti *et al*., 2012).

After the hyperthermia treatment, the cells were left to recover for 24 hours and the effects were ultrastructurally assessed. In the case of 10 nm SPIONs, most of the nanoparticles were located around the cells, only a few inside the cell and no evident differences could be seen between the hyperthermia treated and not-treated cells (Fig. 5C). It is possible that because of the reduced endocytosis, the heat produced by the 10 nm SPIONs placed in alternating magnetic field had dissipated into the cell culture media and did not affect the cell equilibrium. In the case of 100 nm SPIONs hyperthermia treated keratinocytes some differences could be discerned ultrastructurally (Fig. 5D): the number of intracellular SPIONs increased, the cell cytoplasm became much more electron-transparent than in the untreated cells, with large areas of undelimited lysis, and the nuclei contained a lot of euchromatin with many small patches of not so dense heterochromatin. SPIONs could be identified in the cytoplasm and in vesicles and also at cell borders, closely attached to cell membranes. Although the nanoparticle endocytosis process seems

to be more intense, in no cell sections could we find SPIONs internalized by caveolin or clathrin coated membrane pits, which we would expect for the nanoparticles sizes of 10 and 100 nm (Panariti *et al*., 2012).

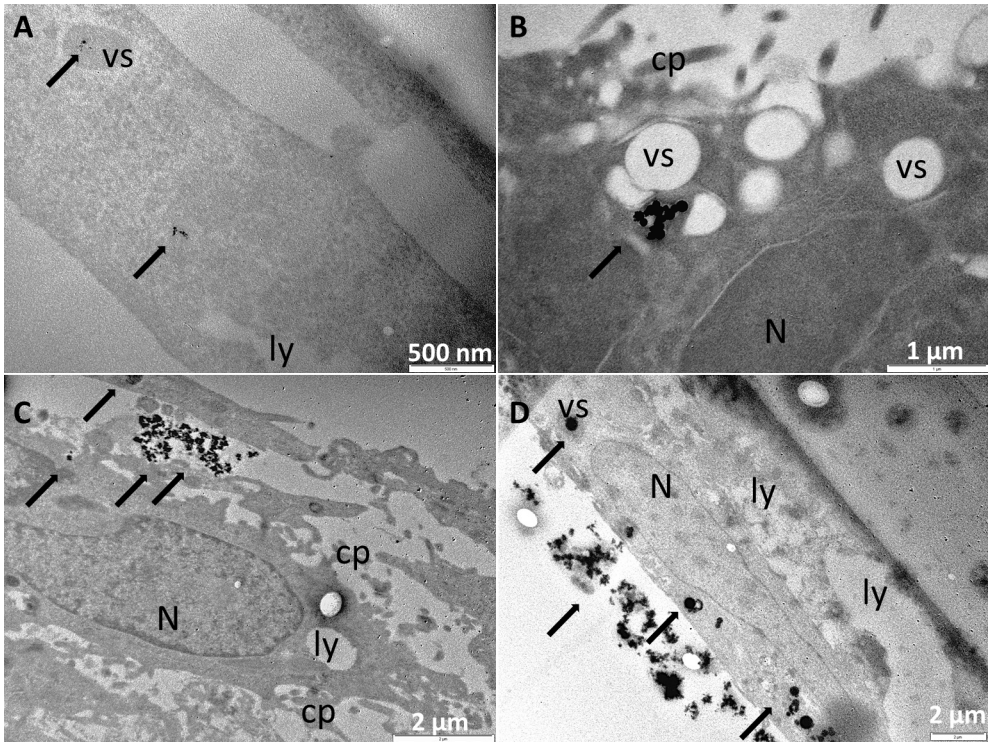


Figure 5. TEM micrographs of normal keratinocytes treated with SPIONs. (A) keratinocytes treated with 10 nm naked SPIONs for 24 hours; (B) keratinocytes treated with 100 nm naked SPIONs for 24 hours; (C) keratinocytes treated by hyperthermia with 10 nm naked SPIONs; (D) keratinocytes treated by hyperthermia with 100 nm naked SPIONs. vs- vesicle, ly - lysis, cp- cytoplasmic process, N- nucleus, arrow - SPIONs

Melanoma cells exposed to 10 and 100 nm SPIONs had a similar response in culture. Cells endocytosed a part of the SPIONs clumps but most of the nanoparticles were retained at the membrane surface (Fig. 6 A and B). The hyperthermia treated melanoma cells showed large areas of lysis in the cytoplasm, which could be located after the presence of the nanoparticles (Fig. 6 C and D).

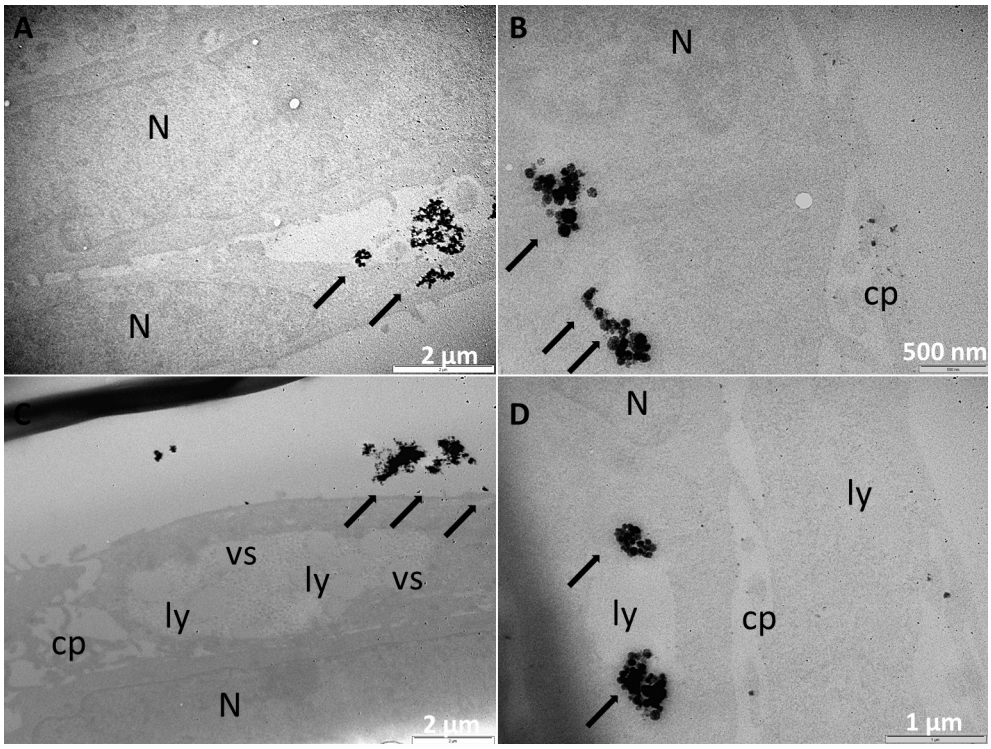


Figure 6. TEM micrographs of malignant melanoma treated with SPIONs. (A) melanoma treated with 10 nm naked SPIONs for 24 hours; (B) melanoma treated with 100 nm naked SPIONs for 24 hours; (C) melanoma treated by hyperthermia with 10 nm naked SPIONs; (D) melanoma treated by hyperthermia with 100 nm naked SPIONs.
vs- vesicle, ly - lysis, cp- cytoplasmic process, N- nucleus, arrow - SPIONs

SPIONs are studied more and more in the present, due to their proved biocompatibility and possibility of biotransformation into natural hemosiderin (Wu *et al*., 2010). Still, studies are controversial *in vitro* (Wahajuddin and Arora, 2012) and *in vivo* (Lee *et al*., 2008). Even if they are labeled as biocompatible, SPIONs still induce an array of cellular and/or medical problems, such as: toxicity by DNA or mitochondrial damage, capillary blockages, oxidative stress and other (Wahajuddin and Arora, 2012). In spite of this, we can find numerous types of iron oxide nanoparticles for sale, and used by physicians today for routine MRI and other applications (Stephen *et al*., 2011; Li *et al*., 2013; Estelrich *et al*., 2015).

Our results showed that 10 nm naked SPIONs are not toxic to normal keratinocytes even at high concentrations (500 µg/mL) but when covered with PEG the

cells' responses indicated mitochondrial stress. Instead, melanoma cells' mitochondrial activity was slightly affected by the 10 nm SPIONs independent of the used concentration and the presence/absence of PEG. PEG seemed to make a difference of action between cell types: keratinocytes had unaffected cell membranes when in contact with 10 nm SPIONs with or without PEG, but melanoma cells released more lactate dehydrogenase when SPIONs were un-covered with PEG. As the TEM analyses showed, these small SPIONs did not enter the cells in great amounts, at least not at the 24 hours' time point.

The 100 nm SPIONs, both covered and un-covered with PEG did not affect mitochondrial or membrane integrity of normal keratinocytes at either of the tested concentrations (0.1 - 500 μg/mL). But when placed in contact with melanoma cells, a relevant decrease in mitochondrial activity was revealed at all concentrations. The hyperthermia treatment affected keratinocytes greatly, and only slightly the melanoma cells (compared to cells not treated with hyperthermia). The improved magnetic hyperthermia response for the 100 nm SPIONs can be attributed to dipolar interaction typical to structured clusters (Coral *et al*., 2016). Another noticeable difference identified was the fact that after the hyperthermia treatment more 100 nm SPIONs were found in both cell types. This could be due to the physical stress induced by the hyperthermia over the cell membranes or due to the fact that, together with the hyperthermia treatment, the total amount of time of cell contact was 48 hours till the endpoint analyses.

Conclusions

In the case on superparamagnetic iron oxide nanoparticles, size matters. It matters for the endocytosis process and for the cells' response to hyperthermia treatment. What also matters is the nanoparticle structure (simple vs. cluster), and the presence of PEG on the surface of these nanoparticles, as some cells might be affected in the absence of PEG (such as melanoma cells membranes, in our case) and other might be affected by its presence (keratinocytes mitochondria, in our case).

Through our study, we showed that different cell types responded differently to hyperthermia treatment, not entirely dependent on the magnetization saturation of the nanoparticles but also on size, structure and surface coverage. These aspects finely tuned for a specific type of cell could make the difference to the final effect of the hyperthermia treatment.

Acknowledgements. This work was supported by the Romanian National Authority for Scientific Research and Innovations, CNCS-UEFISCDI, project number PN2-RU-TE-2014-4-0608 and by the 544644-TEMPUS-1-2013-1-UK-TEMPUS-JPCR Project. We thank A. Sesarman, PhD, for the HaCaT cells and Dumitrita Rugina, PhD, for the access to cell culture infrastructure.

REFERENCES

Atkinson, W. J., Brezovich, I. A., Chakraborty, D. P. (1984) Usable frequencies in hyperthermia with thermal seeds, *IEEE Trans. Biomed. Eng*. **31**:70–75

Bañobre-López, M., Teijeiro, A., Rivas, J. (2013) Magnetic nanoparticle-based hyperthermia for cancer treatment, *Reports of Practical Oncology & Radiotherapy,* **18**(6):397–400

Blasiak, B., van Veggel, F. C. J. M., Tomanek, B. (2013) Applications of Nanoparticles for MRI Cancer Diagnosis and Therapy, *Journal of Nanomaterials,* **2013***:* 12

Chan, F. K. -M., Moriwaki, K., De Rosa, M. J. (2013) Detection of Necrosis by Release of Lactate Dehydrogenase (LDH) Activity, *Methods in molecular biology,* **979**:65-70

Coral, D. F., Mendoza Zélis, P., Marciello, M., del Puerto Morales, M., Craievich, A., Sánchez, F. H., Fernández van Raap, M. B. (2016) Effect of Nanoclustering and Dipolar Interactions in Heat Generation for Magnetic Hyperthermia, *Langmuir*, **32**(5):1201–1213

Craciunescu, I., Petran, A., Liebscher, J., Vekas, L., Turcu, R. (2017) Synthesis and characterization of size-controlled magnetic clusters functionalized with polymer layer for wastewater depollution, *Materials Chemistry and Physics*, **185**:91–97

Das, M., Ansari, K. M., Tripathi, A., Dwivedi, P. D. (2011) Need for safety of nanoparticles used in food industry, *J. Biomed. Nanotechnol.,* **7**(1):13-4

Duan, X., He, C., Kron, S. J., Lin, W. (2016) Nanoparticle formulations of cisplatin for cancer therapy. *WIREs Nanomed Nanobiotechnol*, **8**: 776–791

Estelrich, J., Sánchez-Martín, M. J., Busquets, M. A. (2015) Nanoparticles in magnetic resonance imaging: from simple to dual contrast agents, *International Journal of Nanomedicine*, **10**:1727–1741

Giustini, A. J., Petryk, A. A., Cassim, S. M., Tate, J. A., Baker, I., Hoopes, P. J. (2010) Magnetic Nanoparticle Hyperthermia In Cancer Treatment, *Nano LIFE*, **1:**(01n02)

Kaiser, J. -P., Diener, L., Wick, P. (2013) Nanoparticles in paints: A new strategy to protect façades and surfaces?, *Journal of Physics: Conference Series,* **429**:012036

Karjalainen, P., Pirjola, L., Heikkilä, J., Lähde, T., Tzamkiozis, T., Ntziachristos, L., Keskinen, J., Rönkkö, T. (2014) Exhaust particles of modern gasoline vehicles: A laboratory and an on-road study, *Atmospheric Environment,* **97**:262–270

Kostiv, U., Patsula, V., Šlouf, M., Pongrac, I. M., Škokić, S., Dobrivojević Radmilović, M., Pavičić, I., Vinković Vrček, I., Gajović, S., Horák, D. (2017) Physico-chemical characteristics, biocompatibility, and MRI applicability of novel monodisperse PEG-modified magnetic Fe3O4&SiO2 core–shell nanoparticles, *RSC Adv.,* **7**:8786-8797

Lee, J. -H., Schneider, B., Jordan, E. K., Liu, W., Frank, J. A. (2008) Synthesis of Complexable Fluorescent Superparamagnetic Iron Oxide Nanoparticles (FL SPIONs) and Cell Labeling for Clinical Application, *Advanced Materials,* **20**(13):2512–2516

Li, L., Jiang, W., Luo, K., Song, H., Lan, F., Wu, Y., Gu, Z. (2013) Superparamagnetic Iron Oxide Nanoparticles as MRI contrast agents for Non-invasive Stem Cell Labeling and Tracking, *Theranostics*, **3**(8):595–615

Panariti, A., Miserocchi, G., Rivolta, I. (2012). The effect of nanoparticle uptake on cellular behavior: disrupting or enabling functions?, *Nanotechnology, Science and Applications*, **5**:87–100

Revia, R. A., Zhang, M. (2016) Magnetite nanoparticles for cancer diagnosis, treatment, and treatment monitoring: recent advances, *Materials Today,* **19**(3):157–168

Riss, T. L., Moravec, R. A., Niles, A. L., Duellman, S., Benink, H. B., Worzella, T. J., Minor, L. (2016) Cell Viability Assays, In: *Assay Guidance Manual*, Sittampalam, G. S., Coussens, N. P., Nelson, H., *et al*. (eds.), Eli Lilly & Company and the National Center for Advancing Translational Sciences Bethesda, pp. 1-30

Silva, A. H., Lima, E. Jr., Mansilla, M. V., Zysler, R. D., Troiani, H., Pisciotti, M. L., Locatelli, C., Benech, J. C., Oddone, N., Zoldan, V. C., Winter, E., Pasa, A. A., Creczynski-Pasa, T. B. (2016) Superparamagnetic iron-oxide nanoparticles mPEG350- and mPEG2000-coated: cell uptake and biocompatibility evaluation, *Nanomedicine*, **12**(4):909-19

Smijs, T. G., Pavel, S. (2011) Titanium dioxide and zinc oxide nanoparticles in sunscreens: focus on their safety and effectiveness, *Nanotechnology, Science and Applications,* **4**:95–112

Stephen, Z. R., Kievit, F. M., Zhang, M. (2011) Magnetite Nanoparticles for Medical MR Imaging. *Materials Today*, **14**(7-8): 330–338

Turcu, R., Socoliuc, V., Craciunescu, I., Petran, A., Paulus, A., Franzreb, M., Vasile, E., Vekas, L. (2015) Magnetic microgels, a promising candidate for enhanced magnetic adsorbent particles in bioseparation: synthesis, physicochemical characterization, and separation performance, *Soft Matter,* **11**:1008-1018

Wahajuddin, S. A., Arora, S. (2012) Superparamagnetic iron oxide nanoparticles: magnetic nanoplatforms as drug carriers, *International Journal of Nanomedicine*, **7**:3445–3471

Wu, E. X., Kim, D, Tosti, C. L., Tang, H., Jensen, J. H., Cheung, J. S., Feng, L., Au, W. Y., Ha, S. Y., Sheth, S. S., Brown, T. R., Brittenham, G. M. (2010) Magnetic resonance assessment of iron overload by separate measurement of tissue ferritin and hemosiderin iron, *Annals of the New York Academy of Sciences*, **1202**:115-122

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXII, 1, 2017 (p. 43-55)
doi:10.24193/subbb.2017.1.03

Assessment of the phytoreclamation of an oil-contaminated soil cultivated with *Cynodon dactylon*, *Eleusine indica* and *Eragrostis tenela*

Beckley Ikhajiagbe [1,✉], Boniface O. Edegbai[1], Gloria O. Omoregie[2], and Amenze M. Eweka[1]

SUMMARY. The interactions between *Cynodon dactylon, Eragrostis tenela* and *Eleusine indica* in the phytoremediation of petroleum hydrocarbon were investigated. Top soil was collected from a marked plot and polluted with spent engine oil (SEO) to obtain a constant 5% w/w concentration. Thereafter, the soils were sown with *Cynodon dactylon, Eragrostis tenela* and *Eleusine indica* singly and in combination of two's and three's in separate bowls. The set up was left for three (3) months in a screen house. The results revealed that there were reductions in soil concentrations of total petroleum hydrocarbons, from 26523.76 mg/kg to 19959 mg/kg in the oil-polluted soil. *Bacillus subtilis, Micrococcus* sp.*, Proteus vulgaris* were the prevalent bacteria species found in the soils, while prevalence fungi species included *Aspergillus niger, Geotricium* sp.*, Penicillium* sp., *Rhizopus* sp., *Aspergillus flavus,* and *Fusarium solani*. Morphological parameters of the three grasses were better enhanced when sown singly than when they were in combinations of two's and three's. Remediation was however best when they were sown altogether as one.

Keywords: *Cynodon, Eragrostis*, *Eleusine,* hydrocarbon, rhizoremediation.

Introduction

One of the vulnerabilities associated with oil exploration results mainly from oil spills. In some case, these spills may be accidental; other times they may just result from indiscriminate disposal of both new and used petroleum products. Over the years, snowballing levels of oil spills on both land and aquatic environments continue to pose momentous threat to the environment. This, in fact, has always

[1] *Environmental Biotechnology and Sustainability Research Group, Dept of Plant Biology and Biotech., Univ. of Benin, Benin City, Nigeria.*
[2] *Department of Environmental Management and Toxicology, Fed. Univ. of Petroleum Resources, Effurun, Nigeria*
✉ ***Corresponding author: Beckley Ikhajiagbe**, Environmental Biotechnology and Sustainability Research Group, Dept of Plant Biology and Biotech., Univ. of Benin, Benin City, Nigeria., E-mail: beckley.ikhajiagbe@uniben.edu*

been one of the greatest environmental and health concerns in contemporary oil and Gas Industry. Oil spills can cause enormous damage to the soil, plants and animals as well as cause serious human hazards and destruction of economic and social activities. This implies that if left unchecked, oil spills may spell doom for human populations. The critical goal for cleaning up any contaminated site is to eliminate any current or potential threat to human health and the environment from the chemicals that have been released into the soil, air or water. One of such measures is the reliance on plant populations, or phytoremediation.

Obviously, certain plants are better at removing contaminants than others. Plants that are utilized for contaminated land reclamation must be able to tolerate the types and concentrations of contaminants present. They also must be able to grow and survive in the local climate. Most studies on the phytoremediation of petroleum hydrocarbon (PHC) contaminated soil reported the use of grasses and legumes (Qui *et al*., 1997; Merkl *et al*., 2005; Schwab *et al*., 2006; Kecha varzi *et al*., 2007).

Grass species have far-reaching fibrous root structures, which possess pointedly greater root surface area compared to other species, and have been reported to penetrate the soils to as deepm as 3 meters (Qui *et al*., 1997). This characteristic confers on these grasses some level of resilience and persistence in unfavourable soil conditions. Several other studies have been conducted on the phytoremediative abilities of grasses, including the work of Gunther *et al.* (1996) on rye grass (*Lolium perenne L.*), Reilley *et al*., (1996) on alfafa (*Medicago sativa L.*), tall fescue (*Festuca arundinacea schreb.*), Sudan grass (*Sorghum vulgare L.*) and Switch grass (*Panicum virgatum*).

The degradation of polycyclic aromatic hydrocarbons in contaminated soils by a mix of *Andropogon gerardii, Schizachyrium scoparium, Sorghastrum nutans, Panicum virgatum, Elymus canadensis, Bouteloua curtipendula, Bouteloua gracilis* and *Pascopyrum smithii* was reported by Aprill and Sims (1990). White *et al.* (2006) acknowledged the biodegradation of intractable alkylated PAH compounds in oil-contaminated soil grassed with *Lolium arundinaceum, Lolium multiflorum* and *Cynodon dactylon*. Similarly, Abedi-Koupai *et al.* (2007) have reported the capacity of selected grass species to subsist under harsh environmental conditions occasioned by crude oil pollution.

The study plant species - *Cynodon dactylon, Eleusine indica* and *Eragrostis tenela,* are widely dispersed warm-season grasses in Nigeria, with persistence in oil- and metal-contaminated soils (Anoliefo *et al.,* 2006). In separate studies by Anoliefo *et al.* (2006, 2008); Ikhajiagbe and Anoliefo (2012), these species were persistent within and around workshops owned by automechanics, welders, vulcanizers, generator repairers, and other artisans over a large area spanning more than 10 Local Government Area Councils in MidWestern Nigeria put together. These workshops were usually notable for their deposition of heavy metals and hydrocarbons to soils and the surroundings (Anoliefo *et al*., 2006, 2008; Daniel *et al.*, 2016; Ikhajiagbe and Anoliefo, 2012). This study therefore evaluates the ability of grasses (*Eleusine indica, Eragrostis tenela, Cynodon dactylon*) in the phytoremediation of oil-polluted soil.

Materials and methods

The grasses used for the study were *Eleusine indica, Eragrostis tenela* and *Cynodon dactylon.* These were collected from an open field beside the Faculty of Agriculture, University of Benin, Nigeria, and their identities confirmed at the Department of Plant Biology and Biotechnology Herbarium. The grasses were thereafter regrown in a nursery from tillers.

Garden top soil was collected, air-dried to constant weight and measured into open boxes before amendment with the oil contaminant. The soil was thoroughly mixed with waste engine oil obtained as pooled to get constant 5% w/w oil in soil concentration.

There were 3 sets of experimental boxes (S-box, D-box, and T-box) arranged according to the number of grass species that would be sown thereon. The S-box was designed to cater for only single grass applications. The dimension was 60 cm (L) x 30 cm (B) x 15 cm (H) and would contain 20kg soil. The D-box had a dimension double that of the S-box since it would contain 2 grass species sown together; whereas the T-box was thrice the dimension of S-box. The reason for this disparity dimension of holding boxes was to ensure that plants were exposed to equal amounts of oil in soil. Pollution was done gradually with little soil samples being polluted at a time until the whole sample was thoroughly mixed. After contamination, the soil was left for about two weeks to attenuate.

Eleusine indica, Cynodon dactylon and *Eragrostis tenela,* initially raised from a pre-designated nursery, were sown singly and in combinations of two's and three's in the S-, D- and T-boxes respectively in three replicates each. Three months after plant exposure to oil-polluted soils, total petroleum hydrocarbon contents of soil were determined using GC-2010 (Shimadzu) Gas Chromatograph (GC) equipped with a split/splitless injector and a flame ionization detector (FID) from Agilent Technologies Inc., and according to the method of Dean and Xiong (2000). Remediation efficiency herein defined as percentage reduction in total assayed aliphatic hydrocarbons over the experimental period of three months was determined according to Ikhajiagbe *et al.* (2013). Culturable fungal and bacterial composition of rhizospheric soils of the treatment plants collected and assayed as pooled composite sample respectively was determined according to the procedure proposed by Cheesebrough (2001).

The grasses were also carefully observed for morphological changes including increase in height, stem width, internode length, peduncle length, flag leaf blade length, and flag leaf blade width. Tolerance index was determined at 5 weeks, where plant height was used as the determining parameter. Tolerance index was computed according to Iyagba and Offor (2013);

$$\text{Tolerance index} = \frac{\text{Parameter in contaminant}}{\text{Parameter in control}} \times \frac{100}{1}$$

Results were therefore presented as mean of 5 replications. Least significant differences were used to separate the means at 95% confidence limit.

Results and discussion

Soil contamination ensuing from petroleum exploration activities as well as from indiscriminate disposal of petroleum wastes has become a major source of concern as continuous oil pollution not only threatens food security, but also has negative impact on the health and wellbeing of the environment. In other to address the situation, plants are used in the restoration of contaminated lands. This is even predicated on the fact that the use of plants offers the advantage of being inexpensive, environmentally friendly and not destructive for soil matrix. This study investigated the interactions between *Eleusine indica, Eragrostis tenela,*and *Cynodon dactylon* in the phytoremediation of petroleum hydrocarbon and their plant microbial interactions. *Eleusine indica* and *Eragrostis tenela* has been shown to grow in oil contaminated sites and *Eleusine indica* been able to survive in crude oil and Pb contaminated sites (Anoliefo *et al*., 2006).

Three months after oil-polluted soils were sown with the test grass species singly and in double and triple combinations, there was a 45.65% reduction in TAH in the soil on which *E.* indica was sown, compared to 18.08% reduction in the control (Table 1a,b). TAH remediation efficiency was higher in the soils on which all three plants were sown > single or double > no plant.

Remediation of the compounds was better in the soil with grasses, than soil without. Grasses are generally required for phytoremediation activities particularly because they offer enhanced root zone parameters compared to other plants. Their numerously branched root systems offer them added advantage particularly with enhanced microbial activity and processes (Aprill and Sims, 1990). Accordingly, *Panicum maximum* and *Brachiara brizantha* have been reported to degrade oil and grease in contaminated soils (Merkl *et al*., 2004). Grass species have been previously reported in the remediation of oil-contaminated soils (Shirdam *et al*., 2008; Bordoloi *et al*., 2012; Ikhajiagbe and Anoliefo, 2012). Shirdam *et al.* (2008) studied the outcome of hydrocarbon contamination on selected growth features *Sorghum bicolor* and *Linum usitatissumum*, and reported that the species exhibited significant remediation efficiency even in vastly polluted soil. *Axonopus compressus, C. dactylon,* as well as *E. indica* have also been reported to colonize petroleum hydrocarbon-contaminated soils, with consequent remediation (Bordoloi *et al*., 2012; Ikhajiagbe and Anoliefo, 2012).

It is noteworthy that the restoration of degraded soils by mixed plant communities requires an understanding of the mechanisms responsible for community structure and dynamics (Holmes and Richardson, 1999). Although McCutcheon and Schnoor (2003) reported that *C. dactylon* showed improved performance in remediation of TPH and PAHs in soil where mixed with other grasses, the fact remains that growing more than one plant species at a spot would sometimes lead

to inter or intraspecific competition for nutrients and space. Competition between native plants and invasive species often restricts the success of restoration efforts. In a number of reports, competition among native species have negatively affected the success of restoration projects (Dyer and Rice, 1999; Brooks, 2000; Brown and Rice, 2000; Carlsen *et al.,* 2000; Green and Galatowitsch, 2002). However, in the present study, results showed that remediation of the compounds was better in the soil with *Cynodon dactylon, Eleusine indica* and *Eragrostis tenela* (CEE) altogether. Therefore, the effects of close proximity of planted grasses showed significant remediation. Anoliefo *et al.* (2006) reported earlier that *Cynodon dactylon* and *Eleusine indica* were widely distributed warm-season grasses in many tropical countries like Nigeria; these grasses persevere even in highly polluted soils. There are a number of other reports on the oil-remediating prospects of *C. dactylon* (White *et al*., 2006; Onwuka *et al*., 2012) and *E. indica* (Merkl *et al.,* 2005).

The removal TPH in contaminated soils is assumed to be rhizodegradation, the stimulation of rhizobacteria in the rhizosphere zone to degrade and enhance removal of TPH (Cai *et al*., 2010). An affirmative correlation exists between root biomass production and plants' capability for oil degradation. This is supported by earlier reports of Anoliefo and Ikhajiagbe (2011); Ikhajiagbe and Anoliefo (2011); Ikhajiagbe *et al.* (2012); Ikhajiagbe (2016).

Table 2 provides information on culturable microbial composition of the rhizospheric soils of the test plants after 3 months. Bacteria species which were present in the rhizosphere soil included *Bacillus subtilis, Micrococcus* sp. and *Proteus vulgaris,* whereas fungi species such as *Aspergillus niger, Fusarium solani, Geotricum* sp.*, Penicillium* sp.*, Rhizopus* sp.*,* and *Aspergillus flavus* were also present. Bacteria count after 3 months ranged from 4.2 to 8.0×10^5 cfu/g, compared to 2.4 - 6.9×10^5 cfu/g for fungi. For unpolluted and oil-polluted soils with no plant grown on them, *Bacillus subtilis, Micrococcus* sp. and *Proteus vulgaris* were present. However, *B. subtilis* appeared to be common to all treatments other than *Eragrostis tenela.* The fungi *Aspergillus niger* and *Penicillium* sp. were the most prevalent fungi species in the study after 3 months. Although both *Bacillus subtilis* and *Proteus vulgaris* was reported in the rhizosphere of *Cynodon dactylon*, *Eleusine indica and Eragrostis tenela* (EE), however, in trying to interact rhizospherically, *Bacillus subtilis* may have shown antagonism. However, *Bacillus subtilis* is a hydrocarbon degrading bacterium. The reason why remediation was not successful may be that *Bacillus subtilis* could have been antagonistic to *Micrococcus* sp. and *Proteus vulgaris*. This was similarly suggested According to Radha *et al.* (2010). They reported that *Bacillus subtilis* is antagonistic to *Proteus vulgaris, Candida albican, Staph. aureus, Pseudomonas aureginosa, E.coli* and *Aspergillus niger*. It is therefore suggested that the reason why remediation has been slow may have been the antagonism effect.

Morphological parameters of *Eleusine indica* after 9 weeks of exposure to oil contamination alone or sown with the other two test plants have been presented on Table 3. Before transplanting, the height of *Eleusine indica* was 41.20 cm. Nine

weeks after been sown alone in polluted soil, the height was 44.60 cm, compared to 42.10 cm when sown together with *Eragrostis tenela.* However, it was noticed that *Eleusine indica* grew best when planted alone.

Table 1a.

Total petroleum hydrocarbon contents of soil at 3 months after application of treatments

	1 day after pollution	**No plant**	**ET**	**CD**	**LSD (0.05)**
Nonane	3143.78	2577.90	1949.14	243.79	112.11
Decane	4326.36	3547.62	2682.34	2466.03	212.31
Dodecane	5526.11	4531.41	3426.19	3149.88	423.41
Tetradecane	3276.43	2686.67	2031.39	1867.57	302.31
Hexadecane	39.67	12.69	4.25	33.75	8.54
Octadecane	2543.55	2085.71	1577.00	1119.16	311.97
Nonadecane	835.84	685.39	518.22	702.11	285.65
Eicosane	2041.56	1674.08	449.14	489.97	113.42
Docasane	1053.21	863.63	126.39	326.50	323.23
Tetracosane	783.49	642.46	250.72	102.56	102.43
Hexacosane	237.83	195.02	<0.001	42.84	25.33
Tricosane	303.61	248.96	89.53	59.12	32.04
TAH	24111.44	19751.55	13104.31	10603.27	ND
TVAH	2412.32	207.45	12.98	9.97	ND
TPH	26523.76	19959	13117.29	10613.24	ND
TAHef (%)	---------	18.08	45.65	56.02	ND

TAH total aliphatic hydrocarbons; TVAH total volatile aromatic hydrocarbons; TPH total petroleum hydrocarbons; TAHef TAH remediation Efficiency; ET *Eragrostis tenela,* EI *Eleusine indica,* CD *Cynodon dactylon;* LSD (0.05) least significant difference at 95% confidence limit; ND not determined

Table 1b.

Total petroleum hydrocarbon contents of soil at 3 months after application of treatments

	EI	**CD+ET**	**EI+ET**	**EI+CD**	**EI+CD+ET**	**LSD (0.05)**
Nonane	2137.77	1226.07	185.42	1065.54	69.16	112.11
Decane	2941.92	1687.28	3724.11	1006.45	1384.44	212.31
Dodecane	3757.75	2155.18	3021.83	984.86	1768.36	423.41
Tetradecane	2227.97	584.34	542.72	709.43	1048.46	302.31
Hexadecane	8.21	21.48	17.80	<0.001	1.74	8.54
Octadecane	1220.90	1067.43	743.32	869.64	142.68	311.97
Nonadecane	854.37	483.79	789.53	515.98	521.91	285.65

	EI	CD+ET	EI+ET	EI+CD	EI+CD+ET	LSD (0.05)
Eicosane	799.64	812.05	1183.83	905.43	812.45	113.42
Docasane	74.87	622.71	583.95	428.12	51.74	323.23
Tetracosane	94.12	201.38	41.67	98.04	43.43	102.43
Hexacosane	101.65	89.66	38.22	48.54	<0.001	25.33
Tricosane	21.57	31.14	19.45	65.83	<0.001	32.04
TAH	14240.76	8982.52	10891.85	6697.86	5844.36	ND
TVAH	1.54	1.98	9.65	4.65	0.07	ND
TPH	14242.3	8984.5	10901.5	6702.51	5844.43	ND
TAHef (%)	40.94	62.75	54.83	72.22	75.76	ND

TAH total aliphatic hydrocarbons; TVAH total volatile aromatic hydrocarbons; TPH total petroleum hydrocarbons; TAHef TAH remediation Efficiency; ET *Eragrostis tenela,* EI *Eleusine indica,* CD *Cynodon dactylon;* LSD (0.05) least significant difference at 95% confidence limit; ND not determined

Table 2.

Microbial composition of rhizospheric soils of the treatment plants collected and assayed as pooled composite sample respectively

Sample identity	Bacterial counts ($x10^5$ cfu/g)	Bacterial isolates Identified	Fungal counts ($x10^5$ cfu/g)	Fungal isolates Identified
		(Unpolluted soil after 3 months)		
No plant	4.2	*Bacillus substilis, Micrococcus* sp., *Proteus vulgaris*	3.2	*Aspergillus niger, Geotricum* sp., *Penicillium* sp., *Rhizopus* sp.
		(Oil-Polluted soils after 3 months)		
No plant	5.0	*B. substilis, Micrococcus* sp., *P. Vulgaris*	2.4	*Penicillium* sp.
CD	5.6	*B. substilis, P. vulgaris*	4.0	*A. flavus, Rhizopus* sp.
EI	6.0	*B. substilis*	4.4	*A. niger, Fusarium solani*
ET	8.0	*Micrococcus* sp.	3.9	*A. flavus, Penicillium* sp.
EI+CD	6.9	*B. substilis*	6.9	*F. solani, Penicillium* sp.
CD+ET	5.7	*B. substilis*	3.2	*A. niger, Geotricum* sp., *Rhizopus* sp.
EI+ET	4.8	*B. substilis, P. vulgaris*	3.7	*A. niger, Geotricum* sp
All 3 grasses	6.0	*B. substilis*	3.9	*A. niger, F. solani, Penicillium* sp.

ET *Eragrostis tenela,* EI *Eleusine indica,* CD *Cynodon dactylon*

Plant morphological parameters of *Eragrostis tenela* sown in isolation and in combination with the other test grasses have been presented on Table 4. Plant height of *E. tenela* at the nursery, just before transplanting unto treatment bowls was 21.02 cm. This grew by an additional 12 cm alone (29.70 cm). Sown with *E. indica,* plant height was 26.20 cm, compared to 23.87cm, when sown with both *E.indica* and *C. dactylon* ($p>0.05$). There were no significant differences stem width, internode length, number of leaves per culm, leaf blade length and width and peduncle length. Although foliar chlorosis and necrosis were not reported at nursery stage, the test plant however showed significant evidence of necrosis and chlorosis whether sown alone or in combination with other test plants. This observation was similar to that with *Cynodon dactylon* sown alone or in combination (Table 5). For this plant, there was significant increase in plant height ($p<0.05$) when *C. dactylon* was sown alone (45.01 cm) or with *E. indica* (38.96 cm).

Generally, plant morphological parameters of the test plants were better alone than in combinations or two's and three's. This suggests effects of competition. This is even more interesting because the effects of competition was rather in favour of enhanced remediation than when oil-polluted soils were son with single plant species.

Table 3.

Plant morphological parameters of *Eleusine indica* in the present study at 9 weeks after transplanting

	Before transplanting planting	**Alone**	**With *Eragrostis***	**With *Cynodon***	With *Cynodon* and *Eragrostis*	**LSD (0.05)**
Plant height (cm)	41.20	44.60	42.10	43.90	42.07	6.94
Stem width (mm)	5.30	14.00	7.40	10.13	8.54	4.35
Internode length (cm)	2.80	6.90	4.70	5.30	5.96	2.11
Number of leaves per culm	7.32	11.00	9.02	10.31	9.90	3.41
Culm branching (numbers)	1.50	4.00	2.00	3.76	3.57	1.65
Flag leaf blade length (cm)	10.50	23.50	13.32	17.70	16.65	6.43
Flag leaf blade width (mm)	4.32	4.69	4.47	4.67	4.65	1.01
Number of spikes per culm	0	4.00	3.33	4.00	3.56	1.47
Peduncle length (cm)	3.79	4.36	4.36	4.36	4.14	1.73
Length of longest spike (cm)	0	8.00	6.40	7.51	7.34	3.03
Width of longest spike (mm)	0	3.01	3.30	3.43	3.09	0.92
Number of spikelets	0	71.05	53.43	64.53	50.07	18.63

	Before transplanting planting	**Alone**	**With *Eragrostis***	**With *Cynodon***	With *Cynodon* and *Eragrostis*	**LSD (0.05)**
Culm glabrous (laterally flattened)	+	+	+	+	+	NA
Culm colour (white or silver at base and pale green towards the tip)	+	+	+	+	+	NA
Leaves colour (green)	+	+	+	+	+	NA
Leaf blades linear or lanceolate	+	+	+	+	+	NA
Chlorosis	+	+	+	+	+	NA
Necrotic spots	-	+	+	+	+	NA

+ present, - absent; NA not available

Table 4.

Plant morphological parameters of *Eragrostis tenela* in the present study at 9 weeks after transplanting

	Before transplanting planting	**With *Eleusine***	**With *Cynodon***	**Alone**	With *Cynodon* and *Eleusine*	**LSD (0.05)**
Plant height (cm)	21.02	26.20	29.15	29.70	23.87	6.98
Stem width (mm)	1.00	1.00	1.00	1.03	1.06	0.37
Internode length (cm)	2.40	2.42	2.60	2.67	2.43	1.06
Number of leaves per culm	5.00	5.14	5.33	5.33	5.45	0.95
Culm branching (numbers)	3.00	3.00	3.00	3.00	3.07	0.92
Flag leaf blade length (cm)	2.40	3.31	3.59	4.06	4.03	1.67
Flag leaf blade width (mm)	2.28	3.30	3.80	4.33	3.86	2.04
Number of spikes per culm	19.00	21.30	24.00	24.00	21.98	8.64
Peduncle length (cm)	5.00	5.80	6.20	6.20	6.03	2.34
Length of longest spike (cm)	1.80	1.80	2.20	2.20	1.75	1.00
Width of longest spike (mm)	0.30	0.47	0.50	0.50	0.52	0.22
Culm glabrous (laterally flattened)	+	+	+	+	+	NA
Culm colour (white or silver at base and pale green towards the tip)	+	+	+	+	+	NA
Leaves colour (green)	+	+	+	+	+	NA
Leaf blades linear or lanceolate	+	+	+	+	+	NA
Chlorosis	_	+	+	+	+	NA
Necrotic spots	_	+	+	+	+	NA

+ present, - absent; NA Not available

Table 5.

Plant morphological parameters of *Cynodon dactylon* in the present study at 9 weeks after transplanting

	Before transplanting planting	**With *Eragrostis***	**With *Eleusine***	**Alone**	With ***Eleusine*** and ***Eragrostis***	**LSD (0.05)**
Plant height (cm)	28.60	33.00	38.96	45.01	31.54	9.56
Stem width (mm)	1.20	1.80	1.87	2.00	2.00	1.01
Internode length (cm)	2.72	2.90	3.00	3.00	2.38	1.30
Number of leaves per culm	4.00	4.80	5.00	5.00	4.05	2.17
Culm branching (numbers)	4.70	4.72	5.00	5.00	4.63	2.06
Flag leaf blade length (cm)	4.10	4.60	5.50	5.52	4.95	1.87
Flag leaf blade width (mm)	3.52	3.78	4.00	4.00	4.00	1.70
Number of spikes per culm	5.24	5.93	6.73	6.06	5.92	2.01
Peduncle length (cm)	7.40	7.47	7.50	7.50	7.53	1.47
Length of longest spike (cm)	4.10	5.70	6.30	6.32	5.63	2.12
Width of longest spike (mm)	0.32	0.57	1.03	1.00	1.21	0.24
Culm glabrous (laterally flattened)	+	+	+	+	+	NA
Culm colour (white or silver at base and pale green towards the tip	+	+	+	+	+	NA
Leaves colour (green)	+	+	+	+	+	NA
Leaf blades linear or lanceolate	Linear	+	+	+	+	NA
Chlorosis	-	+	+	+	+	NA
Necrotic spots	-	+	+	+	+	NA

+ present, - absent; NA not available

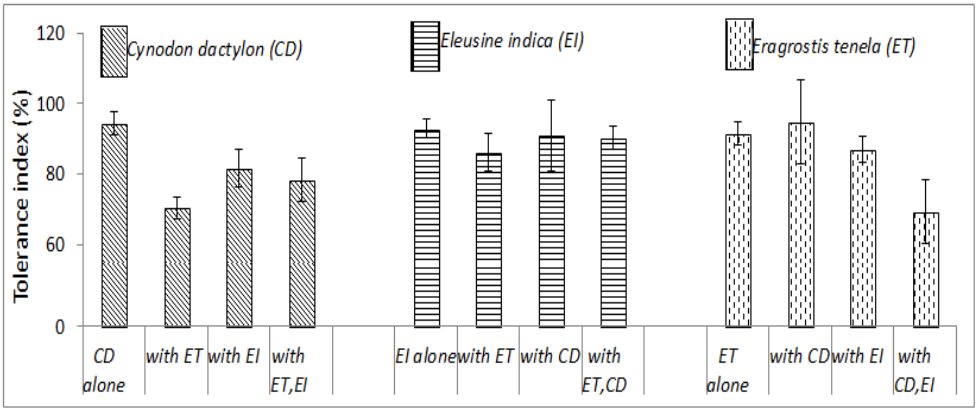


Figure 1. Tolerance index of grasses alone and in combination with other grasses under experimental condition.

Tolerance index for *Cynodon dactylon* alone ubder experimental condition was 94.47%, compared to 92.92% for *E. indica* and 91.58% for *E. tenela* respectively (Fig. 1). The individual grasses showed lower tolerance index in the polluted soils with in association with any other two grasses (i.e. grasses in groups of 3's). in combination of 3's, *E. indica* showed higher tolerance under experimental condition (90.35%).

Conclusions

The interactions among the test plants in the remediation of oil-contaminated soil have been provided in the present study. These plants have therefore demonstrated the ability to survive and provide suitable conditions for rhizobacteria to degrade hydrocarbons whether as single plants in contaminated soils, or in mixed culture.

Acknowledgements. The study was privately funded. The researchers are grateful to Mr. Ben Enebeli, Chief Lab. Technologist at the Department of Plant Biology and Biotechnology, University of Benin, Nigeria for technical and Lab support.

REFERENCES

Abedi-Koupai, J., Ezzatian, R., Vossoughi-Shavari, M., Yaghmae, S., Borghei, M. (2007) The effects of microbial population on phytoremediation of petroleum contaminated soils using tall fescue, *Int. J. Agricult. Biol.,* **9**: 242–246

Anoliefo, G. O., Ikhajiagbe, B., Okonokhua, B. O., Diafe, F. V. (2006) Ecotaxonomic distribution of plant species around auto mechanic workshops in Asaba and Benin City, Identification of oil tolerant species, *African Journal of Biotechnology* **5(19)**: 1757-1762

Anoliefo, G. O., Ikhajiagbe, B., Okonokhua, B. O., Edegbai, B. E., Obasuyi, O. C. (2008) Metal tolerant species distribution and richness in and aroud metal based industries, possible candidates for phytoremediation, *Africa Journal of Environmental Science and Technology* **2 (11)**: 360-370

Anoliefo, G. O., Ikhajiagbe, B. (2011) Plant-microbial interaction in the degradation of crude oil in soil, Synergism in bioremediation, *Nigerian Journal of Life Sciences* **1(1)**: 40-52

Aprill, W., Sims, R. C. (1990) Evaluation of the use of priarie grasses for stimulating polycyclic aromatic hydrocarbon treatment in soil, *Chemosphere* **20**: 253-265

Bordoloi, S., Basumatary, B., Saikia, R., Das, H. C. (2012) *Axonopus copressus* (Sw.) P. Beauv. A native grass species for phytoremediation of hydrocarbon contaminated soil in Assam, India. *Journal of Chemical Technology and Biotechnology, Special Issue, Bioremediation* **87(9):** 1335- 1341

Brooks, M. L. (2000) Competition between alien annual grasses and native annual plants in the Mojave Desert, *American Midland Naturalist* **144:** 92–108

Brown, C. S., Rice, K. J. (2000) The mark of zorro, effects of the exotic annual grass Vulpia myuros on California native perennial grasses, *Restoration Ecology* **8**: 10–17

Cai, Z., Zhou, Q. X., Peng, S. W., Li, K. N. (2010) Promoted biodegradation and microbiological effects of petroleum hydrocarbons by *Impatiens balsamina* L. with strong endurance, *Journal of Hazardous Materials* **177**: 384-389

Carlsen, T. M., Menke, J. W., Pavlik, B. M. (2000) Reducing competitive suppression of a rare annual forb by restoring native California perennial grasslands, *Restoration Ecology* **8**:18–29

Cheesebrough, M. (2001) *District Laboratory Practice in Tropical Countries*, part 2, Cambridge University Press, Cambridge, pp. 355

Daniel, E. S., Musa, J. J., Akos, M. P., Yerima, I. Y., Dada, P. O., Jibril, I., Manta, I. H. (2016) *Assessment of heavy metal pollution in some Nigerian soils: a review*, 37th Annual Conference and Annual General Meeting of the Nigeria Inst. of Agric. Engineers, Minna, Nigeria

Dean, J. R., Xiong, G. (2000) Extraction of organic pollutants from environmental matrices, selection of extraction technique, *Trends in Analytical Chemistry* **19(9)**: 553–561

Dyer, A. R., Rice, K. J. (1999) Effects of competition on resource availability and growth of a California bunchgrass, *Ecology* **80**: 2697–2710

Green, E. K., Galatowitsch, S. M. (2002) Effects of *Phalaris arundinacea* and nitrate-N addition on the establishment of wetland plant communities, *Journal of Applied Ecology* **39**: 134–144

Gunther, T., Dornberger, U., Fritsche, W. (1996) Effects of rye grass on biodegradation of hydrocarbons in soil, *Chemosphere* **33**: 203-215

Ikhajiagbe, B., Anoliefo, G. O. (2011) Impact of substrate amendment on the polyaromatic hydrocarbon contents of a five month old waste engine oil polluted soil, *African Journal of Environmental Science and Technology* **5(10)**: 769-777

Ikhajiagbe, B., Anoliefo, G. O. (2012) Weed biodiversity studies of a waste engine oil-polluted soil exposed at different intervals of natural attenuation and substrate amendment, *Journal of Biological Sciences* **12(5)**: 280 - 286

Ikhajiagbe, B., Anoliefo, G. O., Okoh, H. U., Owenaze, I. (2013) Impact of organic mulching on the enhanced natural attenuation of a petroleum hydrocarbon polluted soil, *Nigerian Society for Experiential Biology Journal* **13(3&4)**: 32 – 42

Ikhajiagbe, B. (2016) Possible adaptive growth responses of *Chromolaena odorata* during heavy metal remediation, *Ife Journal of Science* **18(2)**: 403 – 411

Iyagba, A. G., Offor, U. S. (2013) Phytoextraction of heavy metals by fluted pumpkin (*Telfaria occidentalis* Hooker F.) planted on oil contaminated soil amended with sawdust and siam weed (*Chromolenaodorata*) (L.) R.M King and Robinson) weed, *International Research Journal of Agricultural Sciences and Soil Sciences* **3 (12)**: 422 – 426

McCutcheon, S. C., Schnoor, J. L. (2003) *Phytoremediation: transformation and control of contaminants*, Wiley-Interscience Inc., Hoboken

Merkl, N., Schultze-Kraft, R., Infante, C. (2004) Phytoremediation in the tropics- the effect of crude oil on the growth of tropical plants, *Bioremediation J.* **8**: 177-184

Merkl, N., Schultze-Kraft, R., Infante, C. (2005) Assessment of tropical grasses and legumes for phytoremediation of petroleum contaminated soils, *Water Air Soil Pollut.* **165**: 195 - 209

Onwuka, F., Nwachoko, N., Anosike, E. (2012) Determination of total petroleum hydrocarbon (TPH) and some cations (Na, Ca^{2+} and Mg) in a crude oil polluted soil and possible phytoremediation by Cynodon dactylon L. (Bermuda grass), *J. Environ. Earth Sci.* **2**: 13–17

Qui, X., Leland, T. W., Shah, S. I., Sorensen, D. L., Kendall, E. W. (1997) Field study, grass remediation of for clay soil contaminated with polycyclic aromatic hydrocarbons. *In*: *Phytoremediation of Soil and Water Contaminants*, Kruger, E. L., Anderson, T. A., Coats, J. R. (eds), ACS Symposium Series 664, pp. 186-199

Radha, K. E., Shamsher, K. P., Veerendra, K. B. (2011) Study on Antioxidant activity and strain development of *Bacillus substilis* (MTCC No. 10619), *Journal of Agricultural Technology* **7(6)**: 1693-1703

Reilley, K. A., Banks, M. K., Schwab, A. P. (1996.) Organic chemicals in the rhizosphere, *Journal of Environmental Quality* **25**: 212-219

Shirdam, R, Zand, A. D., Bidhendi, G. N., Mehrdadi, N. (2008) Phytoremediation of hydrocarbon-contaminated soils with emphasis on the effect of petroleum hydrocarbons on the growth of plant species, *Phytoprotection* **89**: 21- 29

White, P. M., Wolf, D. C., Thoma, G. J., Reynolds, C. M. (2006) Phytoremediation of alkylated polycyclic aromatic hydrocarbons in an oil-contaminated soil, *Water Air Soil Pollut.* **169**: 207-220

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXII, 1, 2017 (p. 57-66)
doi:10.24193/subbb.2017.1.04

Changes in metals and polyaromatic hydrocarbon contents of a spent lubrication oil-polluted soil after exposure to sodium azide and hydroxlamine hydrochloride solutions: implications for intrinsic bioremediation

Beckley Ikhajiagbe[1,✉] and Geoffery O. Anoliefo[1]

SUMMARY. The study investigated the changes in heavy metals and polyaromatic hydrocarbon contents of an oil-polluted soil as a result of exposure to sodium azide and hydroxylamine hydrochloride. Measured 5kg of Oil-polluted soils (5%w/w), placed in experimental buckets, were saturated with solutions of sodium azide and hydroxylamine hydrochloride in 3 different concentrations (0.0625, 0.0312 and 0.0156 %v/w) respectively. The entire set up was observed in a well-ventilated Screen House for 3 months. Results showed that experimental concentrations of both mutagenic agents had no significant effect ($p>0.05$) on Fe concentration of soil (998.8 – 1106.2 mg/kg). Although soil levels of Fe exceeded permissible levels by over 5 times, concentrations of Mn, Cd, Ni, and V were below detection limits (<0.001 mg/kg) after application of chemical agents. Hydroxylamine HCl-moistened soil presented enhanced remediative capabilities for chromium (Cr = <0.001 mg/kg) than with sodium azide (Cr = 8.29 - 13.11 mg/kg). Sodium azide did not significantly enhance Cr remediation, compared to the control. Reductions in PAH fractions in the treated soils were better than in the control soils. Efficiency of PAH reduction in the control was 60.47%. application of mutagenic agents to polluted soils at lower to moderate concentrations significantly enhanced remediation efficiency to 80.95 -89.27%. Generally, however, hydroxylamine HCl showed better prospects in the enhancement of remediation (at lower to moderate levels) than did sodium azide.

Keywords: bioremediation, hydrocarbon, hydroxylamine, metals, mutation

[1] *Environmental Biotechnology and Sustainability Research Group, Department of Plant Biology & Biotechnology, Univ. of Benin, Benin City, Nigeria.*

✉ ***Corresponding author: Beckley Ikhajiagbe**, Environmental Biotechnology and Sustainability Research Group, Department of Plant Biology & Biotechnology, Univ. of Benin, Benin City, Nigeria, E-mail: beckley.ikhajiagbe@uniben.edu*

Introduction

Poly aromatic hydrocarbons (PAHs), as environmental pollutants, exist in air, water, sediments and soil. These compounds are also products of transportation, refuse burning, and gasification. Recently, indiscriminate disposal of spent lubrication oil oil has been identified as a significant source of PAHs in the environment (Anoliefo and Edegbai, 2001). PAHs are easily mixable with oil than water. They are toxic, mutagenic and carcinogenic in nature. Lower molecular weight PAH are acutely toxic; similarly, higher molecular PAH, apart from being genotoxic, are not easily biodegradable (Juhasz and Ravendra, 2000). Phenanthrene, for example, induces mutations by causing sister chromatids to interchange and obstructs gap junction intercellular communications. Phenanthrene can be bioaccumulated in aquatic organisms including fish, and then biomagnified up the food chain in humans (Mrozik *et al.*, 2003). In the soil, they hamper crop plant productivity; where in most cases they result in plant death (Ikhajiagbe, 2010)

There are several remediative measures to curtail and contain the spread of these PAHs, among which are those that rely on the primary mechanism of intrinsic remediation or natural attenuation – microbial biodegradation. During biodegradation of organic pollutants, available nutrients are converted by soil microorganisms into beneficial forms of energy and cell production. The biodegrading of contaminants, particularly PAHs, can be considerably boosted when the factors that directly or indirectly affect processes and mechanisms are improved upon. These factors may range from microbial and plant presence as well as other biological and abiotic factors. One of the methods of biological improvements for better performance has been reported to be by mutagenic enhancement (Mshembulla *et al.*, 2012).

Chemicals like sodium azide, hydroxylamine hydrochloride, oryzaline, as well as colchicine have been confirmed to cause mutagenesis. Sodium azide can bring about mutation plants and animals, including microorganisms (Asmahan and Nada, 2006). The ability of sodium azide to enhance crop development of chickpea (Mahesh and Vijay, 2009) and groundnut (Mensah *et al*., 2007) has been reported. Shittu and Ikhajiagbe (2013) reported enhancement of phytoaccumulation of heavy metals in an oil-spiked soil by *Vernonia amygdalina* after exposing the stem cuttings to sodium azide solutions before sowing. Ikhajiagbe and Chijioke-Osuji (2015) enhanced the capacity for Aspillia africana to remediate heavy metals in a spent lubrication oil-polluted soil after exposure to hydroxyl amine hydrochloride. The ability for these chemical agents to enhance plants' survivability in oil-polluted soils have been reported by Ikhajiagbe *et al*. (2013) in rice (*Oryza sativa,* var. FARO-57); Ikhajiagbe and Oshomoh (2014) in *Vigna unguiculata* (var. TVU-3541); Ikhajiagbe *et al*. (2014a) and Ikhajiagbe and Shittu (2015) in *Glycine max;* and Ikhajiagbe *et al.* (2014b) in fluted pumpkin *(Telfairia occidentalis)*.

The argument is whether simple mutagenic agents like these two, which have been reported to enhance plant capabilities by mere contact with plants and their propagules (Mshembula *et al.*, 2012; Omoregie *et al*., 2012; Ikhajiagbe *et al*., 2013) can also confer on soil microorganisms the ability to improve their bioremediation capabilities in oil-polluted soils. Although specific microorganisms are not directly targeted to achieve mutagenesis; it is however hoped that mere application of the chemical agent unto soil might in the long run affect soil microorganisms positively or negatively with regards to remediation rates. The research aim of the study therefore is to investigate to what extent Sodium azide and Hydroxylamine hydrochloride can affect intrinsic bioremediation of a spent lubrication oil-polluted soil.

Materials and methods

Soil contamination with oil

Top soil (0 - 10 cm), of predetermined physicochemical properties (Table 1), was collected from the Ugbowo Campus of the University of Benin, Nigeria. Measured quantity (mass of oil = 250g, specific gravity = 0.846) of spent lubrication oil was added to soil and mixed to get uniform concentration of 5% w/w.

Preparation of mutagenic solution

Measured quantities of sodium azide and hydroxylamine hydrochloride were dissolved in distilled water adjusted to pH 3 using a pH buffer. Sodium azide and hydroxylamine hydrochloride solutions were prepared at three concentrations (0.0625%w/v, 0.0312%w/v and 0.0156%w/v) respectively.

Saturation of polluted buckets with mutagenic solutions

Having previously determined the water-holding capacity of the soil to be 224ml/kg soil, the polluted soils were saturated with the mutagenic solutions using 1000ml of solution per bucket. Buckets were not perforated; the idea was to keep all content within the buckets under experimental condition. The set up was kept in a well ventilated Screen house (temp range = 29.21 ± 2.67 ^{o}C) for three months. Afterwards, soil was analyzed for polyaromatic hydrocarbon contents using the standard methods of Dean and Xiong (2000). Isolation and characterization of bacterial and fungal species was carried out using the methods of Sabba (1995) and Cheesebrough (1998). Heavy metal content of soil was also determined following the methods of SSSA (1971) and AOAC (2005). Soil physicochemical analyses were according the methods of Osuji and Nwoye (2007) and AOAC (2005). The results were subjected to statistical analysis using SPSS 16 ®.

Bioremediation efficiency

This is regarded as the proportion (%) of contaminant concentration that was bioremediated compared to a measured concentration at a start point. This is calculated according as;

$$\text{Efficiency (\%)} = \frac{(\text{c3MAPA} - \text{c1DAPA})}{\text{1DAPA}} \times 100$$

where c3MAPA = contaminant concentration at 3 months after pollution and amendment; c1DAPA = contaminant concentration at the first day following pollution and amendment.

Results and discussion

The physicochemical components of materials used in the study have been presented on Table 1. pH of both soil and spent lubricating oil (SLO) were both slightly acidic. Results as presented on Table 2 showed that the mutagenic agents had no significant effect on Fe concentration of soil (998.8 – 1106.2 mg/kg). These concentrations exceeded permissible levels in soil by over 5 times. Mn, Cd, Ni, and V were below detection limits (<0.001 mg/kg). ecological screening values for these metals were also not exceeded. Ecological screening valus were also not exceeded for Zn, Cu and Pb. However, there were significant reductions in these metals in the treated soils than in the control. Hydroxylamine HCl-treated soil showed better remediative capabilities for chromium (Cr = <0.001 mg/kg) than with sodium azide (Cr = 8.29 -13.11 mg/kg). Compared with the control, there were no significant differences in remediative capacities in the sodium azide-treated soil for chromium.

The effects of sodium azide and hydroxylamine HCl in the intrinsic bioremediation of heavy metals and PAH fractions on a spent lubricating oil-polluted soil has thus been reported. Although no significant changes in Fe concentration of soil was reported, remediation of other heavy metals in this study was significant as reduction was either total (100%) or significantly partial (50 - 98%). Remediation of heavy metals was comparatively better in the hydroxylamine hydrochloride-moistened soil.

Phytotoxicity screening limits for naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, and pyrene were exceeded at the first day when soil was polluted with oil (Table 3). However, after three months, concentrations of the polyaromatic fractions were below limits for naphthalene, acenaphthene, and fluorene. Ecological screening values for phytotoxicity of phenanthrene, anthracene, fluoranthene, and pyrene were still exceeded. Reductions in PAH fractions in the treated soils were better than in the control soils. Remediation efficiencies in the

sodium azide and hydrolyamine hydrochloride-treated soils comparable at lower and moderate concentrations (80.95 – 89.27%), compared to higher concentrations (69.99 – 77.93%). Efficiency of PAH reduction in the control was 60.47%.

Table 1.

Physical and chemical properties of soil and spent lubrication oil (SLO) contamination before commencement of experiment

Parameters	**Soil**	**SLO**		**Soil**	**SLO**
(Physicochemical parameters/heavy metals)			**(PAH content, mg/kg)**		
Ph	6.15	5.98	Naphthalene	<0.001	35.21
Electrical Conductivity (µs/cm)	309.00	NM	Acenaphthylene	<0.001	7.98
Total Org. Matter (%)	0.61	NM	2-bromonaphthalene	<0.001	35.24
Total Nitrogen (%)	0.16	NM	Acenaphthene	<0.001	36.32
Exchangeable Acidity (meq/100 g soil)	0.24	NM	Fluorene	<0.001	42.04
K (meq/100 g soil)	1.40	NM	Phenanthrene	0.85	16.54
Ca (meq/100 g soil)	12.20	NM	Anthracene	<0.001	78.20
Mg (meq/100 g soil)	9.95	NM	Fluoranthene	<0.001	26.41
P (mg/kg)	153.00	NM	Pyrene	<0.001	23.98
Copper, Cu (mg/kg)	<0.001	56.52	Benzo(a)anthracene	<0.001	42.05
Manganese, Mn (mg/kg)	<0.001	29.21	Chrysene	<0.001	115.27
Nickel, Ni (mg/kg)	<0.001	4.15	Benzo(b,j,k)fluoranthene	<0.001	41.68
Vanadium, V (mg/kg)	<0.001	3.95	Benzo(a)pyrene	40.28	129.87
Chromium, Cr (mg/kg)	0.08	14.29	Indeno(1,2,3-cd)pyrene	5.24	198.2
Lead, Pb (mg/kg)	<0.001	30.55	Dibenzo(a,h)anthracene	12.25	46.52
Iron, Fe (g/kg)	183.23	2123.21	Benzo(g,h,i)perylene	19.24	63.25
Zinc, Zn (mg/kg)	3.08	56.22			
Cadmium, Cd (mg/kg)	<0.001	3.65			

NM not measured.

Table 2.

Heavy metal contents of oil-polluted soil after amendment at 3 months after pollution and application of mutagenic agents

Treatments	**Fe**	**Mn**	**Zn**	**Cu**	**Cr**	**Cd**	**Pb**	**Ni**	**V**
					mg/kg				
Control	1106.2^{a}	18.2^{a}	42.2^{a}	38.11^{a}	10.08^{a}	0.97^{a}	22.24^{a}	2.53^{a}	3.91^{a}
					Sodium Azide-treated soil				
0.0156% w/v	1003.2^{a}	<0.001^{b}	3.65^{b}	31.52^{a}	13.11^{a}	<0.001^{b}	8.23^{c}	<0.001^{b}	<0.001^{b}
0.0312% w/v	1054.1^{a}	<0.001^{b}	5.98^{b}	22.03^{b}	9.06^{a}	<0.001^{b}	13.08^{bc}	<0.001^{b}	<0.001^{b}
0.0625% w/v	1035.1^{a}	<0.001^{b}	5.26^{b}	21.30^{b}	8.29^{a}	<0.001^{b}	16.82^{ab}	<0.001^{b}	<0.001^{b}
					Hydroxylamine HCl-treated soil				
0.0156% w/v	1100.6^{a}	<0.001^{b}	4.62^{b}	18.52^{b}	<0.001^{b}	<0.001^{b}	15.38^{b}	<0.001^{b}	<0.001^{b}
0.0312% w/v	1063.3^{a}	<0.001^{b}	4.92^{b}	21.04^{b}	<0.001^{b}	<0.001^{b}	13.26^{bc}	<0.001^{b}	<0.001^{b}
0.0625% w/v	998.8^{a}	<0.001^{b}	5.38^{b}	20.13^{b}	<0.001^{b}	<0.001^{b}	<0.001^{d}	<0.001^{b}	<0.001^{b}

Treatments	Fe	Mn	Zn	Cu	Cr	Cd	Pb	Ni	V
					mg/kg				
ESVp*	NA	100.00	50.00	40.00	1.00	4.00	50.00	30.00	2.00
ESVm*	200.00	100.00	100.00	100.00	10.00	20.00	900.00	90.00	20.00

***Benchmarks available at Efroymson *et al*. (1997); **NA** not available. **LSD** least significant difference; **ESVp** Ecological screening value for phytotoxicity of contaminant; **ESVm** Ecological screening value for toxicity of contaminant to soil microorganisms and soil microbial processes. Means on the same column with similar alphabetic superscripts do not differ from each other ($p>0.05$). $n = 3$.

A good number of polyaromatic hydrocarbon fractions were significantly remediated. Remediation was total (100%) for Acenaphthene, naphthalene, Acenaphthyelene, 2-bromonaphthalene, fluoranthene, chysene, benzo(b,j,k)flouranthene) and partial(60-90%) for phenanthrene, indo(1,2,3-cd)pyrene, dibenzo(a,h)anthracene in 0.0156%w/w sodium azide treatment as confirmed by Ikhajiagbe and Anoliefo (2011).

Table 3.

Polycyclic aromatic contents of oil-polluted soil after amendment at 3 months after pollution

	5 %w/w Oil-Polluted soil at first day of pollution	Control	**Sodium Azide-treated**			**Hydroxylamine HCl-treated**			**ESVp**
			0.0156 %w/w	0.0312 %w/w	0.625 %w/w	0.0156 %w/w	0.0312 %w/w	0.625 %w/w	
Naphthalene	29.25	<0.001	<0.001	<0.001	16.45	14.49	<0.001	15.29	0.10
Acenaphthylene	10.54	1.29	<0.001	<0.001	16.09	14.34	<0.001	<0.001	NA
2-bromonaphthalene	35.21	1.21	<0.001	16.91	17.65	15.30	16.76	16.04	NA
Acenaphthene	35.46	21.68	<0.001	16.25	16.52	14.51	16.06	15.32	20.00
Fluorene	45.22	32.59	14.22	17.02	17.40	15.10	16.67	16.26	30.00
Phenanthrene	5.62	44.84	0.49	0.81	1.76	0.94	0.71	1.03	0.10
Anthracene	29.24	23.25	15.04	18.03	18.53	15.78	17.41	16.99	0.10
Fluoranthene	42.52	17.34	<0.001	<0.001	16.49	<0.001	<0.001	15.82	0.10
Pyrene	36.20	12.20	14.45	17.16	18.06	15.07	16.68	17.06	0.10
benzo(a)anthracene	53.87	23.62	16.94	19.58	22.26	16.67	<0.001	20.36	NA
Chrysene	129.54	3.66	<0.001	<0.001	3.04	<0.001	<0.001	2.43	NA
benzo(b,j,k) fluoranthene	59.48	87.58	<0.001	<0.001	4.76	1.70	<0.001	8.78	NA
benzo(a)pyrene	209.16	40.58	24.30	31.80	60.47	40.49	22.42	42.49	NA
indeno(1,2,3-cd)pyrene	169.54	11.08	0.59	26.63	24.32	2.88	2.67	4.13	NA
dibenzo(a,h) anthracene	52.20	77.94	2.59	3.29	15.63	2.85	7.31	6.91	NA
benzo(g,h,i)perylene	72.65	2.65	20.38	24.83	35.37	23.38	26.14	25.27	NA
Total PAH	1015.70	401.51	109.00	192.31	304.8	193.5	142.83	224.18	1.00
Efficiency (%)	-	60.47	89.27	81.07	69.99	80.95	85.94	77.93	NA

***Benchmarks available at Efroymson *et al*. (1997); **NA** not available; **ESVp** Ecological screening value for phytotoxicity of contaminant.

Results showed microbial toxicity of higher concentrations of hydroxylamine HCl against *Achromobacter* sp. (Table 4). However, the presence of the chemical agent in soil encouraged the growth of *Micrococcus luteus* and *M. roseus.* Similarly, the absence of *Clostridium perfringens* and *Aspergillus flavus* may also suggest toxicity of hydroxylamine HCl to soil microorganism. The fact that the growth certain species of fungi and bacteria did not occur in some treated soil is not enough to prove toxicity of the chemical agents used in the study against the microorganisms. The possibility also exists that perhaps this change may vary from soil to soil as these chemical agents may involve in a number of possible chemical reactions to release toxic or even growth-enhancing substances in the soil. Hence, it is recommended that specific toxicity studies be conducted to further clarify the information provided herein. Further, the availability of heavy metals like Fe beyond ecological screening limits may also account for this change (see Table 2). There were significant reduction in heterotrophic bacteria count in the soils treated with higher concentrations of the chemical agents used in the study ($2.2 – 3.2 \times 10^5$cfu/g), compared to the control (4.3×10^5cfu/g). Percentage hydrocarbon degrading fungi ranged from 60% to absolute, compared to 34.88 – 90.91%.

The study reported differential effects of the chemical agents on microbial availability and processes in the soil; some were antimicrobial at higher concentrations, while others, at lower and moderate concentrations encouraged microbial proliferation. This is usually a precondition to intrinsic remediation of contaminants. However, environmental conditions like availability of nutrient and water also enhance activities of microorganisms in bioremediation. This study records many prevalent microorganisms like *Achromobacter* sp, *Bacillus pumilis*, *Clostridiumperfringens*, *Sarcina*sp, *Pseudomonasaeruginosa*, *Aspergillusniger*, A. *Flavus*, *Penicillium* spp., *P. notatum*, *Mucor* sp., *Fusarium* sp., and *F. solani*.

Table 4.

Microbial composition of treated and control soils at three months after pollution

		Sodium Azide-treated			**Hydroxylamine HCl-treated**		
	Control	0.0156 %w/v	0.0312 %w/v	0.625 %w/v	0.0156 %w/v	0.0312 %w/v	0.625 %w/v
Achromobacter sp.	+	+	+	+	+	+	-
Bacillus pumilis	+	+	+	+	+	+	+
Clostridium perfringens	+	+	+	-	-	-	-
Sarcina sp.	+	+	+	+	+	+	+
Micrococcus sp.	-	-	+	-	-	-	-
M. luteus	-	+		+	+	+	-
M. roseus	-	+	+	-	-	-	+
Pseudomonas aeruginosa	+	+	-	-	-	-	+
Heterotrophic (x 10^5cfu/g)	4.3^{a}	4.0^{a}	3.2^{ab}	2.7^{b}	4.1^{a}	4.0^{a}	2.2^{b}
Hyd. Deg. Bacteria (x 10^5cfu/g)	1.5^{bc}	2.5^{ab}	2.0^{abc}	2.0^{abc}	0.5^{c}	3.2^{a}	2.0^{abc}
% Hyd	34.88	62.50	62.50	74.07	12.19	80.00	90.91

		Sodium Azide-treated			Hydroxylamine HCl-treated		
	Control	0.0156 %w/v	0.0312 %w/v	0.625 %w/v	0.0156 %w/v	0.0312 %w/v	0.625 %w/v
Aspergillus niger	+	+	+	+	+	+	+
A. Flavus	+	+	+	+	-	-	-
A. fumigatus	-	-	-	-	+	-	-
Penicillium sp.	-	+	+	-		+	+
P. notatum	+	-	-	+	+	-	-
Fusarium sp.	-	-	-	+	-	-	-
F. solani	+	-	+	-	-	+	+
Mucor sp.	+	+	-	-	-	+	+
Heterotrophic Fungi (x 10^5cfu/g)	2.9^{ab}	3.0^{a}	2.0^{ab}	1.3^{b}	2.3^{ab}	2.2^{ab}	1.5^{ab}
Hyd. deg. Fungi (x 10^5cfu/g)	1.8^{a}	2.3^{a}	1.2^{a}	0.8^{a}	2.3^{a}	2.0^{a}	1.5^{a}
% Hyd	62.07	76.67	60.00	61.54	100	90.91	100

+ present, - absent, %Hyd Percentage hydrocarbon degraders. n = 3. Means on the same row with similar alphabetic superscripts are similar ($p>0.05$)

These microorganisms might have been involved in the remediation process, considering the fact that their prevalence, even in higher concentrations of SLO in soil, may signify tolerance to these pollutants. The microorganisms identified in this study have been previously reported to belong to the bioremediation microbial consortia by Cerniglia (1992), Ekundayo and Obuekwe (1997), Yogambal and Karegoudar (1997), Remero *et al.* (2001) and April *et al.* (2000). Some microorganisms like *Aspergillu niger* were present all though the experiment. The presence of the fungus was reported in both control and mutagen-treated soils. Also present was the bacterium *Bacillus pumilis*. *Aspergillus niger* metabolize PAHs (Yamazaki *et al.*, 1988). *A. fumigatus* also produces a cytochrome P450 that hydroxylatesbenzo[a]pyrene (Venkateswarlu *et al.*, 1996).

Conclusions

Chemicals agents abound that confer newer and more-improved capabilities to organisms. In the present study sodium azide and hydroxylamine hydrochloride were applied to petroleum hydrocarbon-polluted soil with a view to possibly improving the soil's biological capabilities in reclaiming the oil-degraded soil. Both agents differed in the enhancement remediation efficiencies. Further, lower to moderate levels of hydroxylamine hydrochloride presented better capability to enhance remediative capacities of oil-polluted soil than sodium azide..

Acknowledgements. The researchers are grateful to the Department of Plant Biology and Biotechnology, University of Benin. Benin City, for allowing the use of its Screen House during the study.

REFERENCES

Anoliefo, G. O., Edegbai, B. O. (2001) Effect of spent engine oil as a soil contaminant on the growth of two egg plant species, *Solanum melongen* L and *S. incanum*, *Journal of Agriculture Forestry and Fisheries* **1**: 21-25

AOAC (2005) *Methods of Analysis,* Association of Official Analytical Chemists, Washington D. C, pp. 326

April, T. M., Abbot, S. P., Foght, J. M., Currah, R. S. (2000) Hydrocarbon-degrading filamentous fungi isolated from flare pitsoils in Northern and Western Canada, *Canada Journal Microbiology* **46**: 38-49

Asmahan, A. M., Nada, A. (2006) Effect of gamma irradiation and sodium azide on some economic traits in Tomato, *Saudi Journal of Biological Sciences* **13(1)**: 44-49

Cerniglia, C. E. (1992) Biodegradation of polycyclic aromatic hydro-carbons, *Biodegradation* **3**: 351-358

Cheesebrough, M. (I998) *District laboratory practice in tropical countries*, part II (Microbiology), Cambridgeshire Tropical Health Technology, Cambridge, UK

Dean, J. R., Xiong, G. (2000) Extraction of organic pollutants from environmental matrices, selection of extraction technique, *Trends in Analytical Chemistry* **19**(9): 553–561

Efroymson, R. A., Will, M. E., Suter II, G. W., Wooten, A. C. (1997) *Toxicological Benchmarks for Screening Contaminants of Potential Concern for Effects on Terrestrial Plants*, *1997 Revision*, ES/ER/TM-85/R3.U.S, Department of Energy, Office of Environmental Management, pp. 123

Ekundayo, E. O., Obuekwe, C. A. (1997) Effects of oil spill on soil physico-chemical properties of a spill site in a typicpaledult of Midwestern Nigeria, *Environmental Pollution* **22**: 187-196

Ikhajiagbe, B. (2010) *Synergism in Bioremediation, Phytoassessment of Waste Engine Oil Polluted Soils after Amendment and Bioaugmentation*, Lambert Academic Publishing, Köln, Germany, pp. 276

Ikhajiagbe, B and Anoliefo, G. O. (2011) Impact of substrate amendment on the polyaromatic hydrocarbon contents of a five month old waste engine oil polluted soil, *African Journal of Environmental Science and Technology* **5(10)**:769-777

Ikhajiagbe, B., Ujomonigho, O. E., Okoh, B. E., Agho, E. E. (2013) Effects of sodium azide on the survival, growth and yield performance of rice (*Oryza sativa,* FARO-57 variety) in a hydrocarbon-polluted soil, *The International Journal of Biotechnology* **2(1)**: 28-41

Ikhajiagbe, B., Anoliefo G. O., Mshelbulla P. B., Omoregbee O. (2014a) Effects of sodium azide treatments on plant survival and performance of soy bean (*Glycine max*) in the phytoremediation of a hydrocarbon polluted soil, *Nigeria Journal of Life Sciences* **4 (2)**: 56 -79

Ikhajiagbe, B., Omoregie, U. E., Mshelmbulla, B. P. (2014b) Effects of exposure time to a mutagenic agent on the germination and two leaf characters of pumpkin *(Telfairia occidentalis)* in an oil-polluted soil, Proceedings of the 30th Annual Conf. of Genetics Society of Nigeria, held at the Bishop Kelly Past, Centre, Airport Road, Benin City on 19th -23rd Oct., 2014, pp. 332 – 338

Ikhajiagbe, B., Oshomoh, E. O. (2014) Influence of sodium azide on the germination success of *Vigna unguiculata* (TVU-3541 accession) exposed to water soluble fractions of waste engine oil, *Journal of Laboratory Science* **2(1)**: 26 – 30

Ikhajiagbe, B., Shittu, H. O. (2015) Effects of different concentrations and exposure time of sodium azide on the growth and yield of Glycine max sown in an oil-polluted soil, *UNIBEN Journal of Science and Technology* **3 (1)**: 112 – 126

Ikhajiagbe, B., Chijioke-Osuji, C. C. (2015) phytoaccumulation of heavy metals in a waste engine oil-**polluted** soil by *Aspillia africana* after exposure to hydroxyl amine hydrochloride, *International Journal of Plant & Soil Science* **8(5)**: 1 - 10

Juhasz, L. A., Ravendra, N. (2000) Bioremediation of high molecular weight polycyclic aromatichydrocarbons, a review of the microbial degradation of benzo[a]pyrene, *International Biodeterioration& Biodegradation* **45**: 57-88

Mahesh, P. K., Vijay, S. K. (2011) Effects of sodium azide on yield parameters of chickpea (*Cicerarietinum* L.), *Journal of Phytology* **3(1)**: 39 - 42

Mensah, J. K., Obadoni, B. O., Akomeah, P. A., Ikhajiagbe, B., Ajibolu, J. (2007) The effects of sodium azide and colchicine treatments on morphological and yield traits of sesame seed (Sesame indicum L.), *African Journal of Biotechnology* **6(5)**: 534-538

Mrozik, A., Piotrowska-Seget, Z., Labuzek, S. (2003) Bacterial degradation and bioremediation of polyaromatic hydrocarbon, *Pol. J. Environ.Stud*. **12**: 15 - 25

Mshembula, B. P., Mensah, J. K., Ikhajiagbe, B. (2012) Comparative assessment of the mutagenic effects of sodium azide on some selected growth and yield parameters of five accessions of cowpea – Tvu-3615, Tvu-2521, Tvu-3541, Tvu-3485 and Tvu-3574, *Archives of Applied Science Research* **4 (4)**: 1682-1691

Omoregie, U. E., Mensah, J. K., Ikhajiagbe, B. (2012) Germination response of five varieties treated with sodium azide, *Research Journal of Mutagenesis* **4 (1)**: 14 - 22

Osuji, L.C., Nwoye, I. (2007) An appraisal of the impact of petroleum hydrocarbons on soilfertility, The Owaza experience*, Africa Journal of Agricultural Research* **2**: 318-324

Romero, M. C., Hammer, E., Cazau, M.C., Arambarri, A. M. (2001) Selection of autochthonous yeast strains able to degrade biphenyl*, World Journal of Microbiology and Biotechnology* **17**: 591-594

Sabba, R. N. S (1995) *Soil Microbiology, Soil Micro-organism and Plant Growth*, Oxford Publisher, New Delhi, pp. 509

Shittu, H. O., Ikhajiagbe, B. (2013) Phytoaccumulation of heavy metals in an oil-spiked soil by *Vernonia amygdalina* after exposure to sodium azide solutions, *Nigeria Journal of Life Sciences* **3 (2)**: 169 – 179

SSSA (1971) *Instrumental Methods for Analysis of Soil and Plant Tissue*, Soil Science Society of America, Corporated, Wisconsin, U.S.A, pp. 27-32

Venkateswarlu, K., Marsh, R. M., Faber, B., Kelly, S. L. (1996) Investigation of cytochrome P450 mediated benzo[a]pyrene hydroxylation in *Aspergillus fumigatus*, *Journals of Chemical Technology and Biotechnology* **66**: 139-144

Yamazaki, Y., Hayashi, Y., Hori, N., Mikami, Y. (1988) Microbial conversion of β-myrceneby *Aspergillusniger*, *Agric. Biol. Chem.* **52**: 2921 - 2922

Yogambal, R. K., Karegoudar, T. B. (1997) Metabolism of polycyclic aromatic hydrocarbons by *Aspergillus niger*, *Indian Journal of Experimental Biology* **35**: 1021 – 1023

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXII, 1, 2017 (p. 67-85)
doi:10.24193/subbb.2017.1.05

Same karstic substratum, different aquatic communities? Case study: three water bodies from western Romania

Anca Mihaela Ciorca[1], Laura Momeu [1,✉] and Karina Paula Battes [1]

SUMMARY. We investigated the phytoplankton, periphyton and microcrustacean communities developing on similar limestone substratum, in three karstic lakes: Iezerul Ighiel (Alba County), Dracului and Ochiul Beiului (Caraș-Severin County), during 2014 and 2016. Species richness was significantly higher in the lake greater in size for both algae and microcrustaceans, consistent with the species-area hypothesis. Forty algal taxa and only one microcrustacean species were common in all three lakes, even if comparable physico-chemical characteristics were recorded. Relatively similar saprobic conditions were shown by indicator species, while trophic state differed at some extent. Since current factors existing in the three environments were relatively similar (limestone substratum, physico-chemical parameters, water source etc.), the dissimilarities found in the plankton and periphytic communities were best explained by long-term factors like geographical isolation or the strength of disturbances.

Keywords: current and long-term factors, karstic lakes, microcrustaceans, planktonic and benthic algae, similarity.

Introduction

Does similar karstic substratum sustain similar assemblages of algae and crustaceans? This question was firstly asked for terrestrial habitats (McCune and Allen, 1985), but it can also be applied to aquatic communities (Jenkins and Buikema, 1998). Karstic lakes include water bodies formed through a process of chemical dissolution of rocks, most commonly composed of carbonate or sulphate minerals, but also chloride minerals (Löffler, 2004). Lakes in karst terrain are distributed all over the world, from China to Northern and Central America, from the Alps to the Balkan Peninsula, and Romania.

[1] *Babeş-Bolyai University, Faculty of Biology and Geology, Department of Taxonomy and Ecology,, 5-7 Clinicilor Street, 400006, Cluj-Napoca, Romania*
✉ ***Corresponding author: Laura Momeu**, Babeş-Bolyai University, Faculty of Biology and Geology, Department of Taxonomy and Ecology, 5-7 Clinicilor Street, 400006, Cluj-Napoca, Romania*
E-mail: lmomeu@yahoo.com

The karstic lakes considered for the present study are located in two protected areas in western Romania. Lake Iezerul Ighiel, one of the largest permanent karstic lakes in Romania, is included in the Iezerul Ighiel IUCN Natural Reserve, category IV, from the Trascău Mountains, part of the Apuseni Mountains (Pop and Măhăra, 1965). The other two karstic water bodies, Lake Dracului and Ochiul Beiului are located in the Cheile Nerei – Beuşniţa National Park (IUCN protected area, category II), from the Aninei Mountains, south-western Romania (Pisota and Trufaş, 1971).

Algal and microcrustacean communities were the focus of the present paper. Algal communities comprise a high range of organisms with a wide spectrum of morphological, behavioral and physiological traits (Udovič *et al*., 2016). Phytoplankton represents the most important primary producer in pelagic food webs. The community composition is influenced by the physico-chemical conditions present in the aquatic ecosystem in which they develop, especially nutrient availability (Litchman and Klausmaier, 2008). Phytobenthos or periphyton represents the group of photosynthetic algae that are adapted to live on different substrates, developing a high range of morphological adaptations (Pan *et al*., 2016). Both types of algal communities have been intensively used in assessing the ecological state of the water all over the world, because their impact on the functioning of the ecosystem is high (Litchman and Klausmaier, 2008).

Microcrustaceans (Arthropoda Crustacea: Branchiopoda Cladocera; Maxillopoda Copepoda), together with protozoans and rotifers, represent zooplankton community that occupies central positions of lentic food webs in most freshwater habitats, from temporary pools to large lakes (Kobayashi *et al*., 2009). Cladocerans are mostly planktonic filtrators and detritus grazers, with only a few predator species, while copepods include herbivore, omnivore and carnivorous species (Błędzki and Rybak, 2016). Microcrustaceans play important roles in controlling the abundances of phytoplankton, nutrient recycling or lake trophic condition indicators. They are also used in water quality assessment, climatic change analyses, phylogenetic research, toxicology etc. (Błędzki and Rybak, 2016).

Previous studies conducted in Lake Iezerul Ighiel focused on geographical and abiotic parameters (Pop and Măhăra, 1965; Decei, 1981; Duma, 2009; Mihăiescu *et al.,* 2012), palaeohydrological aspects dealing with the controversial genesis of the lake (Haliuc *et al.*, 2017) and biotic communities (Decei, 1981, Momeu *et al.*, 2015). In Lake Dracului and Ochiul Beiului, no previous research was found.

Thus, the aim of the present study was to investigate if similar karstic substrata sustained similar algal and microcrustacean assemblages in the three lakes: Iezerul Ighiel, Dracului and Ochiul Beiului. The present study represents the first attempt to compare phytoplankton, periphyton and microcrustacean communities from different karstic lakes in Romania and to explain the dissimilarities recorded. 27 algal taxa was first cited for the Romanian flora in the study area (Cărăuş, 2017).

Material and methods

Lake Iezerul Ighiel (46°10'47.2"N; 23°21'48.4"E) is located in the central-eastern limestone plateau Ciumerna, on the Ighiu Valley, tributary to the Ampoi River (Pop and Măhăra, 1965), in a 365 ha area of strict protection of forests and meadows, representing the buffer zone for the lake. Lake Iezerul Ighiel is one of the most typical permanent karst lakes in our country (Fig. 1; Table 1). The lake is recharged during spring and precipitation events by torrents, but the water level fluctuates, with minimums in summer and winter (Pop and Măhăra, 1965; Duma, 2009).

Lake Dracului (46°51'52.3"N; 21°48'46"E) and Ochiul Beiului (44°56'09"N; 21°47'20.9"E) are two smaller water bodies formed on limestone, located in the National Park Cheile Nerei-Beușnița, in the homonymous Natural Reserve, stretching on an area of 3081.3 ha (Pișotă and Trufaș, 1971). They are both permanent systems (Fig. 1; Table 1). Ochiul Beiului is fed by several springs that refresh the water continuously. The water temperature is almost constant and it does not freeze during winter (Pișotă and Trufaș, 1971).

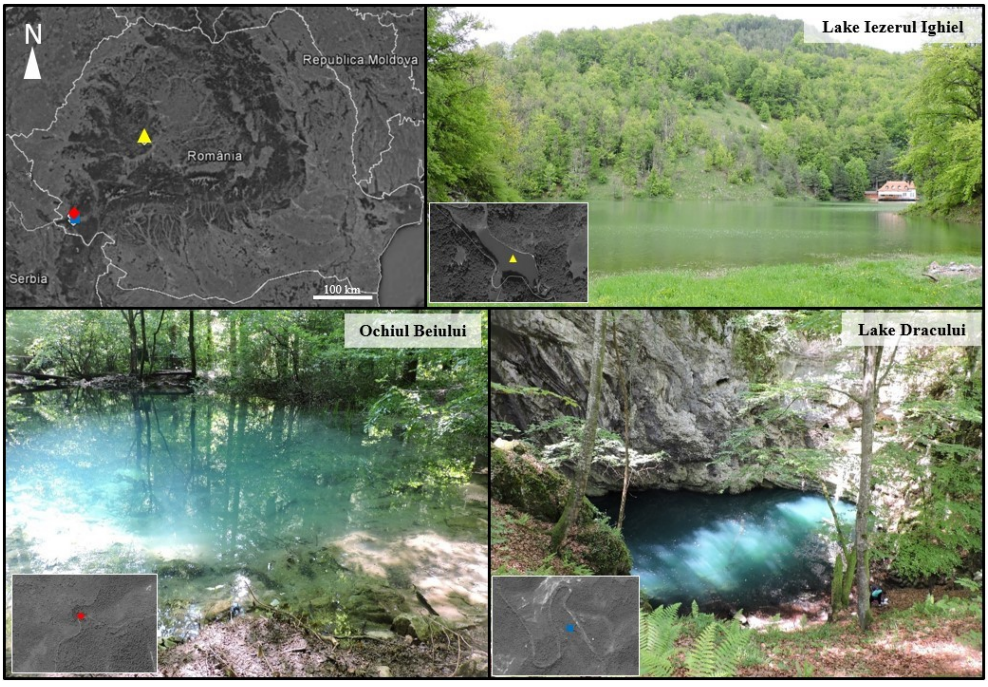


Figure 1. Location of the studied areas: Lakes Iezerul Ighiel, Dracului and Ochiul Beiului

Table 1.

Main physico-geographical characteristics of the three water bodies (depth and area according to Pop and Măhăra (1965), Pişotă and Trufaş (1971); abbreviations: SP - spring; SU - summer; AU – autumn)

Site code	Site name	Altitude (m)	Area (m^2)	Maximum depth (m)	Average depth (m)	Investigated biotopes	Substratum
IG_SP IG_SU IG_AU	Lake Iezerul Ighiel	915	52605	9	4.2	pelagial and littoral	rocks, silt, submerged macrophytes
DR_SP	Lake Dracului	215	700	9.3	4.63	littoral	rocks, logs, dead organic matter, submerged macrophytes
OB_SP	Ochiul Beiului	292	284	3.6	1.5	littoral	moss, rocks, sand, submerged macrophytes

Plankton samples from Lake Iezerul Ighiel were collected by boat from three sampling sites, in spring, summer and autumn 2014 – 2016 (May, August and November 2014; May, July and October 2015; May and July 2016). Phytoplankton data were averaged from the samples taken in 2015 and 2016, while for zooplankton, 2014 and 2016 data were considered. Only one sampling point for phytoplankton was considered for Lake Dracului, and one for Ochiul Beiului, in May 2016. Periphyton samples were collected from the banks in all three water bodies.

The physical and chemical parameters were recorded in the field: water temperature, dissolved oxygen, pH and conductivity (measured *in situ* with portable meters YSI 52 and HI98129).

Plankton samples were collected with a 20 μm mesh size net in case of phytoplankton and a 50 μm mesh size one for microcrustaceans. The benthic algae were sampled by scraping the hard substratum, by collecting the sediment using a pipette or by collecting submersed macrophytes. All samples were preserved in the field in 4% formaldehyde. Identifications were made to the species level in case of algae (Krammer and Lange-Bertalot, 1986, 1988, 1991; Ettl, 1983) and microcrustaceans (cladocerans: Negrea, 1983; adult copepods: Damian-Georgescu, 1963; 1966; 1970; Einsle, 1993; Janetzky *et al*., 1996).

Relative abundance, expressed as percentages, was calculated for algal and microcrustacean communities. For phytoplankton and periphyton roughly 400 individuals were counted in one drop of water, from every sample, at 40x magnification. In case of low number of individuals / sample, the entire surface of the slide was counted. Dominant algal taxa causing water blooms were identified. Since taxa other than Bacillariophyta dominated in some cases, counts were performed in wet mounts instead of fixed ones, specific for diatoms. This is why some diatom taxa were identified only to the genus level, in order to avoid erroneous results. In case of colonial taxa, the whole colony was

considered as one during counts, except for *Dinobryon* sp., where single cells were found in most cases. Relative abundance for microcrustaceans was estimated from counts ranging from 35 to 350 individuals / sample. Only adult copepods were identified to the species level; copepodites and nauplii were assigned proportionally to the adult copepod individuals found in the samples. Frequency was also calculated.

Similarity was considered for both algal and microcrustacean communities: Jaccard and Dice indices for qualitative information; and Bray-Curtis index for quantitative data. Diversity was calculated for phyto- and zooplankton samples, based on Shannon-Wiener index and the Equitability (Washington, 1984).

Multivariate data analyses were performed in order to visualize the data. Principal Component Analysis (PCA) (Jolliffe, 1986) was used to show the aggregation of sampling locations depending on abiotic variables and the aggregation of microcrustacean species in the three water bodies. Correspondence Analysis (CA), another ordination method (Hennebert and Lees, 1991), was used to single out the most dominant phytoplankton and periphyton species in each lake, and to depict the ecological status of the lakes based on indicator species. XLSTAT Version 2017.19.03.44468 and PAST version 3.14 were used for similarity, diversity and multivariate analyses.

To assess the trophic state of the water bodies based on algal communities, the following indices were used: alpha-eutrophicity index, gamma-eutrophicity index (Oltean, 1977); Nygaard compound index (1949), the Q index of eutrophy (Järnefelt, 1951) and the diatom index (Stockner, 1972). The saprobic indicator values of certain algal species were considered, following Rott *et al.* (1997), Hindak (1978), Sládeček (1973) and Van Dam *et al*. (1994). The organic pollution index (Palmer, 1969) was also used. The indication values for water saprobity and trophic state for the most abundant microcrustaceans were considered, according to Sládeček (1973), Damian-Georgescu (1963, 1966, 1970) and Negrea (1983).

Results and discussion

Physical and chemical parameters

Several physico-chemical parameters have been measured *in situ* in order to compare the three karstic aquatic ecosystems (Fig. 2). Average values from all available data were calculated; temperature and dissolved oxygen were measured in a vertical profile, while for conductivity, pH and transparency only one value was recorded.

The values of the main physico-chemical parameters were comparable between the three karstic lakes, showing similar conditions. Higher temperature values were recorded in summer in Lake Iezerul Ighiel (18.7°C), a normal characteristic of the dimictic lakes from the northern hemisphere. In spring however, the temperature values were homogenous in all three studied areas (10.67-11.63°C).

High conductivity values were caused by the limestone bedrock characteristic to karstic lakes, due to the weathering of the substrate. Our results agree with previous studies in Lake Iezerul Ighiel (Mihăiescu *et al*., 2012). The highest values of conductivity

and dissolved oxygen were recorded in Ochiul Beiului (630 µS/cm and 10.29 mg/L, respectively), probably because of the permanent water flow through the water body, nutrient and oxygen rich (Fig. 2). Similar high water conductivity values were reported in several karst rheocrene springs in southern Poland (Wojtal and Sobezyk, 2012). Typically for karst areas, pH was circumneutral to slightly alkaline, with slightly higher values in spring and autumn in Lake Iezerul Ighiel (7.51 and 7.55, respectively). Lake Dracului and Ochiul Beiului had clear waters, with transparency values of 3 m, while the transparency of Lake Iezerul Ighiel did not exceed 1.85 m (Fig. 2), probably due to frequent algal blooms.

The range of variation for the main physico-chemical parameters measured for the present study are in accordance with the literature: the dissolved oxygen values are similar to the ones recorded in several karst lakes from Croatia (Stanković *et al.*, 2011; Udovič *et al.*, 2016); slightly alkaline pH was found in several subalpine karstic lakes from China (Pan *et al.*, 2016) etc.

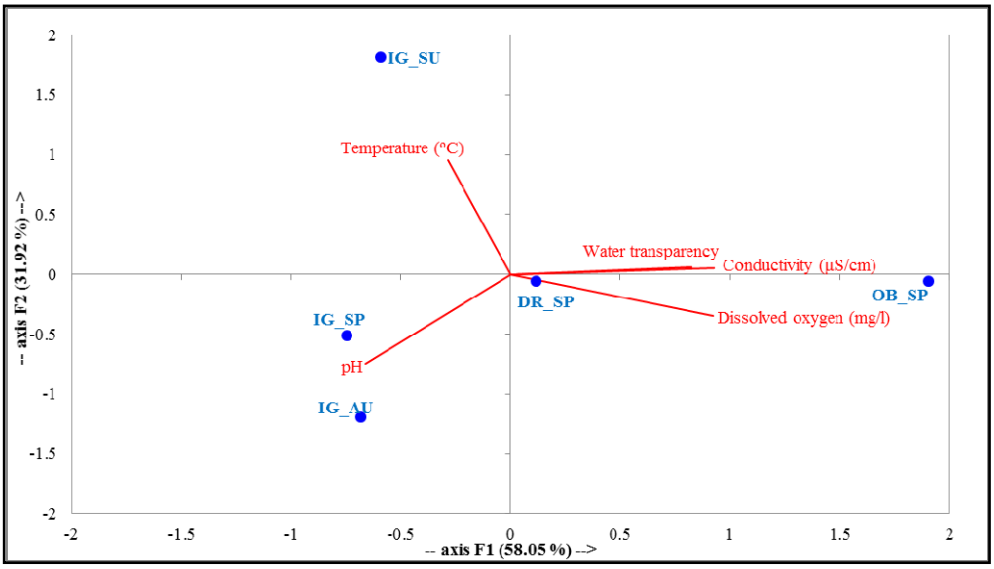


Figure 2. Principal Component Analysis (PCA) biplot (axes F1 and F2: 89.97 %) for the sampled lakes (abbreviations as in Table 1) and their aggregation based on physical and chemical parameters (average values for all available data from 2014 to 2016 were used)

Phytoplankton and periphyton

A total number of 288 taxa were identified in 2015 and 2016 from Lake Iezerul Ighiel (Bacillariophyta: 41%, Chlorophyta: 38%, Cyanophyta: 9%, Euglenophyta: 6%, Chrysophyta: 3%, Xanthophyta: 2% and Dinophyta: 1%). The total number of taxa differed with the season, from 200 taxa in spring, to 202 and 104 in summer and

autumn, respectively. Only 49 taxa were identified in spring 2016 in Lake Dracului (Bacillariophyta: 88%, Cyanophyta: 8%, Chlorophyta: 2% and Xanthophyta: 2%). The same number of taxa were identified in spring 2016 in Ochiul Beiului (Bacillariophyta: 88%, Cyanophyta: 8% and Chlorophyta: 4%). Fourteen taxa were common in all three lakes, such as: *Amphora montana*, *Asterionella formosa*, *Cocconeis placentula*, *Cyclotella iris*, *Gomphonema olivaceum*, *Navicula cincta* etc.

Dominant phytoplankton and periphyton taxa were discriminated by calculating the average abundances of species in spring, summer and autumn from Lake Iezerul Ighiel and in spring from Lakes Dracului and Ochiul Beiului. Due to the low number of phytoplankton taxa in these two lakes, the average abundances were calculated only for the periphyton samples.

The Correspondence Analysis for phytoplankton from Lake Iezerul Ighiel shows the specific grouping of the samples according to their seasonal variation (Fig. 3). Water blooms were caused by the dominant taxa in spring: *Fragilaria nanana*

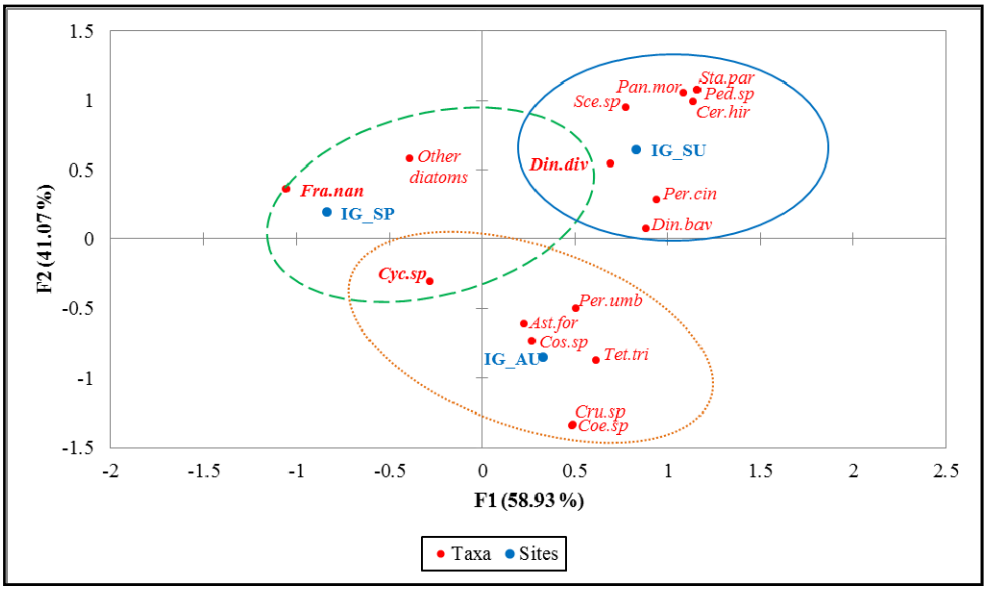


Figure 3. Correspondence Analysis (CA) plot (axes F1 and F2: 74.34%) showing the aggregation of phytoplankton samples; average abundances considered for Lake Iezerul Ighiel (abbreviations as in Table 1; *Ast.for – Asterionella formosa*; *Cer.hir – Ceratium hirundinela*; *Coe.sp – Coenococcus* sp.; *Cos.sp – Cosmarium* sp.; *Cru.sp – Crucigeniella* sp.; *Cyc.sp – Cyclotella* sp.; *Din.bav – Dinobryon bavaricum var medium*; *Din.div – Dinobryon divergens*; *Fra.nan – Fragilaria nanana*; *Pan.mor – Pandorina morum*; *Ped.sp – Pediastrum* sp.; *Per.cin – Peridinium cinctum*; *Per.umb – Peridinium umbonatum*; *Sce.sp – Scenedesmus* sp.; *Sta.par – Staurastrum paradoxum*; *Tet.tri – Tetrastrum triangulare*; Other diatoms – *Fragilaria* sp.; *Navicula* sp.; *Nitzschia* sp.

and *Cyclotella* sp. (with *Cyclotella iris* being the most abundant). *Dinobryon divergens* dominated in summer. Consistent with the typical diatom peaks of phytoplankton during spring and autumn (Willén, 2000), *Cyclotella* sp. dominated the autumn samples (again *Cyclotella iris* being the most abundant) (Fig. 3). In 2014, *Asterionella formosa* and *Cyclotella iris* were the taxa responsible for water blooms (Momeu *et al.*, 2015). This rapid shift in dominant diatom species showed the unique dynamics of Lake Iezerul Ighiel. The domination of centric diatoms in spring and autumn was also observed in deep karst lakes in Croatia (Udovič *et al*., 2016). The most abundant *Cyclotella iris*, followed by *C. ocellata*, *C. distinguenda* (Momeu *et al.,* 2015) represented the common taxa, as did *Dinobryon* sp. in summer (followed by *Peridinium cinctum*) (Udovič *et al*., 2016).

The Correspondence Analysis for periphyton from Lake Iezerul Ighiel, Lake Dracului and Ochiul Beiului shows the specific grouping of the samples according to their seasonal variation and to the specific aquatic ecosystem (Fig. 4). Diatoms dominated the periphyton communities from Lake Dracului and Ochiul Beiului, with *Gomphonema* sp. abundant in both. In Ochiul Beiului however, the dominant taxa was *Cocconeis placentula* (with a few variations like: *C. plancentula* var. *euglypta* and var. *linearis*).

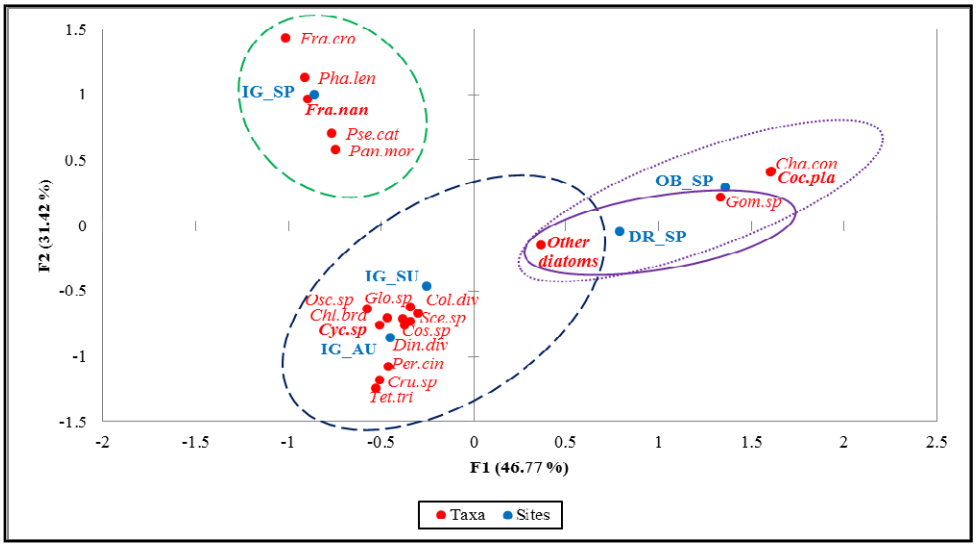


Figure 4. Correspondence Analysis (CA) plot (axes F1 and F2: 77.93 %) showing the aggregation of periphyton samples average abundances considered from the three waterbodies; abbreviations as in Table 1; *Cha.con* - *Chamaesiphon confervicolus*; *Chl.bra* - *Chlamydomonas braunii*.; *Coc.pla* - *Cocconeis placentula*; *Col.div* - *Coleochaete divergens*; *Cos.sp* - *Cosmarium* sp; *Cru.sp* - *Crucigeniella* sp; *Cyc.sp* - *Cyclotella* sp.; *Din.div* - *Dinobryon divergens*; *Fra.nan* – *Fragilaria nanana*; *Fra.cro* - *Fragilaria crotonensis*; *Glo.sp* - *Gloeotila* sp.; *Gom.sp* - *Gomphonema* sp.; *Osc.sp* - *Oscillatoria* sp.; *Pan.mor* - *Pandorina morum*; *Per.cin* – *Peridinium cinctum*; *Pha.len* - *Phacotus lenticularis*; *Pse.cat* - *Pseudanabaena catenata*; *Sce.sp* - *Scenedesmus* sp; *Tet.tri* – *Tetrastrum triangulare*; Other diatoms – *Achnanthes* sp., *Fragilaria* sp., *Navicula* sp., *Nitzschia* sp.

Diatoms, chlorophyceae and cyanophyceae were found in Lake Iezerul Ighiel periphyton. The spring samples separate themselves from the other two seasons (Fig. 4), with *Fragilaria nanana, F. crotonensis*, *Phacotus lenticularis* and *Pandorina morum* among the most abundant taxa. In the other two seasons, *Cyclotella* sp., *Dinobryon divergens*, *Tetrastrum triangulare*, *Gloeotila* sp. and *Coleochaete divergens* dominated.

Consistent with the data from nine karstic lakes in Jiuzhaigou Nature Reserve (Pan *et al*., 2016), taxa like: *Pseudanabaena* sp., *Leptolyngbya* sp., *Oscillatoria* sp., *Achnanthes minutissima* and *Denticula tenuis* were also frequent in the periphyton samples from Lake Iezerul Ighiel.

From the total number of taxa identified in the three studied areas, 130 taxa are known to have a cosmopolitan distribution: *Cocconeis placentula, Pandorina morum, Navicula cincta* etc. However, 25 taxa are described from mountain areas: *Anomoeoneis vitrea, Chroococcus subnudus, Cymbella affinis, Diatoma mesodon, Gonatozygon brebissonii, Neidium binodeforme, Leptolyngbya fontana* etc. A number of 41 true planktonic taxa were also identified: *Ankyra lanceolata, Asterionella formosa*, *Peridinium cinctum*, *Trachelomonas hispida* f. *minor* etc. Several other species have different physico-chemical preferences, like neutral to alkaline pH: *Cosmarium regnellii*, *Fragilaria crotonensis*, *Navicula oblonga* etc.; low water temperature: *Nitzschia hantzschiana*, *Gomphonema acuminatum* etc.

Consistent with the nature of the karst substrate, 22 calciphile taxa were found: *Aphanocapsa parietina*, *Achnantes flexella*, *Aulacoseira crenulata*, *Chamaesiphon confervicolus*, *Cymbella tumidula*, *Eunotia arcus*, *Gomphonema clavatum*, *Ophiocytium arbusculum*, *Phormidium foveolarum* etc.

According to Cărăuş (2017), 27 algal taxa from all the sampling sites are first cited for Romania: *Aphanocapsa hyalina*, *Astasia hypolimnica* (Fig. 5), *Bitrichia longispina*, *Characium strictum*, *Characium substrictum*, *Chlamydomonas braunii*, *Chlamydomonas*

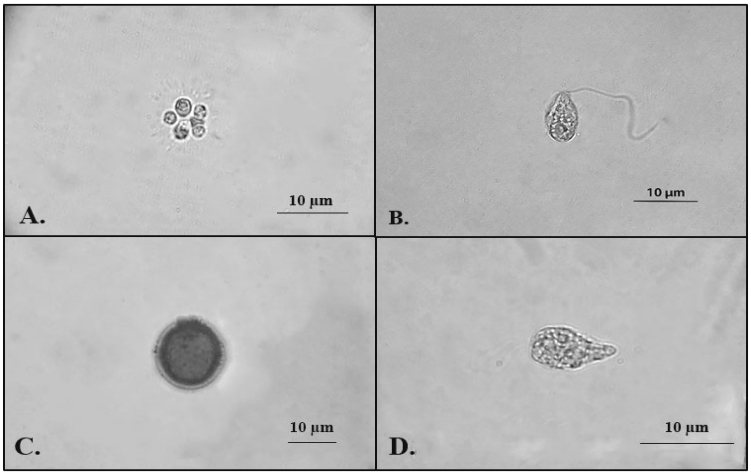


Figure 5. New taxa cited for Romania: A. *Radiococcus wildemannii*, B. *Astasia hypolimnica*, C. *Trachelomonas lomnickii*, D. *Pyramimonas fasciata*

pseudagloë, *Chlamydonephris impressa*, *Cosmarium taxichondriforme* var. *nudum*, *Diplostauron angulosum*, *Dysmorphococcus pseudovariabilis*, *Euglena chlamydophora*, *Geminella planctonica*, *Leptolyngbya fontana*, *Navicula laevissima* var. *perhibita*, *Pyramimonas fasciata* (Fig. 5), *Radiococcus wildemannii* (Fig. 5), *Radiosphaera minuta*, *Scherffelia bichlora*, *Sphaerellopsis ignava*, *Sporotetras pyriformis*, *Stichococcus minutissimus*, *Stokesiella acuminata*, *Thorakochloris nygaardii*, *Trachelomonas hispida* f. *minor*, *Trachelomonas lomnickii* (Fig. 5), *Tribonema taeniatum*.

Microcrustaceans

Twelve cladoceran species and five copepod species were identified in Lake Iezerul Ighiel in 2014 and 2016 (Fig. 6). With 38% from the microcrustacean community in terms of average relative abundances from all available data, *Cyclops vicinus* (Ulianine 1875) represented the most abundant taxa, followed by *Acanthodiaptomus denticornis* Kiefer 1932 (31%) and *Daphnia* sp. (12% for *D. galeata* and *D. rosea*, considered as one group). For copepods, adults and immature stages were considered together. On the other hand, the cladocerans *Alonella nana (Baird 1843)* and *Bosmina longirostris (O. F. Muller 1776),* species with lower relative abundances, recorded high frequency values (exceeding 67% from the total number of available samples).

Only copepods were found in Lake Dracului in spring 2016. Out of the 5 species identified in this lake, *Eudiaptomus zachariasi* (Poppe, 1886) recorded the highest relative abundance (62%), followed by *Cyclops strenuus* Fischer, 1851 (32%). Ochiul Beiului had no true plankton community, since only 2 cyclopoid copepodites were found in spring 2016 (Fig. 6).

Microcrustaceans are usually ubiquitous (Błędzki and Rybak, 2016), and similar taxa can be found in water bodies located in different karstic regions. In fact, several common taxa were found in similar studies conducted in a limestone lake from Poland: *B. longirostris, D. galeata, C. vicinus* (Ślusarczyk, 2003) or in an alpine karst lake from Austria: *Chydorus sphaericus, D. rosea, A. denticornis, Eucyclops serrulatus* (Jersabek and Schabetsberger, 1996). A few common taxa were also found in gypsum karstic lakes from the Balkans: *B. longirostris, C. vicinus* in 3 Croatian lakes (Stanković *et al*., 2011) and *Alona* sp*., B. longirostris, Ceriodaphnia pulchella* in a Greek monomictic lake (Chalkia *et al*., 2012). Moreover, very different habitats were also characterized by some common taxa, like *B. longirostris, Ceriodaphnia* sp*, Daphnia* sp. in an ephemeral karst lake from USA (Kelley *et al*., 2000) or *B. longirostris, C. strenuus* in a pseudokarstic lake formed in the depositions of a retreating glacier in North Italy (Obertegger *et al*., 2010).

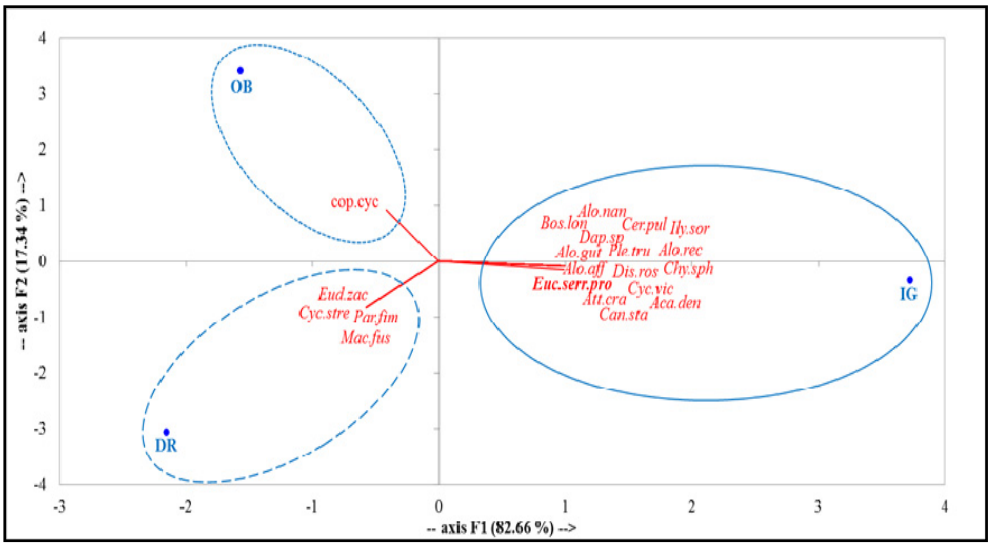


Figure 6. Principal Component Analysis (PCA) biplot (axes F1 and F2: 100 %) showing microcrustacean taxa found in Lakes Iezerul Ighiel, Dracului and Ochiul Beiului; average abundances considered from all available data; abbreviations as in Table 1; cladocerans: *Alo.aff – Alona affinis; Alo.gut – Alona guttata; Alo.rec – Alona rectangula; Alo.nan – Alonella nana; Bos.lon – Bosmina longirostris; Cer.pul – Ceriodaphnia pulchella; Chy.sph – Chydorus sphaericus; Dap.sp. – Daphnia galeata* and *D. rosea; Dis.ros – Disparalona rostrata; Ily.sor – Ilyocryptus sordidus; Ple.tru – Pleuroxus truncatus*; copepods: *Aca.den – Acanthodiaptomus denticornis; Att.cra – Attheyella crassa; Can.sta – Canthocamptus staphylinus; Cyc.stre – Cyclops strenuus; Cyc.vic – Cyclops vicinus; Euc.serr.pro - Eucyclops serrulatus proximus; Eud.zac – Eudiaptomus zachariasi; Mac.fus – Macrocyclops fuscus; Par.fim – Paracyclops fimbriatus*; cop.cyc – cyclopoid copepodites.

Diversity and assessment of ecological status of the lakes

The Shannon-Wiener index and the Equitability were calculated to assess diversity of planktonic algal and microcrustacean communities. Phytoplankton diversity was impossible to calculate in Lake Dracului and Ochiul Beiului, due to the low number of taxa. Similarly, microcrustacean diversity was null in Ochiul Beiului, since only 2 immature copepod individuals were found in the water column. The values of diversity indices were calculated from the average abundances found in each season for algae and microcrustaceans; all present taxa were considered, even if they were not included in counts.

Significant differences were recorded in species richness for the three karstic lakes, with values as different as 148 algal taxa (11 microcrustacean species) in Lake Iezerul Ighiel compared to only 49 (5 microcrustaceans) in Lake Dracului and 49 (0

microcrustaceans) in Ochiul Beiului in spring 2016. Significant positive correlation was found between the number of algal taxa and lake area (Pearson r = 0.999; p = 0.004). These findings are consistent with the species-area hypothesis formulated by MacArthur and Wilson (1967), stating that species richness results from a balance of immigration and extinction, larger areas having higher number of species.

Higher species richness could imply higher Shannon-Wiener diversity, as shown in Fig. 6 for microcrustaceans. Overall, Shannon-Wiener values were comparable to those reported in the literature from similar limestone lakes, not exceeding 1.5 (Stanković *et al.*, 2011). Direct relationships between number of taxa and lake area or lake depth were also previously found in the literature, for several karstic lakes from Spain (Armengol and Miracle, 1999).

Moreover, different diversity values were recorded for different seasons. For phytoplankton for example, summer communities had the higher diversity value, due to frequent water blooms caused by diatoms in spring and autumn that lowered the diversity score (Fig. 7). Similar results were found in the literature (Steward and Wetzel, 1986). For microcrustaceans, higher diversity was recorded in autumn, a typical situation, recorded in the literature as well (Armengol and Miracle, 1999). However, the equitability was generally low (<0.6), for algae and microcrustaceans alike, showing that only few species could thrive in these karstic environments.

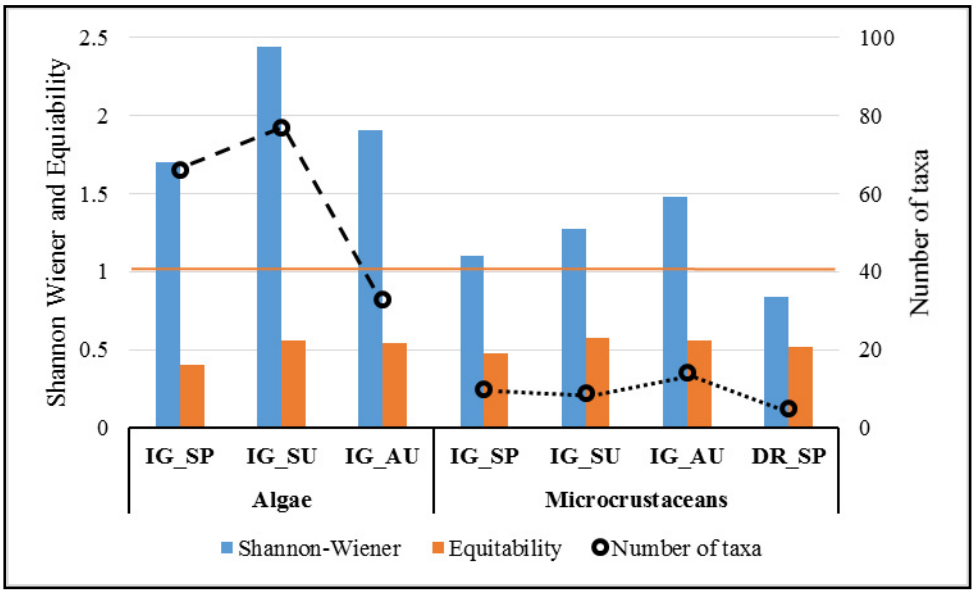


Figure 7. The diversity of the plankton communities in Lakes Iezerul Ighiel and Dracului; abbreviations as in Table 1; dotted lines: number of taxa; solid line: maximum equitability value.

Algal and microcrustacean communities used in assessing the ecological status of the karstic lakes considered for the present study gave coherent results in case of water saprobity, organic pollution and trophicity (Fig. 8). The correspondence analysis considered sums of algal and microcrustacean taxa that indicated different saprobity and trophicity levels; for Ochiul Beiului only algae were used.

Eutrophic conditions were depicted by the highest number of indicator taxa in all three karstic lakes (Fig. 8). All phytoplanktonic indices calculated for Lake Iezerul Ighiel showed eutrophic conditions: Nygaard's compound index (Willén, 2000) of 4.04 and Stockner diatom index (Stockner, 1972) of 2.14. From the total number of phytoplanktonic taxa, 18 recorded the Q indicator value (Järnefelt, 1951) higher than 1, indicating eutrophy. Trophic phytoplankton indices according to Oltean (1977) showed an on-going eutrophication process in Lake Iezerul Ighiel, since a clear transition was recorded from alpha-eutrophic conditions in spring 2014 to gamma in summer 2015 and 2016. These findings are consistent with previous results based on algae (Momeu *et al.,* 2015), or solely on total phosphorus, indicating oligotrophic conditions in 2010 (Mihăiescu *et al.,* 2012). Possible explanations could be the on the one hand the continuous tributary inputs into the lake and on the other hand the anthropic influences like camping near the lake shore, fishing and swimming.

Numerous periphyton, and not phytoplankton taxa, indicated eutrophic conditions in all karstic water bodies, probably due to the accumulation of nutrients in lake sediments. This is why most algal taxa depicted eutrophic conditions in Lake Dracului and Ochiul Beiului. But, according to Oltean (1977) phytoplankton communities had low species richness and abundances, indicating oligotrophic conditions, as did Stockner diatom indices (Stockner, 1972), which did not exceed one.

Indicator taxa, algae and microcrustaceans alike, showed relatively clean waters with respect to saprobity (oligosaprobic to β-mesosaprobic conditions) in Lake Iezerul Ighiel (Fig. 8). In terms of organic pollution, 14 out of the 20 indicator algal genera, included in the Palmer genus index (Palmer, 1969) were identified in this lake: *Ankistrodesmus, Chlamydomonas, Closterium, Cyclotella, Euglena, Gomphonema, Melosira, Navicula, Nitzschia, Oscillatoria, Pandorina, Phacus, Phormidium and Scenedesmus*. Similarly, 10 indicator taxa for organic pollution, from a total of 20, were found: *Ankistrodesmus falcatus, Cyclotella meneghiniana, Gomphonema parvulum, Navicula cryptocephala, Nitzschia acicularis, Nitzschia palea, Oscillatoria limosa, Oscillatoria tenuis, Pandorina morum* and *Scenedesmus quadricauda*. The values of the organic pollution index, both at genus and at species levels (28 and 34, respectively) indicated slightly high organic pollution in the water.

The high number of species indicating β-mesosaprobic conditions in Lake Dracului and Ochiul Beiului can be explained by the input of organic materials in form of leaves, twigs or logs from the forest surrounding the lakes.

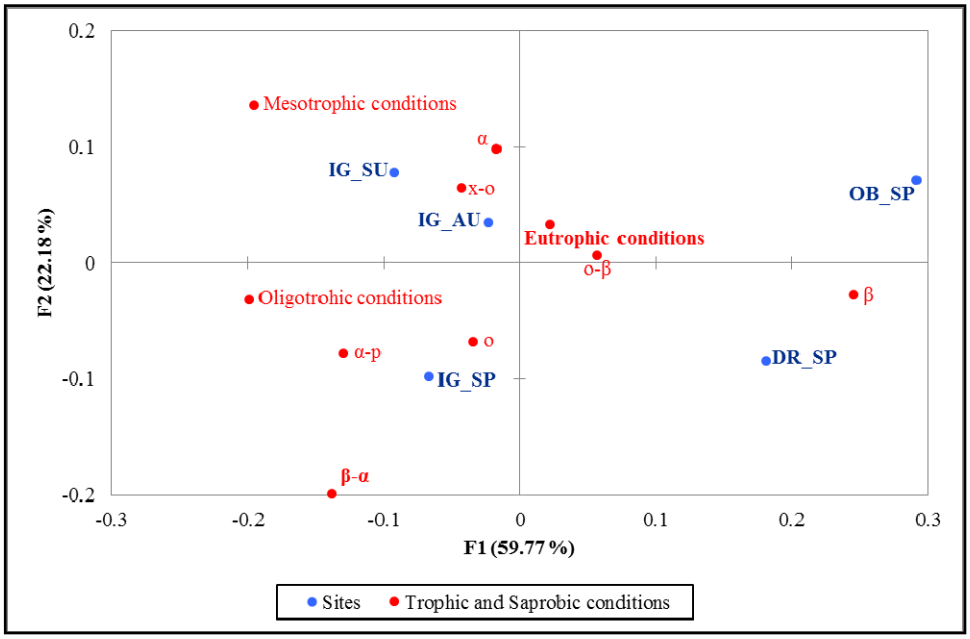


Figure 8. Correspondence Analysis (CA) plot (axes F1 and F2: 81.95 %) showing the aggregation of sampling sites with the number of algal taxa and microcrustacean species with indicator value; abbreviations as in Table 1; saprobic conditions indicated by taxa: x: xenosaprobic; o: oligosaprobic; β: β - mesosaprobic; α: α - mesosaprobic; p: polysaprobic; intermediate classes also depicted.

Similarity of aquatic communities between the three karstic lakes

Does similar karstic substratum sustain similar assemblages of algae and crustaceans? This was the initial question of the study. For freshwater microcrustaceans for example, since they consist of many ubiquitous species, capable to adapt to different habitats in various geographic locations (Błędzki and Rybak, 2016), similar conditions in limestone karstic lakes might mean similar communities. However, very different microcrustacean assemblages were identified in the three karstic lakes (Fig. 6). The similarity based on Dice index recorded just 10%, due to the one common copepod species, *Eucyclops serrulatus proximus* (Lilljeborg 1901), while the Bray-Curtis index revealed no similarity at all. This was also the case for algae, where Jaccard and Bray-Curtis indices revealed a low similarity of only 10 % between the algal community from Lake Iezerul Ighiel and the communities from the other two lakes: Lake Dracului and Ochiul Beiului, which sustain more similar assemblages (30-40%), due to the 23 common algal taxa.

These results are consistent with previous literature: Jenkins and Buikema (1998) analyzed the colonization of 12 similar experimental ponds, which sustained different zooplankton communities in terms of structure (species richness, density and biomass), but no clear differences were detected in case of community function (production, respiration, nutrient regeneration rates).

In accordance with the analyses made by McCune and Allen (1985) in coniferous forests, two types of factors influencing the qualitative and quantitative structure of aquatic communities that develop in similar sites were identified: (1) **current factors**, that characterize the site at present in terms of geo-morphology, climate, hydrology, physical-chemical properties etc.; and (2) **long-term factors**, that characterize the history of the site, long- or short-lasting processes with influences on the present status of the community (Fig. 9).

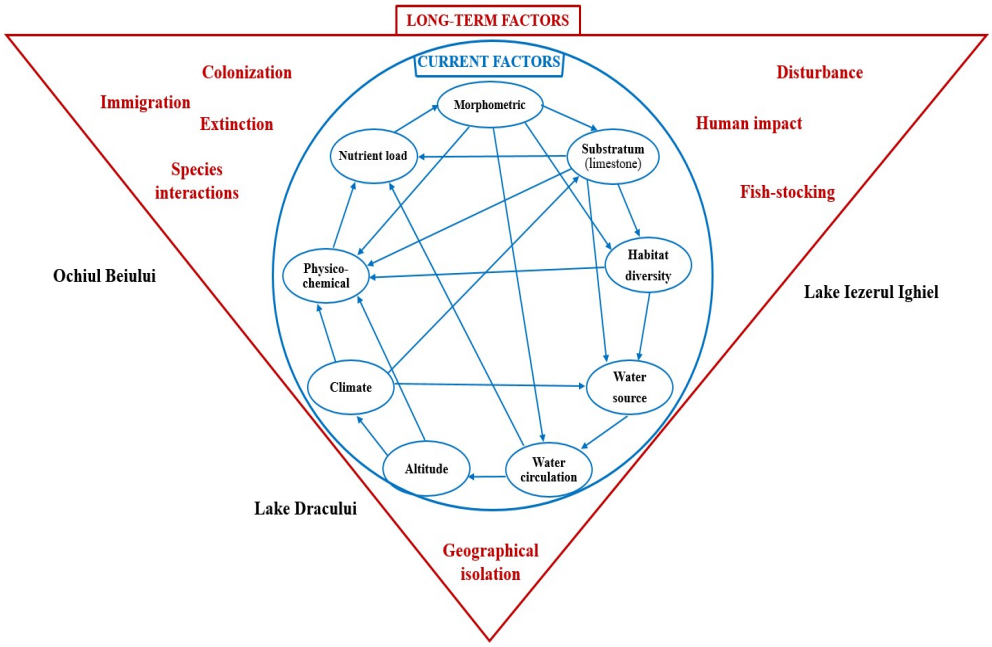


Figure 9. Schematic view of current vs. long-term factors inflencing the assemblages from the three karstic lakes considered

Most current factors characterize similar environments in the three karstic lakes considered for the present study: similar limestone substratum, high conductivity, dissolved oxygen or transparency values, relatively high habitat diversity including pelagic and littoral regions (rocks, sand, vascular and non-vascular plants, dead organic matter), water feeding from springs or surface run-off, water circulation,

shading etc. There are however important differences in morphometry: lake area and volume are much higher for Lake Iezerul Ighiel. On the other hand, all long-term factors separate the three lakes. Geographical isolation that in turn influences the colonization ability and immigration/extinction rates is much higher for Lake Dracului and Ochiul Beiului, where no surface water inputs were described. The disturbances are higher for Lake Iezerul Ighiel, in form of constant tourism pressure: camping, bathing, fishing and fish-stocking. From this point of view, the higher diversity recorded here can be linked to the intermediate disturbance characteristic to the region (consistent with the intermediate disturbance hypothesis, described by Connell, 1978). Species interactions like competition and predation could also explain the disimilarities from the three karstic lakes.

Conclusions

Three karstic lakes were analysed considering the physico-chemical parameters, algal and microcrustacean communities during 2014 and 2016. Even if similar karstic conditions were recorded in Lakes Iezerul Ighiel, Dracului and Ochiul Beiului, dissimilar communities were recorded, for algae and microcrustaceans alike, in terms of species richness, average abundances and diversity.

Thus, even if most abiotic parameters like limestone substratum or physico-chemical values (the current factors) unify the three karstic environments, long-term parameters like geographical isolation and disturbance divide them, Lake Iezerul Ighiel having highly different assemplages of algae and microcrustaceans compared to Lake Dracului and Ochiul Beiului.

Acknowledgements. Authors are grateful to R.N.P. Romsilva and to the administration of the National Park Cheile Nerei-Beuşniţa R.A. for allowing access to the lakes and to Orsolya Tünde László and Miruna Dosa for microcrustacean counts.

REFERENCES

Armengol, X., Miracle, M. R. (1999) Zooplankton communities in doline lakes and pools, in relation to some bathymetric parameters and physical and chemical variables, J. *Plankton Res.*, **21**(12):2245-2261

Błędzki, L. A., Rybak, J. I. (2016) *Freshwater Crustacean Zooplankton of Europe: Cladocera & Copepoda (Calanoida, Cyclopoida) Key to species identification, with notes on ecology, distribution, methods and introduction to data analysis*, Springer, pp. 917

Cărăuş, I. (2017) Algae of Romania: a distributional checklist of actual algae, Version 2.4, *Stud. Cerc. Biol., Bacău*, **7**:1-1002

Chalkia, E., Zacharias, I., Thomatou, A. A., Kehayias, G. (2012) Zooplankton dynamics in a gypsum karst lake and interrelation with the abiotic environment, *Biologia*, **67**(1):151-163

Connell, J. H. (1978) Diversity in tropical rain forests and coral reefs, *Science*, **199**:1302-1310

Damian-Georgescu, A. (1963) *Crustacea, Copepoda, Fam. Cyclopidae (forme de apa dulce), Fauna Republicii Populare Române*, IV(6), Ed. Acad. R.P.R., București, pp. 205

Damian-Georgescu, A. (1966) *Crustacea, Copepoda, Calanoida (forme de apă dulce). Fauna Republicii Socialiste România*, IV(8), Ed. Acad. R.S.R., București, pp. 130

Damian-Georgescu, A. (1970) *Crustacea, Copepoda, Harpacticoida (forme de apă dulce). Fauna Republicii Socialiste România*, IV(11), Ed. Acad. R.S.R., București, pp. 248

Decei, P. (1981) *Lacuri de munte. Drumeție și pescuit*, Ed. Sport-turism, București

Duma, S. (2009) The impact of mining activity upon the aquatic environment in the southern Apuseni Mountains, *Romanian Review of Regional Studies*, **V**(1):51-66

Einsle, U. (1993) *Crustacea, Copepoda, Calanoida und Cyclopoida, Susswasserfauna von Mitteleuropa*, 8/4-1, Gustav Fischer Verlag, Germany, pp. 208

Ettl, H. (1983) Chlorophyta 1. Phytomonadina, In: *Süßwasserflora von Mitteleuropa*, Ettl, H., Gerloff, J., Heynig, H., Mollenhauer, D. (eds.), 9, Gustav Fischer Verlag, Stuttgart, pp. 1–807

Haliuc, A., Vereș, D., Brauer, A., Hubay, K., Hutchinson, S. M., Begy, R., Braun, M. (2017) Palaeohydrological changes during the mid and late Holocene in the Carpathian area, central-eastern Europe, *Global Planet. Change*, **152**:99–114

Hennebert, M., Lees, A. (1991) Environmental gradients in carbonate sediments and rocks detected by correspondence analysis: examples from the Recent of Norway and the Dinantian of southwest England, *Sedimentology*, **38**:623-642

Hindak, F. (ed.) (1978) Sladkovodné Riasy, Ed. Slovenské Pedagogické nakladatel, Bratislava, pp. 728

Janetzky, W., Enderle, R., Noodt, W. (1996) *Crustacea: Copepoda: Gelyelloida und Harpacticoida, Sussvasserfauna von Mitteleuropa*, 8/4-2, Gustav Fischer Verlag, Stuttgart, pp. 227

Järnefelt, H. (1951) Beobachtungen über die Vertikalverteilung des Planktons, *Verh. Int. Ver. Limnol.*, **11**:213-218

Jenkins, D. G., Buikema, A. L. (1998) Do similar communities develop in similar sites? A test with zooplankton structure and function, *Ecol. Monogr*., **68**(3):421-443

Jersabek, C. D., Schabetsberger, R. (1996) Limnological aspects of an alpine karst lake with extreme changes in water level, *Limnologica*, **26**(1):1-13

Jolliffe, I. T. (1986) *Principal Component Analysis*. *series in statistics*, Springer-Verlag

Kelley, R., Jack, J., Fant, M. (2000) A survey of the physiochemical characteristics and the zooplankton communities in an ephemeral karst lake. *Aquat. Ecol*., **34**(1):77-89

Kobayashi, T., Shiel, R. J., King, A. J., Miskiewicz, A. G. (2009) Freshwater zooplankton: diversity and biology, In: *Plankton: a guide to their ecology and monitoring for water quality,* Suthers I. M., Rissik, D. (eds.), CSIRO Publishing, Collingwood Australia, pp. 157-179

Krammer, K., Lange-Bertalot, H. (1986) *Bacillariophyceae. 1. Teil: Naviculaceae. Süsswasserflora von Mitteleuropa*, 2/1, Spektrum Akademischer Verlag, Heidelberg, pp. 876

Krammer, K., Lange-Bertalot, H. (1988) *Bacillariophyceae. 2. Teil: Bacillariaceae, Epithemiaceae, Surirellaceae. Süsswasserflora von Mitteleuropa,* 2/2, Spektrum Akademischer Verlag, Heidelberg, pp. 611

Krammer, K., Lange-Bertalot, H. (1991) *Bacillariophyceae. 3. Teil: Centrales, Fragilariaceae, Eunotiaceae. Süsswasserflora von Mitteleuropa*, 2/3, Spektrum Akademischer Verlag, Heidelberg, pp. 599

Litchman, E., Klausmeier, C. A. (2008) Trait-based community ecology of phytoplankton, *Annu. Rev. Ecol. Evol. S*., **39**:615-639

Löffler, H. (2004) The origin of lake basins, In: *The Lakes Handbook, volume 1, Limnology and limnetic ecology*, O'Sullivan, P. E., Reynolds, C.S. (eds.), Blackwell Publishing, 8-60

Wilson, E. O., MacArthur, R. H. (1967) *The theory of island biogeography*, Princeton, NJ.

McCune, B., Allen T. F. H. (1985) Will similar forests develop on similar sites? *Can. J. Botany,* **63**:367-376

Mihăiescu, R., Pop, A. I., Mihăiescu, T., Muntean, E., Beldean, S., Muntean, N., Alhafez, L., Ozunu A. (2012) Physico–chemical characteristics of the karst lake Ighiu (Romania), *Environmental Engineering and Management Journal*, **11**(3):623-626

Momeu L., Ciorca, A., László, O. T., Segedi, C., Battes, K.P., Cîmpean M., (2015) The karstic lake Iezerul Ighiel (Transylvania, Romania): its first limnological study, *Studia UBB Biologia*, **60**(2): 39-60

Negrea, Ş. (1983*) Cladocera. Fauna Republicii Socialiste România,* IV(12), Ed. Acad. R.S.R., Bucureşti, pp. 399

Nygaard, G. (1949) *Hydrobiological studies in some ponds and lakes, Part II - The quotient hypothesis and some new or little known phytoplankton organisms*. Kongel. Danske. Videns. Selskab. Biol. Skrifter, Bd. 7 number 1, pp. 293

Obertegger, U., Borsato, A., Flaim, G. (2010) Rotifer–crustacean interactions in a pseudokarstic lake: influence of hydrology, *Aquat. Ecol*., **44**(1):121-130

Oltean, M. (1977) În legătură cu aprecierea gradului de troficitate al apelor stagnante pe baza structurii fitoplanctonului, *Hydrobiol*., **15**:97-102

Pan, Y., Wang, L., Cao, Y., Pang, W., Wang, Q., Zhu, Z., Zhang, X., Deng, G. (2016) Variation of benthic algal assemblages among habitats in subalpine Karstic Lakes and implications for bioassessment, *Hydrobiol.*, **777**(1):183-196

Palmer, C. M. (1969) A composite rating of algae tolerating organic pollution, *J. Phycol*., **5**: 78-82

Pişota, I., Trufaş, V. (1971) *Hidrologia R. S. România*, *Vol. 2: Lacurile României*, Centrul de multiplicare al Universităţii din Bucureşti, Bucureşti

Pop, G., Măhăra, G. (1965) Lacul Ighiu, Studiu fizico-geografic, *Natura Seria Geogr.Geol*., **4**: 40-44

Rott, E., Hofmann, P. G., Pall, K., Pfister, P., Pipp, E. (1997) *Indikationslisten für Aufwuchsalgen in Österreichischen Fliessgewässern,* Teil 1: *Saprobielle Indikation*, Bundesministerium für Land- und Forstwirtschaft, Wien, pp. 248

Sládeček, V. (1973) System of water quality from the biological point of view, *Arch. Hydrobiol. Ergebn. Limnol*., **7**:1-218

Ślusarczyk, A. (2003) Limnological study of a lake formed in limestone quarry (Kraków, Poland). I. Zooplankton community, *Pol. J. Environ. Stud.*, **12**(4):489-494

Stanković, I., Ternjej, I., Mihaljević, Z., Furač, L., Kerovec, M. (2011) Crustacean plankton community (Crustacea: Copepoda and Cladocera) in gypsum karst lakes and their relation to abiotic parameters, *Hydrobiol*., **666**(1):145-153

Steward, A. J., Wetzel, R. G. (1986) Cryptophytes and other microflagellates as couplers in planktonic community dynamics–Arch, *Hydrobiol*, **106**:1-19

Stockner, J. (1972) Paleolimnology as a means of assessing trophication, Verhandlungen der internationale, *Vereinigung fur theoretische und angewandte Limnologie*, **18**: 1018-1030

Udovič, M. G., Cvetkoska, A., Žutinić, P., Bosak, S., Stanković, I., Špoljarić, I., Mršić, G., Borojević, K. K., Ćukurin, A., Plenković-Moraj, A. (2017) Defining centric diatoms of mo st relevant phytoplankton functional groups in deep karst lakes, *Hydrobiol.*, **788**(1):169-191

Van Dam, H., Mertens, A., Sinkeldam, J. (1994) A coded checklist and ecological indicator values of freshwater diatoms from the Netherlands, *Aquat. Ecol.*, **28**(1):117-133

Washington, H. G. (1984) Diversity, biotic and similarity indices - A review with special relevance to aquatic ecosystems, *Water Res*., **18**(6): 653-694

Willén, E. (2000) Phytoplankton in water quality assessment–an indicator concept, In: *Hydrological and limnological aspects of lake monitoring*, John Wiley & Sons, 57, pp. 80

Wojtal, A. Z., Sobczyk, Ł. (2012) The influence of substrates and physicochemical factors on the composition of diatom assemblages in karst springs and their applicability in water-quality assessment, *Hydrob.*, **695**(1): 97-108

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXII, 1, 2017 (p. 87-100)
doi:10.24193/subbb.2017.1.06

=== REVIEW ===

Heart rate variability: a practical review for the beginner

Ștefan-Claudiu Mirescu[1,2,✉], Andreea Loredana Ciocoiu[1], Leontin David[1] and Corneliu Tarba[1]

SUMMARY. Heart rate variability (HRV) refers to beat-to-beat interval changes on the electrocardiogram (ECG) and is considered a noninvasive measure of the autonomic balance. Although classical ECG is a well-known and standardized clinical method, HRV developed abruptly due to the evolution of computerized data acquisition systems in cardiology (digital electrocardiographs) and their software programs. HRV can be evaluated by visual methods (the beat-to-beat tachogram, the histogram and the Poincaré diagram) or by statistically computed parameters. Its applications include sympathovagal balance evaluation, monitoring of different neuropathies and contribution to survival prediction after cardiac acute events. Studies state that HRV can also be used as a method for rapid screening of some autonomic and cardiac diseases, along with other diagnostic procedures.

Keywords: autonomic nervous system, electrocardiogram, heart rate variability, sympathovagal balance.

Introduction

Heart rate variability (HRV) is a simple and noninvasive method that describes oscillations in the intervals between consecutive heart beats (Camm *et al*., 1996; Gardim *et al.*, 2014; Karim *et al*., 2006). On a standard electrocardiogram (ECG) trace, the maximum uphill deflection of a normal QRS complex is at the peak of the

[1] *Babeș-Bolyai University, Faculty of Biology and Geology, Department of Molecular Biology and Biotechnology, 5-7 Clinicilor Str., Cluj-Napoca, Romania*
[2] *Regional Institute of Gastroenterology and Hepatology „Octavian Fodor", Cluj-Napoca, Romania*
✉***Corresponding author: Ștefan-Claudiu Mirescu**, Babeș-Bolyai University, Faculty of Biology and Geology, Department of Molecular Biology and Biotechnology, 5-7 Clinicilor Str., Cluj-Napoca*
E-mail: claudiu.mirescu@ubbcluj.ro

R-wave. The interval between two consecutive R waves is called the R-R interval (Karim *et al*., 2006). Using computerized methods, R-R intervals can be extracted from a short-term (minimum 5 minutes) or long-term (24 hours or more) ECG trace and HRV parameters can be calculated.

On a more in-depth perspective, HRV is the variation of the period between consecutive heartbeats (Fig. 1), basically influenced by time and entirely dependent on the extrinsic regulation of the heart rate (HR), measuring the balance between the actions of the sympathetic mediators (epinephrine and norepinephrine) and the parasympathetic one (acetylcholine) released by nerve fibers, on both sinus and atrioventricular nodes, which leads to an increase or a decrease, respectively, in the heart rate as well as a secondary effect on the atrioventricular conduction (Karim *et al*., 2006).

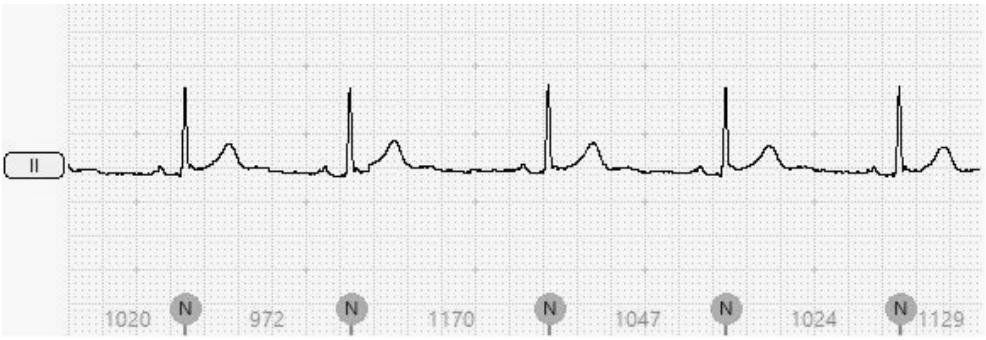


Figure 1. An ECG signal (DII lead) with R-R intervals in milliseconds. The letter "N" underneath every R wave designates that the corresponding beat is considered normal (it is not an ectopic beat)

Therefore, it can be said that HRV is a marker of the cardiovascular autonomic function (Camm *et al.*, 1996) and a powerful indicator of the relationship between psychological and physiological processes, also being an empirical and theoretical support for the emergence of HRV as an important marker for regulated emotional responding (Appelhans and Luecken, 2006).

As from an historical point of view, the Ancient Greeks were the first ones to acknowledge the importance of measuring HR, but it was not fully understood until 1733, when Rev. Stephen Hales managed to establish a connection between the variation of the pulse and the respiration, considering them directly proportional. Also, with the first record of the respiratory sinus arrhythmia (RSA) made by Carl Ludwig in 1847 and the measurement of the ECG (1855), followed by the apparition of the digital processing techniques, the HRV analysis has become more relevant, mainly, in the diagnosis of coronary heart diseases (Billman, 2011).

Recording of HRV

Basically, any method that records beat-to-beat intervals is suitable for HRV. Two classical methods for recording heart signals can be used for HRV purposes: photopletismography (PPG) and electrocardiography (ECG), each with its own conveniences and drawbacks.

PPG is a noninvasive optical technique used for measuring changes in blood circulation, mainly at skin level (Pilt *et al*., 2013). The PPG sensor consists of a light emitting diode (LED), which emits red or infrared radiation, and a photodetector (PD). The LED and the PD are placed on opposite sides of a finger (Pilt, 2013; Mirescu, 2015). The absorbance of the light emitted by the LED is proportional to the blood flow in the tissue between the LED and the PD (Singh, 2013). Thus, the amplitude of the signal captured by the PD is proportional to the volume of the blood that passes through the tissue, reciprocating the alternation between cardiac systole and diastole. Therefore, the PPG signal is composed of cyclic inflections and deflections, bearing a systolic peak that can be identified for HRV purposes (Fig. 2) (Elgendi *et al*., 2011; Mirescu and Harden, 2012; Elgendi, 2012).

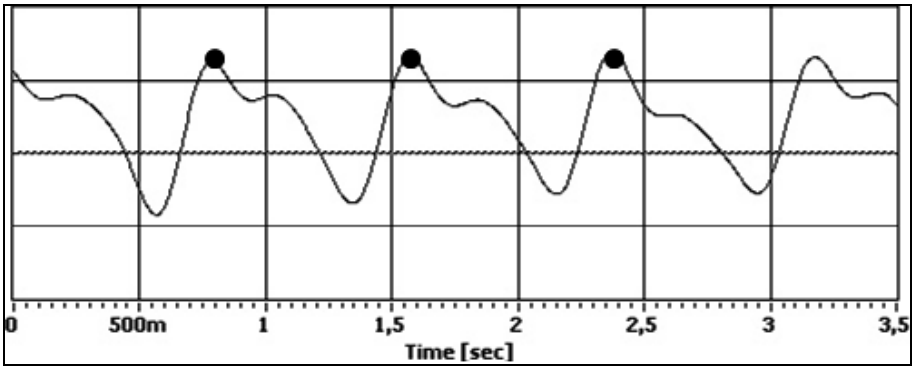


Figure 2. A finger PPG signal. The black dots mark the systolic peaks; the intervals between these peaks are further used for variability analysis

The circuit for a simple PPG device is easy to design: apart from the two optical diodes, it requires two voltage dividers (one for the LED and one for the PG), a voltage source (a low power battery) and an analog-to-digital converter, which can be a computer soundcard (Mirescu, 2015) or a microcontroller-based prototyping board. The signal is processed in order to extract beat-to-beat intervals used for HRV analysis.

This method is comfortable for the subject and convenient for the examiner. The only interface between the device and the subject is a clip on the finger. However, a significant disadvantage of PPG is the high sensitivity to artifacts, especially movement artifacts (Mirescu, 2014).

On the other hand, the ECG is a much more reliable method in detecting beat-to-beat intervals used for variability analysis, despite the more complex experimental setting and instruments. For ECG trace recording, a digital computer-connected electrocardiograph is needed, *e.g.* Neurosoft Poly-Spectrum-8®. This device functions as a classical 12-lead ECG device, but as a result of its software capabilities, it can be also used for extracting R-R intervals.

Because measurements of HRV require the detection of each heartbeat (Acharya, 2006), the software used must meet this requirement.

HRV parameters and plots are calculated from the R-R intervals. This can be accomplished by the ECG acquisition software or by a different one. One of the most used software equipment for this purpose is Kubios HRV Analysis, released by the Department of Physics from University of Kuopio, Finland. It is a very intuitive and easy to use freeware application program which requires a text file containing R-R intervals as input. The intervals can be measured in seconds or milliseconds.

Kubios HRV analysis is a powerful instrument which calculates time-domain parameters, frequency-domain parameters and nonlinear parameters, and also plots the tachogram, the histogram, the power-spectrum density and the Poincaré diagram. It also comes with a comprehensive instruction manual (Fig. 3) (Tarvainen, 2006).

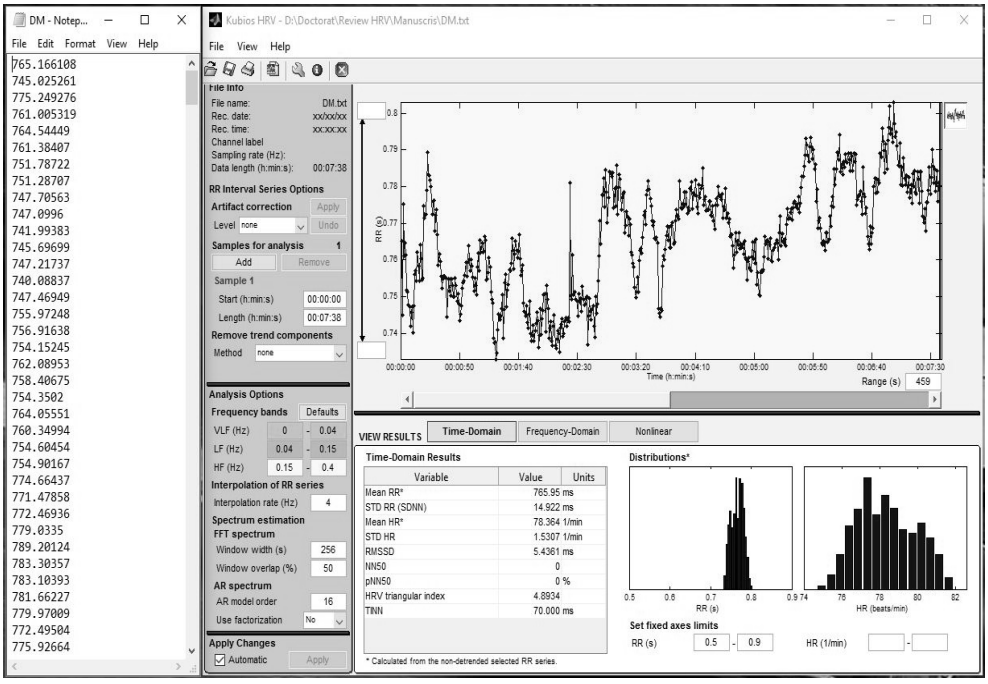


Figure 3. Left – A text file containing the R-R intervals in milliseconds; right – the main interface of *Kubios HRV Analysis*

The software supports beat-to-beat intervals and offers the users the possibility of converting the results within a HRV recording into a PDF report which can be used to have a more detailed examination. In addition, Kubios HRV allows the users to save the recording results as an ASCII file text, which can be opened by MS Excel, or some other file extensions, such as Matlab or MAT-file for ulterior editing (Tarvainen *et al*., 2006).

HRV visual descriptors

One of the fastest and most accessible approaches to HRV interpretations consists of the HRV visual descriptors. Usually, they include (1) the tachogram of R-R intervals; (2) the histogram of R-R intervals; (3) the Poincaré plot.

The tachogram of R-R intervals is an oscillatory curve produced when R-R intervals are plotted on a time scale (von Borell, 2007)(Carvajal, 2005). In a similar manner, momentary heart rates derived from R-R intervals can be plotted on a time scale, with the same scientific value. The tachogram of R-R intervals is a powerful visual aid in HRV interpretation, because it allows fast comparison between two subjects (usually a control subject and a patient with a certain disease). For example, Fig. 4 illustrates the difference between the tachogram of a normal healthy subject and a patient with advanced type II diabetes mellitus, both plotted on similar scales. It can be seen that the normal tachogram has frequent inflections and deflections, corresponding to a substantial HRV, while the diabetic tachogram is almost flat; hence, the diabetic patient has a lower HRV compared to the healthy subject. Historically, the tachogram is the first developed method for HRV analysis, not necessarily requiring a digital acquisition method for the ECG signal. Its simplicity still makes it one of the most reliable visual methods for HRV analysis.

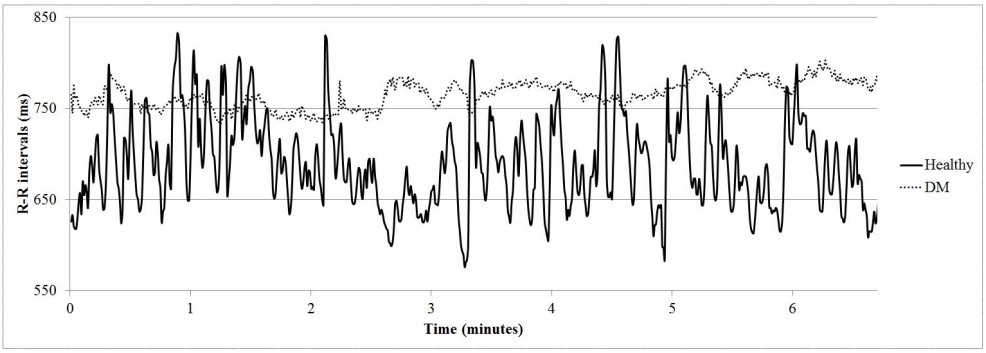


Figure 4. A comparison between a tachogram derived from a healthy subject (continous line) and one derived from a diabetic patient (dotted line).

The histogram of the R-R intervals is a statistically derived HRV plot. Essentially, the histogram is a bar graph representation of the number of beats contained in a certain interval (Acharya, 2006; Cam *et al.*, 1996; von Borell, 2007). As for interpretation, a high HRV is translated into a larger distribution of the R-R intervals on the histogram, describing a normal (Gaussian) distribution (Fig. 5).

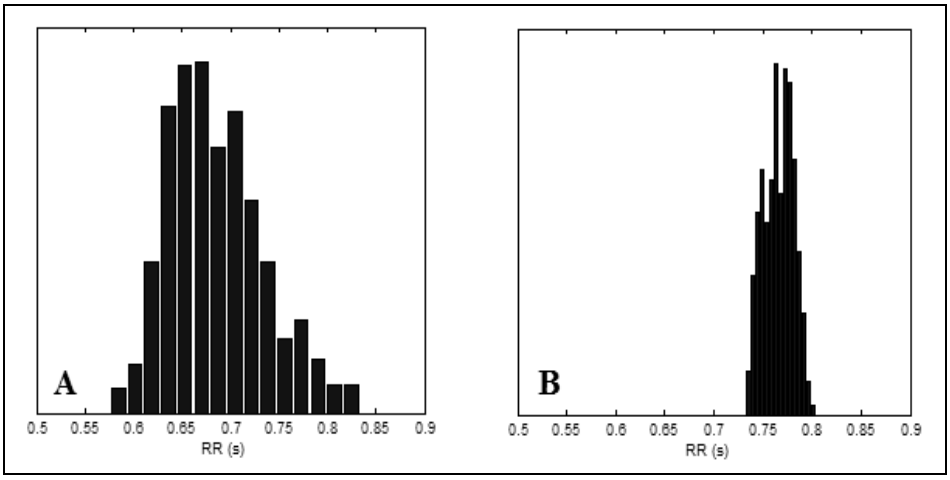


Figure 5. Histogram of R-R intervals in a healthy subject (A) vs. histogram of R-R intervals in a diabetic patient (B).

The Poincaré plot is considered the most powerful visual descriptor of HRV, being a quantitative-visual method extracted from non-linear dynamics. A Poincaré plot is a scattergram representing every R-R_{i+1} interval as a function of RR_i. The result is a comet-shaped scattergram (Fig. 6). An increased variability is depicted by a large area of the distribution of the points, and vice versa.

Despite its value as a visual descriptor, the indexes extracted from it have been proven not to provide additional information to that obtained by other methods (Milagro, 2016; Brennan, 2001). It is also one of the few HRV methods that have been tested in clinical settings, allowing the detection of patterns resulting from non-linear processes, otherwise not observable (Corbi, 2013).

The Poincaré plot is also useful in real-time detection and classification of cardiac ectopic beats (Thalange, 2010).

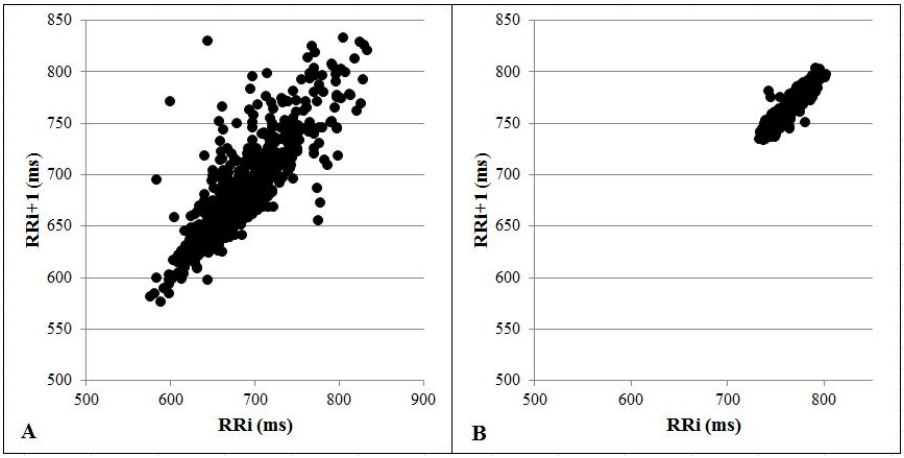


Figure 6. The Poincaré plot of a healthy subject (A) vs. the Poincaré plot of a diabetic patient (B).

HRV parameters

Although helpful, the visual descriptors of HRV are not enough to characterize the behavior of the variability of cardiac rhythm. Complex mathematical and statistical models were developed in order to extract HRV parameters from R-R intervals arrays. These parameters fall into three categories: time-domain parameters, frequency-domain parameters and nonlinear dynamics parameters. The most commonly used parameters in HRV studies and their significance are summarized in Table I.

Time-domain parameters are the simplest to quantify and are based on the instantaneous heart rate (Camm *et al., 1996)*. They include statistical parameters like the mean R-R interval, the standard deviation of the R-R intervals, the mean heart rate and the standard deviation of the mean heart rates (Camm *et al.*, 1996; Karim *et al*., 2006; Tsai *et al*., 2014; Gardim *et al.*, 2014).

Another independent descriptor of HRV is NN50, which represents the number of consecutive R-R intervals that differ by more than 50 milliseconds. A derivative of NN50 is pNN50, which is the percent NN50 from the total number of intervals. out of the total R-R intervals (Camm *et al.*, 1996; Karim *et al*., 2006; Medeiros, 2010; Henry *et al*., 2010).

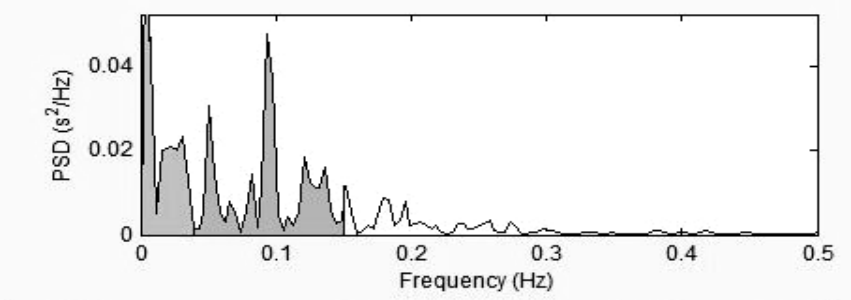
Baseline width of the minimum square difference triangular interpolation of the highest peak of the histogram of all R-R intervals is the TINN Index (ms), which is also an independent marker of HRV (von Borell, 2007; Acharya, 2006).

Frequency-domain methods are spectral methods for the analysis of HRV and imply the use of complex mathematical algorithms (the fast-Fourier transform – FFT). Power spectral density analysis provides information on how power distributes as a function of frequency (Camm *et al.*, 1996).

Power spectral density analysis usually separates the cyclic modifications of HRV into three components, whose interpretation is controversial (ChuDuc *et al*., 2013):

- High frequency (HF) component, from 0.15 Hz to 0.04 Hz;
- Low frequency (LF) component, from 0.04 Hz to 0.15 Hz;
- Very low frequency (VLF), from 0.003 Hz to 0.04 Hz (Fig. 7).

Until recently, most authors accepted that HF reflects the activity of the parasympathetic nervous system (vagal tonus) and LF is a reflection of the sympathetic activity (ChuDuc *et al*., 2013; Jaiswal *et al*., 2013). However, there are studies that contradict this hypothesis, revealing that LF/HF ratio does not accurately measure cardiac sympathovagal balance (Billman, 2013).



Frequency Band	Peak (Hz)	Power (ms^2)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0039	909	36.7	
LF (0.04-0.15 Hz)	0.0938	1137	45.9	72.6
HF (0.15-0.4 Hz)	0.1523	428	17.3	27.4
Total		2475		
LF/HF		2.654		

Figure 7. Power-spectrum density of HRV components according to the mentioned frequency intervals

For accurate frequency-domain measurements, certain recommendations must be taken into account. According to most authors, it is suggested that the duration of the recording should be at least two-times the wavelength of the lowest frequency component (Aubert *et al*., 2003). Thus, the minimum duration for the assessment of

the HF component (0.15 Hz) should be at least 13.3 seconds and for the LF – at least 50 seconds. However, many authors that focused their research on short-term HRV recommend a minimum duration of 5 minutes (Aubert *et al*., 2003; Paritala, 2009; Nunan *et al*., 2010).

Nonlinear dynamics methods of HRV analysis mainly refer to the Poincaré plot parameters and to entropy parameters. They have proven their prognostic value in clinical setting, but their physiological origin is not very well established (Camm *et al*., 1996; Weippert, 2014).

The standard descriptors of the Poincaré plot are SD1, which represents the fast R-R variability, and SD2, which describes the longer-term variability. Studies showed that SD1 reflects mainly parasympathetic activity, while SD2 reflects both sympathetic and parasympathetic contributions to the heart rate (Fig. 8) (Makivić, 2013; Melillo, 2011).

Among the entropy parameters, Sample Entropy (SampEn) is the most popular, due to its high correlation with traditional HRV indices (Weippert, 2014). SampEn quantifies the probability that sequences of patterns in a data set that are initially closely related remain close in the next incremental comparison, within a specified tolerance (Henry, 2010).

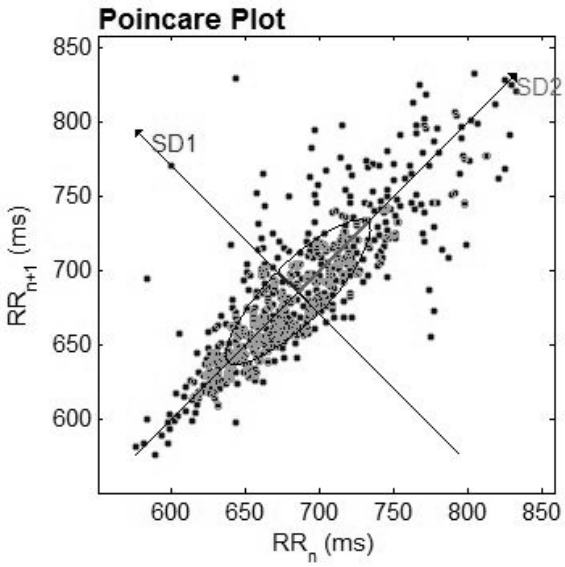


Figure 8. A typical Poincaré plot with the short and long axis standard deviations (SD1 and SD2, respectively)

Clinical significance of HRV measurements

Many studies have attested the role of HRV measurements in health and disease. Most of them have been performed on conditions that influence the autonomic balance. A significant number of studies assessed HRV in different autonomic nervous system (ANS) dysfunctions.

HRV and myocardial infarction. One of the first applications of HRV in clinical practice was the use of HRV parameters as risk predictor after acute myocardial infarction (MI). In MI survivor patients, an increase in the sympathetic tone has been demonstrated, which is transient and ceases after a few weeks. Given the fact that the increase in the sympathetic tonus (thus, a decreased HRV) predisposes to malignant ventricular arrhythmias, it has been concluded that a prolonged decreased HRV is an independent risk factor for death after MI (Stys *et al*., 1998; Niakan *et al*., 1986; Balcıoğlu *et al.*, 2015).

HRV and diabetes. Many studies associated global decreased HRV with diabetes mellitus (DM) and its complications. In a study performed on DM type I young patients, Jaiswal *et al*. (2013) discovered that SDRR, an independent descriptor of HRV, was 10 ms lower among patients, compared to non-DM subjects ($p = 0.003$) (Jaiswal *et al.*, 2013). Similar results were obtained by Gardim *et al*. (2014) on DM type I children (Gardim *et al.*, 2014). According to many authors, decreased HRV in diabetic patients (both type I and type II) is not only associated with poor prognosis, but also precedes autonomic neuropathy (Camm *et al.*, 1996). In another study, Orlov *et al*. (2012) concluded that HRV is a measure of both small and large autonomic nerve fibers functionality and, therefore decreased HRV is present in both early and late phenotypes of diabetic neuropathy (Orlov *et al*., 2012).

Table 1.

Commonly used HRV parameters (Camm *et al.,*, 1996; von Borell, 2007)

Parameter	Unit	Significance
Time-domain parameters		
Mean RR	ms	Average of consecutive beats intervals
STD RR	ms	Standard deviation of consecutive beats intervals
Mean HR	beats/min	Average heart rate
SD HR	beats/min	Standard deviation of heart rate averages
NN50	#	Number of consecutive heartbeats with a difference of at least 50 ms
pNN50	%	Percent of consecutive heartbeats with a difference of at least 50 ms
TINN	ms	The baseline of the intervals histogram

Parameter	Unit	Significance
Frequency-domain parameters		
LF/HF	-	Ratio between low frequency spectral power and high frequency spectral power
Nonlinear dynamics parameters		
SD1	ms	Standard deviations according to the Poincaré plot of the intervals between heartbeats
SD2	ms	
ApEn	-	Approximate entropy of the stationary signal
SampEn	-	The likelihood that runs of patterns that are close to each other will remain close in the next incremental comparisons
Correlation dimension	-	Entropy parameters
DFA α1	-	
DFA α2	-	

HRV and depression. Many clinical trials associated depression with imbalances of the ANS, which are quantified by HRV measurements. Depression is correlated with an increase in the sympathetic tone, which may lead to other cardiac diseases (ChuDuc *et al.*, 2013). These findings were as well correlated to depression in childhood (Sharma *et al.*, 2011). Changes in the respiratory sinus arrhythmia, also measurable by HRV, are likewise linked to depressive disorders (Sinatra *et al.*, 2011; Bylsma *et al*., 2014; Assad *et al*., 2012).

Cardiac arrhythmias, although not a direct expression of the autonomic nervous system activity, can be investigated by HRV, especially by nonlinear dynamics methods (entropy methods and the Poincaré diagram) (Qu and Weiss, 2006; Thalange and Mergu, 2010).

Most ectopic beats can be noticed on the Poincaré diagram. Mainly any deviation from the cloud appearance of the points distribution (regardless of the area of the cloud) reflects ectopic beats (atrial or ventricular premature contractions). Atrial fibrillation appears as a multi-cloud Poincaré plot. Special care has to be taken to subjects showing a prominent respiratory sinus arrhythmia, which is characterized by an asymmetrical appearance of the Poincaré plot cloud (Brennan *et al*., 2001). However, when analyzing time-domain and frequency-domain parameters, ectopic beats have to be eliminated, in order to obtain relevant values of the parameters (Mirescu and Harden, 2012; Mirescu, 2015).

Conclusions

HRV proved to be a valuable instrument in clinical studies, as well as for research purposes. As a result of the rapid development of computerized ECG acquisition systems and software, HRV brings about an accessible and easy-to-use set of parameters in evaluating the autonomous nervous system balance. Albeit many studies have been performed, the intimate mechanisms of HRV origin are not completely understood and some applications are yet to be investigated.

REFERENCES

Appelhans, M., Luecken, L. (2006) Heart Rate Variability as an Index of Regulated Emotional Responding, *Rev. Gen. Psychol.* **10**(3):229–40

Assad, S, Ding, F., Fu, N, Xu Y. (2012) *Correlating Heart Rate Variability with Mental Fatigue*, Worchester Polytechnic Institute, Massachusetts

Aubert, A., Seps, B., Beckers, F. (2003) Heart Rate Variability in Athletes *Sports Medicine* **33**(12):889–919

Balcıoğlu, A., Müderrisoğlu, H. (2015) Diabetes and Cardiac Autonomic Neuropathy: Clinical Manifestations, Cardiovascular Consequences, Diagnosis and Treatment, *World J. Diabetes* **6**(1):80–91

Billman, G. (2011) Heart rate variability - a historical perspective, *Front. Physiol*. doi: 10.3389/fphys.2011.00086

Billman, G. (2013) The LF/HF Ratio Does Not Accurately Measure Cardiac Sympatho-Vagal Balance, *Front. Physiol.*, **4**:1–5

Brennan, M., Palaniswami, M., Kamen, P. (2001). Do Existing Measures of Poincaré Plot Geometry Reflect Nonlinear Features of Heart Rate Variability?, *IEEE Rev Biomed Eng*., **48**(11):1342–47

Bylsma, L., Salomon, K., Taylor-Clift, A., Morris, B., Rottenberg, J. (2014) Respiratory Sinus Arrhythmia Reactivity in Current and Remitted Major Depressive Disorder *Psychosom. Med.* **76**(1):66–73

Carvajal, R., Wessel, N., Vallverdú, M., Caminal, P., Voss., A. (2005) Correlation Dimension Analysis of Heart Rate Variability in Patients with Dilated Cardiomyopathy, *Comput. Methods Programs Biomed.* **78**(2):133–40

Chevalier, G., Sinatra, S. (2011) Emotional Stress, Heart Rate Variability, Grounding, and Improved Autonomic Tone: Clinical Applications, *Integr. Med. Clin. J.,* **10**(3):16–22

ChuDuc, H., NguyenPhan, K., NguyenViet, D. (2013) A Review of Heart Rate Variability and Its Applications, *APCBEE Procedia* **7**:80–85

Corbi, G., D'Addio, G., Russo, G., Rengo., G., Pinna, G., Maestri, R., Rengo, F., Ferrara, N. (2013) A Non-Linear Analysis of Heart Rate Variability in Stroke Patients, *Mathematics and Computerns in Contemporary Science,* 117–22

Elgendi, M., Jonkman, M., De Boer, F. (2010) Heart Rate Variability Measurement Using the Second Derivative Photoplethysmogram, Proceedings of the Third International Conference on Bio-Inspired Systems and Signal Processing

Elgendi, M., Jonkman, M., DeBoer, F. (2011) Heart Rate Variability and the Acceleration Plethysmogram Signals Measured at Rest *CCIS* **127**:266–77

Gardim, C., de Oliveira, B., Bernardo A, Gomes, R., Pacagnelli, F., Lorençoni, R., Vanderlei, L. (2014) Heart Rate Variability in Children with Type 1 Diabetes Mellitus, *Revista Paulista de Pediatria* **32**(2):279–85

Henry, B., Minassian, A., Paulus, M., Geyer, M., Perry, W. (2010) Heart Rate Variability in Bipolar Mania and Schizophrenia, *J. Psychiatr. Res.* **44**(3):168–76

Jaiswal, M., Urbina, E. M., Wadwa, R. P., Talton, J. W., D'Agostino, R. B., Hamman, R. F., Fingerlin, T. E., Daniels, S., Marcovina, S. M., Dolan, L. M., Dabelea, D. (2013) Reduced Heart Rate Variability among Youth with Type 1 Diabetes: The SEARCH CVD Study, *Diabetes Care* **36**(1):157–62

Karim, N., Hasan, J., Ali S. (2016) Heart Rate Variability – A Review, *Medi. Biol. Eng. Comput.* **44**(12):1031–51

Makivić, B., Nikić, D., Willis, S. (2013) Heart Rate Variability (HRV) as a Tool for Diagnostic and Monitoring Performance in Sport and Physical Activities, *J. Exerc. Physiol. Online* **16**(3):103–31

Mandeep, S., Nagpal, S. (2013) Features Extraction in Second Derivative of Finger PPG Signal: A Review, *IJCSC* **4**(2):1–5

Camm, A. J., Malik, M., Bigger, J. T., Breithardt, G., Cerutti, S., Cohen, R. J., Coumel, P., Fallen, E. L., Kennedy, H. L., Kleiger, R. E., Lombardi, F. (1996) Heart rate variability. Standards of measurement, physiological interpretation, and clinical use. *Eur. Heart J.*, **17**(3):354-381

Medeiros, J. (2010) Development of a Heart Rate Variability Analysis Tool, Plux, Engenharia de Biosensores

Melillo, P., Bracale, M., Pecchia, L. (2011) Nonlinear Heart Rate Variability Features for Real-Life Stress Detection. Case Study: Students under Stress due to University Examination, *Biomed. Eng. Online* **10**(1):96

Milagro, F. (2016) Poincaré Plot Analysis And Graphical User Interface Development For The Study Of Heart, Master of Science Thesis, Faculty of Computing and Electrical Engineering

Mirescu, C. (2015) Computer Sound Card Used as Analog-to-Digital Converter in a Teaching Physiology Laboratory, *Studia UBB Biologia*, **60**(2):85-88

Mirescu, C., Harden, S. (2012) Photopletismography as a Potential Alternative to Electrocardiography for Recording Heart Rate Intervals Used in Variability Analysis, *J. Med. Life* **5**(Special Issue):123-128

Mirescu, C., Harden, S. (2012) Nonlinear Dynamics Methods for Assessing Heart Rate Variability in Patients With Recent Myocardial Infarction, *Romanian J. Biophys.* **22**(2):117–24

Mirescu, C., Petrescu, M., Petrescu, F., Mirescu, N., David, L. (2014) Challenges in implementing heart rate variability testing in a family medicine practice: strengths, pitfalls and caveats, *Studia UBB Biologia*, **59**(2):105-113

Niakan, E., Harati, Y., Comstock, J. (1986) Diabetic Autonomic Neuropathy, *Metabolism* **35**(3):224–234

Nunan, D., Sandercock, G., Brodie., A. (2010) A Quantitative Systematic Review of Normal Values for Short-Term Heart Rate Variability in Healthy Adults *PACE - Pacing and Clinical Electrophysiology* **33**(11):1407–17

Orlov, S., Bril, V., Orszag, A., Perkins, B. (2012) Heart Rate Variability and Sensorimotor Polyneuropathy in Type 1 Diabetes, *Diabetes Care* **35**(4):809–16

Paritala, S. (2009) *Effects of Physical and Mental Tasks on Heart Rate Variability*,Thesis for Master of Science, Kakatiya University, India

Pilt, K., Ferenets, R., Meigas, K., Lindberg, L., Temitski, K., Viigimaa, M. (2013) New Photoplethysmographic Signal Analysis Algorithm for Arterial Stiffness Estimation, *Scientific World J.* 2013:1–10

Qu, Z., Weiss, J. (2006) Dynamics and Cardiac Arrhythmias, *J. Cardiovasc. Electrophysiol.* **17**(9):1042–49

Sharma, R., Balhara, Y., Sagar, R., Deepak, K., Mehta, M. (2011) Heart Rate Variability Study of Childhood Anxiety Disorders, *J. Cardiovasc. Dis. Res.* **2**(2):115–22

Stys, A., Stus, T. (1998) Current Clinical Applications of Heart Rate Variability, *Clin. Cardiol.* **21**:719–24

Tarvainen, M., Niskanen, J. (2006) *Kubios HRV Analysis, version 2.0 beta, User's Guide*, University of Kuopio, Finland

Thalange, A., Mergu, R. (2010) HRV Analysis of Arrhythmias Using Linear - Nonlinear Parameters, *Int. J. Comput. Appl.* **1**(12):75–80

Tsai, J., WenChun, C., Jou, S., Lin, C. (2014) Heart Rate Variability and Meditation with Breath Suspension, *Biomed. Res. India* **25**(1):6–10

Von Borell, E., Langbein, J., Després, G., Hansen, S., Leterrier, C., Marchant-Forde, J., Marchant-Forde, R., Minero, M., Mohr, E., Prunier, A., Valance, D., Veissier, I. (2007) Heart Rate Variability as a Measure of Autonomic Regulation of Cardiac Activity for Assessing Stress and Welfare in Farm Animals - A Review, *Physiol. Behav.* **92**(3):293–316

Weippert, M., Behrens, M., Rieger, A., Behrens, K. (2014) Sample Entropy and Traditional Measures of Heart Rate Dynamics Reveal Different Modes of Cardiovascular Control during Low Intensity Exercise, *Entropy* **16**(11):5698–5711

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXII, 1, 2017 (p. 101-114)
doi:10.24193/subbb.2017.1.07

=== REVIEW ===

Sources and mechanisms of combined heavy-metal and antibiotic resistance traits in bacteria

Claudia Țugui[1], Edina Szekeres[1,2] and Andreea Baricz[2,✉]

SUMMARY: Nowadays, antibiotic resistance poses a great threat to the health of the individuals worldwide. In this context, scientific interest on how bacteria adapt in stress-related environmental conditions like those enriched in heavy metals and how the heavy-metal adaptive mechanism influence the antibiotic resistance is increasing. It was noted that the simultaneous use of heavy metals and antibiotics in agriculture and aquaculture might positively impact the dissemination of the antibiotic resistance genes in the environment. Current knowledge on the sources of simultaneous pollution with heavy metals and antibiotics, the co-occurrence of heavy-metal and antibiotic resistance traits in bacteria altogether with physiological mechanism underlying this phenomenon are overviewed.

Keywords: agriculture, antibiotic resistance, aquaculture, heavy metal resistance.

Introduction

Besides the wide use in the clinical field to treat a large variety of diseases caused by bacterial infections, antibiotics are also applied as growth promoters in agriculture and aquaculture. At the same time, high concentrations of heavy metals in soils and waters of agricultural interest might originate either from anthropogenic

[1] *Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania.*
[2] *National Institute of Research and Development for Biological Sciences (NIRDBS), Institute of Biological Research, Cluj-Napoca, Romania.*
✉ ***Corresponding author: Andreea Baricz*** *National Institute of Research and Development for Biological Sciences (NIRDBS), Institute of Biological Research, Cluj-Napoca, Romania.*
E-mail: andreea.baricz@icbcluj.ro

activities (such as mining, smelting, waste disposal, vehicle exhaust, sewage sludge, pesticides, fertilizers application) (Binggan and Linsheng, 2010) or natural causes (Tchounwou *et al.*, 2012). Co-existence of heavy metals and antibiotics occurs in various environments such as human and mammalian guts, livestock and animal manure, poultry and fish farms (aquaculture) etc. (Fig. 1) (Heuer *et al*., 2009; Chen *et al.*, 2015).

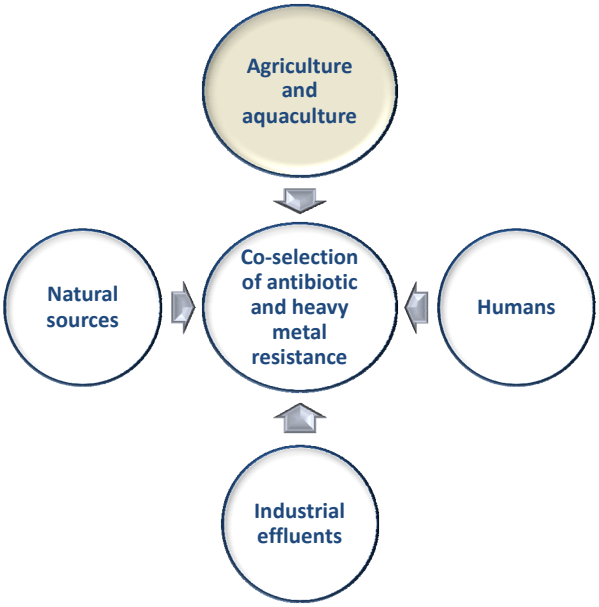


Figure 1. The sources of combined antibiotic and heavy metal contamination in natural environments. The agriculture and aquaculture (emphasized in gray background) are most frequent sources for simultaneous antibiotic and metal pollution.

Besides their various toxic effects on human health (Tchounwou *et al.*, 2012), presence of elevated levels of heavy metals (mostly divalent Cu^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+} but also zerovalent Hg^{0} and trivalent/hexavalent $Cr^{3+/6+}$) in the environment was shown to stimulate the enrichment of indigenous organisms (mainly *Bacteria*) which bear antibiotic resistance genes (Baker-Austin *et al.*, 2006). Stressful conditions created by the co-existence of heavy metals and antibiotics triggers the onset of adaptive mechanisms that include, for example, extracellular immobilization or efflux pumps helping the affected microbial cells to entrap or expel toxic compounds. Additionally, manifold increase of antibiotic resistance genes was correlated with the high concentration of the heavy metals in the environment (Chen *et al.*, 2015).

Understanding the mechanisms underlying co-selection of heavy-metal and antibiotic resistance capabilities in microorganisms of medical and environmental relevance is crucial for understanding the patterns of their dispersal and persistence in various natural or antropic environments (Resende *et al.*, 2012). Moreover, such knowledge would allow i) designing of strategies to monitor and/or prevent spreading of toxic antibiotics and heavy metals; ii) bioprospecting of microbes to aid in xenobiotics and metal bioremediation.

The present review discusses recent advances on the links between heavy metal and antibiotic resistance and the bacterial mechanisms underlying the simultaneous adaptation to both types of pollutants.

The use of heavy metals in agriculture and aquaculture

Heavy metals are extensively and widely used in agriculture due to their capacity to improve the efficiency of animal growth, quality of the meat production and the fitness of the animals providing a healthier trait (Zhang *et al.*, 2012). Copper (Cu^{2+}) used as copper sulfate ($CuSO_4$) is well known as biocidal compound with bactericidal and fungicidal effects. Therefore, it is used in foot-bath in milking yards to treat digital dermatitis that can lead to lameness, unless untreated, in dairy cattle (Thomsen *et al.*, 2015). Copper sulfate is also used in piggery and poultry units as growth promoter (Zhang *et al.*, 2012; Yazdankhah *et al.*, 2014). Furthermpre, it is used on laying hen for lowering the cholesterol content in eggs (Karimi *et al.*, 2011). Zinc (Zn^{2+}) used as zinc white (ZnO) is added in pig feed (24 - 33 mg/ kg) diet for preventing and curing parakeratosis and stimulate growth (Hill *et al.*, 1986; Forrest, 2012). For poultry units zinc acts like a growth promoting factor influencing skeletal and feather development and also the reproduction system (Forrest, 2012). It was recognized that feeding 20-35 mg Zn/kg will meet the daily diet requirement of the chicks (Underwood and Suttle, 1999). Arsenic (As^{3+}), although well known for its toxicity, is used in animal feed to help in gaining weight thus enhancing the feed efficiency (Zhang *et al.*, 2012). Arsenic derivatives are also used as medicine against external parasites and protozoa (Banu *et al*., 1982). Arsenic confers pigmentation of the animals improving their aspect (Li and Chen*,* 2005; Jackson *et al.*, 2003). Although cadmium ($Cd^{2=}$) is not added in animal feed, it is spread as residuals in agriculture mineral supplements (e.g., in phosphates, zinc sulfate, zinc oxide etc.) because of close chemical similarity with Zn^{2+} (Plum *et al.*, 2010).

The rapid growth of population in the last century resulted in sharp increase in the demand of seafood products (fish and edible invertebrates). Intensive seafood farming (or *aquaculture*) uses formulated feeds containing antibiotics, antifungals and other pharmaceuticals in addition to application of pesticides and disinfectants. Moreover, human and animal faeces are often used as source of food in aquaculture (Sapkota *et al.*, 2008). As a consequence of their addition in the mineral supplements to stimulate growth, heavy metals such as Zn, Cu, Fe, Mn, Pb, Cd, etc. accumulate in tissues (liver, muscle, shell) of farmed animals (Wu and Yang, 2011).

Pollution and accumulation of heavy metals in the marine environment

The main sources of coastal heavy metal pollution may include ship dismantling (Hossain and Islam, 2006), mining, industrial and urban discharge through polluted rivers (Morillo *et al.*, 2004; Gao and Chen, 2012). Heavy metals tend to enrich in the coastal and deep sea sediments, subsequently entering the food chain through bioaccumulation and biomagnification, and thus affecting the marine ecosystems (Gao and Chen, 2012; Kacar and Kocyigit, 2013). The presence of heavy metals in wild seafood (shrimps, shellfish and finfish) raises great health concern for the consumers. Even the fishes in cage aquaculture contain high levels of heavy metals. When exposed for 28 days with daily feed of 0.01 mg Cu/L or 0.016 mg Pb/L, carp (*Cyprinus carpio*) accumulate up to 21.53 mg Cu/kg and 7 mg Pb/kg, respectively (Salami *et al.*, 2008).

Microbial diversity of heavy metal contaminated aquatic sediments

Diversity of microbial communities associated with (usually mixed) heavy metal (and other pollutants)-contaminated marine sediments was explored revealing that most frequently retrieved *Bacteria* belonged to Proteobacteria (γ-, δ- and ε-Proteobacteria), Firmicutes and Bacteroidetes phyla (Gillan *et al.*, 2005; Zhang *et al.*, 2008; Quero *et al.*, 2015), whereas archaeal communities were dominated by Methanobacteria and Methanomicrobia (Quero *et al.*, 2015). Moreover, increase in the diversity and abundance of genes involved in heavy metal tolerance were reported in metal-contaminated aquatic (riverine) sediments by GeoChip 5.0 functional gene array (Jie *et al.*, 2016). Among all heavy metals, cadmium was found to be most toxic, whereas lead was generally best tolerated in representatives of bacterial communities investigated from metal-contaminated samples tested for metal susceptibility (Table 1).

Table 1.

Toxic metal susceptibility of bacterial representatives of microbial communities associated with sediments contaminated with heavy metals

Source	Metal sensibility ranking	Reference
Golden Horn Estuary	Cu>Mn>Ni>Zn>Pb>Cd>Fe	Altug and Balkis, 2009
Iskenderun Bay	Cd>Cu>Cr>Pb>Mn	Matyar *et al.*, 2008
Eastern Aegean Sea	Hg>Cd>Cr>Zn>Cu>Co>Ni>As>Pb	Kacar and Kocyigit, 2013
The Eastern Harbour, Egypt	Cd>Co>Zn>Ni>Hg>As>Cu>Pb	Sabry *et al.*, 1996
Equatorial Indian Ocean	Cd>Zn>Cu>Pb	Devika *et al.*, 2013

Antibiotics and their use in agriculture and aquaculture

Antibiotics have been largely used in agriculture and aquaculture for decades. They were seen as very effective especially by protecting livestocks from pathogens and, thus, usually administered as preventive measure (Jassim and Limoges, 2014). Classes of antimicrobials like tetracyclines, macrolides, beta-lactams, streptogramins, and sulfonamides may be used at different times during the life cycle of poultry, cattle, and swine (Sarmah *et al.*, 2006). For example, by 2012, 16% of all lactating dairy cows in the U.S. received antibiotic therapy for clinical mastitis each year, but nearly all dairy cows received intramammary infusions of prophylactic doses of antibiotics following each lactation to prevent and control mastitis, mostly with penicillins, cephalosporins, or other beta-lactam drugs. Also, beef calves that enter feedlots were treated with antibiotics for clinical respiratory disease. Moreover, therapeutic antibiotic doses have been administered to healthy calves to prevent outbreaks. To ensure growth, calves are fed a nourishment supplemented with tylosin, a macrolide drug, to prevent liver abscesses. Growing swine were commonly treated with tetracyclines or tylosin for growth promotion and disease prevention (Landers *et al.*, 2012).

Large amounts of antibiotics were used in fish feed for prophylactic and therapeutic purposes before being replaced by vaccines (Seiler and Berendonk, 2012). Oxytetracycline and chloramphenicol were two of the most used antibiotics in aquaculture (Sapkota *et al.*, 2008). Extensive use of multiple antibiotics in aquaculture led to the rise of cross-resistance to a certain type of antibiotic and to multi-drug resistance. For example, in farmed shrimp, infections with vibrios multiresistant to penicillin, tetracycline, and ampicillin were reported. In addition, the most common cross-resistance was observed in penicillin-cephalothin relation (Costa *et al.*, 2015).

Factors that drive the accumulation and spread of antibiotic and heavy-metal resistant bacteria

Both heavy metal- and antibiotic-resistant bacterial strains are disseminated into the environment through animal manure (Chen *et al.*, 2015). Natural fertilizers from farms used for agricultural purposes may contribute to the enrichment of the genes responsible of antibiotic resistance (or 'resistome') in soil (Forsberg *et al.*, 2012) and also to the dissemination of resistance genes in the environment. The *resistome* was defined as the entire pool of antibiotic determinants and their precursors in a microbial community (Wright, 2007). Humans also play an important role in the dissemination of the antibiotic resistance genes by consuming antibiotics to treat bacterial infections. The extensive use of antibiotics by humans led to an increased resistance in the human-associated bacterial resistome. Moreover, hospitals contribute to

the uncontrolled dispersal of antibiotics by discharging insufficiently treated wastewater into rivers or lakes (Matyar *et al.*, 2010; Amador *et al.*, 2015, Țugui *et al.*, 2015; Szekeres *et al.*, 2017). As a consequence, the emergence of bacteria with multiple antibiotic resistance and increased virulence became a global issue (Walker *et al.*, 2009; Guidos, 2011).

By co-existing in the same environment, heavy metals can influence the resistance to antibiotics (Chen *et al*, 2015) and are able to co-select for different types of antibiotic resistance, thus influencing survival of certain bacteria in a more polluted environment. For instance it has been noticed that resistance to Zn/Cd co-occurs with aminoglycoside/macrolide resistance. Also copper in the form of $CuSO_4$ is related to higher ampicillin resistance in soil samples (Berg *et al*., 2005). It has been stated that metals like copper, zinc, antimony, cobalt, nickel, cadmium, iron, and mercury are all co-selectors for strain resistance. Antibiotics like tetracycline, chloramphenicol, ampicillin, and gentamicin have a strong relation with various heavy metal resistance (Table 2). On the other hand, three types of resistance genes for arsenic, copper and silver cluster separately from antibiotic resistance genes on plasmids making it possible to select among each other. This fact suggests that these MRGs are less likely to select for antibiotics (Pal *et al*., 2015).

Mechanisms of metal tolerance and its influence on the antibiotic resistance

The proliferation of antibiotic resistance can be determined by heavy metal contamination of the environment , as there are clear indications on the coupling of resistance mechanisms against antibiotics and heavy metals. The co-selection mechanisms include *cross-resistance* and *co-resistance*.

Cross-resistance occurs on mobile elements (plasmids, transposons) and inquires genes encoding for generic detoxifying mechanism like efflux pumps which non-specifically reduce intracellular concentrations of both metals and antibiotics. On the other hand, **co-resistance** involves separate genes which are integrated on the same genetic element (Knapp *et al.*, 2011).

Microbial strategies to overcome toxic levels of heavy metals

Through evolution, bacteria has developed different adaptive strategies to tolerate elevated metal levels. Generally, three mechanism of heavy metal resistance are known in prokaryotes (*Bacteria* and *Archaea*) (Silver, 1996; Nies, 2003; Voica *et al.*, 2016):

- passive extracellular or intracellular ***sequestration*** of toxic metals either by extracellular polymeric substances or intracellular compounds such as polyphosphates;

- ***detoxification*** through chemical neutralization of intracellular ions by enzymes (for example by the activity of mercury reductase encoded by *merA* gene, Seiler and Berendok, 2012);
- active ***extrusion*** of toxic ions by efflux systems (or 'efflux pumps'). An example in this case is Czc which is a cation/proton antiporter mediating resistance to divalent metal ions like Cd^{2+}, Zn^{2+}, and Co^{2+} by expelling metals from the cytoplasm through the inner and outer membrane to the surrounding environment (Silver, 1996; Nies, 2003; Baker-Austin *et al.*, 2006; Seiler and Berendok, 2012).

Table 2.

Co-occurence of heavy metals and antibiotic resistance in various bacterial strains

Heavy metal	**Antibiotic categories /Generic names**	**Source**
Cu	tetracycline	soil bacteria Scotland(a), Fecal *Enterococci* (b)
	carbapenem	*Pseudomonas aeruginosa* (c)
	vancomycin	*Enterococcus faecium* (d)
	chloramphenicol	bacteria isolated from ship *(*e)
	erythromycin	soil bacteria Scotland (a), *Enterococcus faecium* (d)
Hg	sulfonamide	*Salmonella enterica* (f)*,* Fecal Gram-negative bacteria (g)
	chloramphenicol	*Salmonella enterica* (f)*,* Fecal Gram-negative bacteria(g)
	ampicillin	soil bacteria Kenya (h), *Salmonella enterica* (f), Fecal Gram-negative bacteria (g)
	streptomycin	*Salmonella enterica* (f), Fecal Gram-negative bacteria (g)
	augmentin	soil bacteria Kenya (h)
Zn	norfloxacin	soil bacteria Kenya(h)
	augmentin	soil bacteria Kenya (h)
	gentamicin	soil bacteria Kenya h)
	ampicillin	soil bacteria Kenya (h)
	carbapenem	*Pseudomonas aeruginosa* (c)
Cr	tetracycline	soil bacteria Scotland (a)
	carbapenem	soil bacteria Scotland (a)
Fe	tetracycline	soil bacteria Scotland (a)
	ciprofloxacin	*Escherichia coli* (i)
Ni	tetracycline	soil bacteria Scotland (a)
Cd	augmentin	soil bacteria Kenya (h)
	ampicillin	soil bacteria Kenya (h), bacteria isolated from ship (e)

(a)-Knapp *et al*., 2011
(b)-Amachawadi *et al.*, 2013
(c)-Li *et al.,* 2012
(d)-Wales *et al*., 2015
(e)-Kacar and Kocyigit, 2013
(f)-Baker-Austin *et al*., 2006
(g)-Wireman *et al*., 1997
(h)-Budambula and Kinyua, 2013
(i)-Mehi *et al*., 2014

Main mechanisms of antibiotic resistance in bacteria

Antibiotics may be combated by bacteria and archaea through three mechanisms (Kohanski *et al.*, 2010; D'Costa *et al.*, 2011; Wright and Poinar, 2012):

- by changing the target of the antibiotic compound as in Gram-positive and some Gram-negative bacteria, where antibiotics like amynoglicosides usually interact with the 30S ribosomal subunit blocking the protein synthesis and affecting the translation of mRNA (Tsai *et al*., 2013);
- by inactivating the antibiotics using enzymes; for example AmpC beta – lactamases (a hydrolase) conferring resistance for different classes of beta-lactams;
- by active export of antibiotics through the efflux *m*ulti*d*rug-*r*esistance (MDR) pumps (i.e. MexAB, AcrAB and TolC are among the most thoroughly investigated efflux systems). Gram-negative bacteria that have higher prevalence of efflux pumps are resistant to many antibiotics like beta-lactams, cephalosporins, fluoroquinolones and gentamicin (Fair and Tor, 2014).

Common antibiotic and metal resistance systems

Strategies to respond the toxic effect of both heavy metals and antibiotics present in the environment were demonstrated in bacteria. Antibiotic and metal resistance systems were found to work in similar manner. For example, the passive mechanisms may involve the lowering of membrane permeability or sequestration. It was observed that the mutants of *Serratia marcescens* deficient in porins (outer membrane proteins, Omp's) acquired resistance for antibiotics like ciprofloxacin, tetracycline, chloramphenicol, beta-lactams (Ruiz, 2003). The sequestration of both drugs and heavy metals represents another common resistance system. Bacteria are able to sequestrate metal ions either by extracellular polymeric substances or intracellular polyphosphates (Voica *et al*., 2016). Similarly, *E. coli* was capable to sequestrate antibiotics with the help of polypeptides (del Castillo *et al*., 1991).

The active mechanisms for AB-HM detoxification may include secondary and primary efflux systems. For example, the efflux of drugs and metals has been hypothesized to co-work for pollutants like Cu, Cd, Zn, Co, tetracycline, chloramphenicol (Nies, 2003; Levy, 2002). ATPases (like the CadA or ArsA cadmium or arsenic resistance ATPases) may also play significant roles in combined antibiotic-metal resistance (Silver, 1996).

In some cases, single enzymes function as efflux pumps for multiple metals and antibiotics (Stepanauskas *et al*., 2006). For example, DsbA-DsbB of *Burkholderia cepacia* KF1 is an efflux system formed of membrane-bound oxidoreductase (DsbB) and a periplasmic disulfide oxidoreductase subunit DsbA. This MDR system confers resistance to Cd and Zn and to a variety of beta-lactam antibiotics like kanamycin, erythromycin, novobiocin and ofloxacin (Hayashi *et al*., 2000).

Plasmid vs. chromosome - different types of resistance genes and their localization

In a recent work, Pal *et al.* (2015) stated that co-selection of antibiotic resistant bacteria occurs when antibiotics and heavy metals are simultaneously present in the surroundings of bacteria possessing determinants for both types of resistance or tolerance. Genetic determinants for these capabilities were found to be both chromosome- and plasmid-borne. Resistance to sulfonamides, beta-lactams, aminoglycosides, and tetracyclines are coded by antibiotic resistance genes (ARGs) found both on plasmids and chromosomes. In addition chromosomal ARGs are involved in macrolide and chloramphenicol resistance. Metal resistance genes (MRGs) code for detoxification of mercury, cadmium, arsenic, and copper, whereas chromosomal MRGs are mostly responsible of arsenic, copper and chromium resistance (Pal *et al.*, 2015). Interestingly, MRGs were found to belong to a larger family of genes responsible for both biocide (e.g. disinfectants such as quaternary ammonium compounds, peroxides, acriflavines, etc.) and biocide/metal resistance genes (BMRGs). However it was shown that, when MRG's and ARG's co-occur on plasmids, the first tend to promote horizontal gene transfer (HGT) of antibiotic resistance genes through co-selection (Pal *et al*., 2015).

In polluted environments bacterial isolates carried notably higher proportion of plasmids with genes and conjugative systems (Pal *et al*., 2015).

Strategies for dissemination of metal/antibiotic resistance bacteria

Large bacterial dissemination is not possible without mechanisms to ensure the survival of descendant cells. One mechanism is called 'toxin-antitoxin system' and has a huge impact on the dissemination of bacteria containing MRGs. This system stabilizes plasmids in their host by killing daughter cells that do not inherit the plasmids (Gerdes *et al*., 1986). This strategy helps bacteria to grow resistance faster. Instead of letting the environmental pressure to select fit individuals, bacteria might be able to regulate its fitness beyond environmental control.

An interesting evolutionary curiosity is the size of the plasmid and its potential to be transferred by HGT. Considering the fact that not all plasmids can be transferred to other bacteria, conjugative plasmids are larger than non-conjugative ones. In the study by Pal *et al.* (2015) it has been discovered that in a colony, 57% of plasmids carrying ARGs and BMRGs (were conjugative and their size was under 20 kb. Smaller plasmid (<10 kb) were able to contain only ARGs whereas plasmids over 250 kb contain BMRGs.

Conclusions

Human activities, especially through the land, animal or fish farming (i.e. agriculture and aquaculture-related activities) enrich and promote the heavy metal and antibiotic resistance in bacteria. In addition, industrial areas and other areas subjected to a high anthropic impact are supplementary sources for heavy metal pollution thus potentiating the metal and antibiotic co- and cross-resistance in natural environments. Heavy metals might favor the spread of the antibiotic resistance genes, making the bacteria fitter to survive in an antibiotic and heavy metal-polluted environment.

Acknowledgments Completion of this work was supported by the EnviroAMR project, grant number 3499/20.05.2015, financed through the EEA 2009–2014 Financial Mechanism under the RO04 programme – Reduction of hazardous substances.

REFERENCES

Altug, G., Balkis, N. (2009) Levels of some toxic elements and frequency of bacterial heavy metal resistance in sediment and sea water, *Environ. Monit. Assess.,* **149**:61–69

Amachawadi, R. G., Scott, H. M., Alvarado, C. A., Mainini, T.R., Vinasco, J., Drouillard, J. S., Nagarajaa, T. G. (2013) Occurrence of the transferable copper resistance gene tcrb among fecal Enterococci of U.S. feedlot cattle fed copper-supplemented diets, *Appl. Environ. Microbiol*., **79**:4369–4375

Amador, P. P., Fernandes, R. M., Prudêncio, M. C., Barreto, M. P., Duarte, I. M. (2015) Antibiotic resistance in wastewater: occurrence and fate of Enterobacteriaceae producers of class A and class C β-lactamases, *J. Environ. Sci. Health A Tox. Hazard Subst. Environ. Eng.*, **50**:26-39

Baker-Austin, C., Wright, M. S., Stepanauskas, R., McArthura, J. V. (2006) Co-selection of antibiotic and metal resistance, *Trends Microbiol*., **14**:176-182

Banu, C., Preda, N. Vasu, S. (1982) *Produsele alimentare și inocuitatea lor*, Ed. Tehnică, București, pp. 404-420 [In Romanian]

Berg, J., Tom-Petersen, A., Nybroe, O. (2005) Copper amendment to agricultural soil selects for bacterial antibiotic resistance in the field, *Lett. Appl. Microbiol.*, **40**:146–151

Binggan, W., Linsheng, Y. (2010) A review of heavy metal contaminations in urban soils, urban road dusts and agricultural soils from China*, Microchem. J*., **94(2)**:99-107

Budambula, N. M. L., Kinyua, D. M. (2013) Antibiotic resistance of metal tolerant bacteria isolated from soil in Juja, Kenya, *Conference Paper: JKUAT Scientific, Technological and Industrialization Conference* November 2013, Nairobi

Chen, S., Li, X., Sun, G., Zhang, Y., Su, J., Ye, J. (2015) Heavy metal induced antibiotic resistance in bacterium LSJC7, *Int. J. Mol. Sci.*, **16**:23390-23404

Costa, R. A., Araújo, R. L., Souza, O. V., Silva dos Fernandes, R. H. ,Vieira, R. H. (2015) Antibiotic-Resistant Vibrios in Farmed Shrimp, *Biomed. Res. Int*., 1-5

D'Costa ,V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W. L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H. N., Wright, G. D. (2011) Antibiotic resistance is ancient, *Nature,* **477**:457-461

delCastillo, I., Vizán, J. L., Rodríguez-Sáinz, M. C., Moreno, F. (1991) An unusual mechanism for resistance to the antibiotic coumermycin A1, *Proc. Natl. Acad. Sci U. S. A.,* **88(19)**:8860-8864

Devika, L., Rajaram, R., Mathivanan, K. (2013) Multiple Heavy Metal and Antibiotic Tolerance Bacteria Isolated from Equatorial Indian Ocean, *Int. J. Microbiol. Res.,* **4**: 212-218

Fair, R. J., Tor, Y. (2014) Antibiotics and Bacterial Resistance in the 21st Century, *Perspect. Medicin. Chem*., **6**:25–64

Forrest, H. N. (2012) History of zinc in agriculture, *Adv. Nutr*. **3**:783–789

Forsberg, K. J., Reyes A., Wang, B., Selleck, E. M., Sommer, M. O. A., Dantas, G. (2012) The shared antibiotic resistome of soil bacteria and human pathogens, *Science,* **337**: 1107–1111

Gao, X., Chen, C. T. A. (2012). Heavy metal pollution status in surface sediments of the coastal Bohai Bay, *Water Res.,* **46**:1901-1911

Gerdes, K., Rasmussen, P. B., Molin, S. (1986) Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells, *Proc. Natl. Acad. Sci. U. S A*., **83(10)**:3116–3120

Gillan, D. C., Danis, B., Pernet, P., Joly, G., Dubois, P. (2005) Structure of sediment-associated microbial communities along a heavy-metal contamination gradient in the marine environment, *Appl. Environ. Microbiol*., **71**:679–690

Guidos, R. J. (2011). Combating Antimicrobial Resistance: Policy Recommendations to Save Lives. *Clin. Infect. Dis*., **52**:397–428

Hayashi, S., Abe, M., Kimoto, M., Furukawa, S., Nakazawa, T. (2000) The DsbA-DsbB Disulfide Bond Formation System of *Burkholderia cepacia* Is Involved in the Production of Protease and Alkaline Phosphatase, Motility, Metal Resistance, and Multi-Drug Resistance*, Microbiol. Immunol*., **44(1)**:41-50

Heuer, O. E, Kruse, H., Grave, K., Collignon, P., Karunasagar, I., Angulo, F. J. (2009) Human health consequences of use of antimicrobial agents in aquaculture, *Clin. Infect. Dis.,* **49**:1248–1253

Hill, D. A., Peo, E. R., Lewis, A. J., Crenshaw, J. D. (1986) Zinc-amino acid complexes for swine, *J. Anim. Sci*., **63**:121–30

Hossain, M. M. M., Islam, M. M. (2006) Ship breaking activities and its impact on the coastal zone of Chittagong, Bangladesh: Towards sustainable management, *Young Power in Social Action (YPSA)*, *Chittagong, Bangladesh*, 12-16

Hui, L., Yi-Feng, L., Williams, B. J., Blackwell, T. S., Can-Mao, X. (2011) Structure and function of OprD protein in Pseudomonas aeruginosa: From antibiotic resistance to novel therapies, *Int. J. Med. Microbiol*., **302(2)**:10

Jackson, B. P., Bertsch, P. M., Cabrera, M. L., Camberato, J. J., Seaman, J. C., Wood, C. W. (2003) Trace element speciation in poultry litter, *J. Environ. Qual*., **32**:535–540

Jassim, S. A. A., Limoges, R. G. (2014) Natural solution to antibiotic resistance: bacteriophages 'The Living Drugs', *World J. Microbiol. Biotechnol.,* **30(8)**:2153–2170

Jie, S., Li, M., Gan, M., Zhu, J., Yin, H., Liu, X. (2016) Microbial functional genes enriched in the Xiangjiang River sediments with heavy metal contamination, *BMC Microbiol.,* **16**:179

Jones, H. C., Tuckman, M., Murphy, E., Bradford, P. A. (2006) Identification and Sequence of a tet(M) Tetracycline Resistance Determinant Homologue in Clinical Isolates of Escherichia coli, *J. Bacteriol*., **188(20)**:7151–7164

Kacar, A., Kocyigit, A. (2013) Characterization of heavy metal and antibiotic resistant bacteria isolated from Aliaga Ship Dismantling Zone, Eastern Aegean Sea, Turkey, *Int. J. Environ. Res.,* **7**:895-902

Karimi, A., Sadeghi, G., Vaziry, A. (2011) The effect of copper in excess of the requirement during the starter period on subsequent performance of broiler chicks, *J. Appl. Poult. Res.,* **20**:203-209

Knapp, C. W., McCluskey, S. M., Singh, B. K., Colin, D. C., Hudson, G., Graham, W. D. (2011) Antibiotic Resistance Gene Abundances Correlate with Metal and Geochemical Conditions in Archived Scottish Soils, *PLoS Onev.,* **6(11)**:1-6

Kohanski, M. A., Dwyer, D. J., Collins, J. J. (2010) How antibiotics kill bacteria: from targets to networks, *Nature Revs. Microbiol*., **8**:423–435

Landers, T. F., Cohen, B., Wittum, T. E., Larson, E. L. (2012) A Review of Antibiotic Use in Food Animals: Perspective, Policy, and Potential, *Public Health Rep*., **127**(1), 4–22

Levy, S. B. (2002) Active efflux, a common mechanism for biocide and antibiotic resistance*, J. Appl. Microbiol*., **92**:65–71

Li, Y. X., Chen, T. B. (2005) Concentrations of additive arsenic in Beijing pig feeds and the residues in pig manure, *Res. Conserv. Recy*., **45**:356–367

Matyar, F., Akkan, T., Uçak, Y., Eraslan, B. (2010) *Aeromonas* and *Pseudomonas*: antibiotic and heavy metal resistance species from Iskenderun Bay, Turkey (northeast Mediterranean Sea), *Environ. Monit. Assess.,* **167**:309–320

Matyar, F., Kaya, A., Dinçer, S. (2008) Antibacterial agents and heavy metal resistance in Gram-negative bacteria isolated from seawater, shrimp and sediment in Iskenderun Bay, Turkey, *Sci. Total Environ.,* **407**:279-285

Mehi, O., Bogos, B., Csörgő, B., Pál, F., Nyerges, A., Papp, B., Pál, C. (2014) Perturbation of Iron Homeostasis Promotes the Evolution of Antibiotic Resistance, *Mol. Biol. Evol*., **31(10)**:2793–2804

Morillo, J., Usero, J., Gracia, I. (2004) Heavy metal distribution in marine sediments from the southwest coast of Spain. *Chemosphere,* **55**:431-442

Munita, J. M. Arias, C. A. (2016) Mechanisms of Antibiotic Resistance, *Microbiol Spectr.,* **4**(2):10

Nies, D.H. (2003) Efflux-mediated heavy metal resistance in prokaryotes*, FEMS Microbiol. Rev*., **27**:313-319

Pal, C., Bengtsson-Palme, J., Kristiansson, E., Larsson, D. J. (2015) Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential, *BMC Genomics*, **16**:964

Plum, M. L., Rink, L., Hajo, H. (2010) The Essential Toxin: Impact of Zinc on Human Health, *Int. J. Environ. Res. Public Health,* **7(4)**:1342–1365

Quero, G. M., Cassin, D., Botter, M., Perini, L., Luna, G. M. (2015) Patterns of benthic bacterial diversity in coastal areas contaminated by heavy metals, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), *Front. Microbiol.,* **6**: 1053

Resende, J. A., Silva, V. L., Fontes, C. O., Souza-Filho, J. A., de Oliveira, T. L. R., Coelho, C. M., César, D. E., Diniz, C. G. (2012) Multidrug-resistance and toxic metal tolerance of medically important bacteria isolated from an aquaculture system, *Microbes. Environ.,* **27**:449–455

Ruiz, N. (2003) The role of *Serratia marcescens* porins in antibiotic resistance, *Microb. Drug Resist*., **9**:257–264

Sabry, S. A., Ghozlan, H. A., Abou-Zeid, D. M. (1997) Metal tolerance and antibiotic resistance patterns of a bacterial population isolated from sea water , *J. Appl. Microbiol.,* **82**:245-252

Salami, I. R., Rahmawati, S., Sutarto, R. I., Jaya, P. M. (2008) Accumulation of heavy metals in freshwater fish in cage aquaculture at Cirata Reservoir, West Java, Indonesia, *Ann. N. Y. Acad. Sci*., **1140**:290-6

Sapkota, A., Sapkota, A. R., Kucharski, M., Burke, J., McKenzie, S., Walker, P., Lawrence, R. (2008) Aquaculture practices and potential human health risks: current knowledge and future prioritie, *Environ. Int.,* **34**:1215-1226

Sarmah, A. K., Meyer, M. T., Boxall, A. B. (2006) A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment, *Chemosphere,* **65(5)**:725-59

Seiler, C., Berendonk, T. U. (2012) Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Role and prevalence of antibiosis and the related resistance genes in the environment, *Front. Microbiol.,* **3**: 399

Silver, S. (1996) Bacterial heavymetal resistance: new surprises, *Ann. Rev. Microb*., **50**: 753–789

Stepanauskas, R., Glenn, T. C., Jagoe, C. H., Tuckfield, R. C., Lindell, A. H., King, C. J., McArthur, J. V. (2006) Coselection for microbial resistance to metals and antibiotics in freshwater microcosms, *Environ Microbiol.,* **8(9)**:1510-1514

Szekeres, E., Baricz, A., Chiriac, C. M., Farkaş, A., Opris, O., Soran, M. L., Andrei, A. S., Rudi, K., Balcázar, J. L., Dragos, N., Coman, C. (2017) Abundance of antibiotics, antibiotic resistance genes and bacterial community composition in wastewater effluents from different Romanian hospitals, *Environ. Poll.*, http://doi.org/10.1016/j.envpol.2017.01.054

Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., Sutton, D. J. (2012) Heavy metals toxicity and the environment, *EXS*, **101**:133–164

Thomsen, P. T. (2015) Short communication: Efficacy of copper sulfate hoof baths against digital dermatitis—Where is the evidence?, *J. Dairy Sci.,* **98**:2539-2544

Tsai, A., Uemura, S., Johansson, M., Puglisi, E., Marshall, R., Colin, C., E., Korlach, J., Ehrenberg, M., Puglisi, D. J (2013) The impact of aminoglycosides on the dynamics of translation elongation, *Cell Rep.,* **3**(2):497–508

Ţugui, C.G., Vlădăreanu, I., Baricz, A., Coman, C. (2015) Detection of beta-lactamase resistance genes in a hospital chlorinated wastewater treatment system. *Studia UBB Biologia*, **60**:33-38

Underwood, E. J., Suttle, N. F. (1999) Zinc. In: Mineral nutrition of livestock. 3rd ed. *CAB International New York*, 477–512

Voica, D. M., Bartha, L., Banciu, H. K., Oren, A. (2016) Heavy metal resistance in halophilic Bacteria and Archaea, *FEMS Microbiol. Lett.,* **363(14)**:1-9

Wales, D. A., Davies, R. H. (2015) Co-Selection of Resistance to Antibiotics, Biocides and Heavy Metals, and Its Relevance to Foodborne Pathogens, *Antibiotics (Basel),* **4(4)**: 567–604

Walker, B., Barrett, S., Polasky, S. Galaz, V., Folke, C., Engström, G., Daily, G. (2009) Looming global-scale failures and missing institutions, *Science,* **325**:1345-1346

Webber, M. A., Piddock, L. J. V. (2013) The importance of efflux pumps in bacterial antibiotic resistance, *J. Antimicrob. Chemother.,* **51(1)**:9-11

Wireman, J., Liebert, C. A., Smith, T., Summers, A. O. (1997) Association of mercury resistance with antibiotic resistance in the gram-negative fecal bacteria of primates, *Appl Environ Microbiol*., **63(11)**:4494–4503

Wright, G. D. (2007) The antibiotic resistome: the nexus of chemical and genetic diversity, *Nature Revs. Microbiol.,* **5**:175-186

Wright, G. D., Poinar, H. (2012) Antibiotic resistance is ancient: implications for drug discovery, *Trends Microbiol.,* **20**:157-159

Wu, X. Y., Yang, Y. F. (2011) Heavy metal (Pb, Co, Cd, Cr, Cu, Fe, Mn and Zn) concentrations in harvest-size white shrimp *Litopenaeus vannamei* tissues from aquaculture and wild source, *J. Food Comp. Anal.,* **24**:62-65

Yazdankhah, S., Rudi, K., Bernhoft, A. (2014) Zinc and copper in animal feed – development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin, *Microb. Ecol. Health Dis*., **25**:10

Zhang, F., Li, Y., Yang, M., Li, W. (2012) Content of Heavy Metals in Animal Feeds and Manures from Farms of Different Scales in Northeast China, *Int. J. Environ. Res. Public Health,* **9**:2658-2668

Zhang, W., Ki, J. S., Qian P. Y. (2008) Microbial diversity in polluted harbor sediments I: bacterial community assessment based on four clone libraries of 16S rDNA, *Estuar. Coast. Shelf. Sci.* **76**:668–681

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXII, 1, 2017 (p. 115-119)
doi:10.24193/subbb.2017.1.08

=== SHORT COMMUNICATION ===

Acute effects of electronic cigarette smoking on heart rate variability

Ştefan-Claudiu Mirescu[1,2]✉, Beatrice Andreea Caitas[1], Sergiu Holhoş[1], Corneliu Tarba[1] and Leontin David[3]

SUMMARY. Heart rate variability (HRV) refers to a temporal variation between consecutive heart beats and is considered a measure of the balance between the sympathetic and the parasympathetic nervous system. HRV is also one of the few methods which can evaluate the acute effects of smoking on the autonomous nervous system. The aim of our study was to evaluate and compare HRV parameters during regular cigarette and electronic cigarette smoking. The study was performed on 15 regular cigarette smokers and 15 electronic cigarette smokers, and time-domain, frequency-domain and nonlinear parameters of HRV were recorded. When smoking regular cigarettes, the subjects had lower values of HRV parameters than before smoking, whereas electronic cigarette smokers described higher HRV parameters values for all HRV parameters. Our results regarding the behavior of HRV parameters during regular cigarette smoking are consistent with the literature, but the evaluation of the sympathovagal balance during electronic cigarette smoking was performed for the first time in our study.

Keywords: electronic cigarette, heart rate variability, smoking.

Introduction

Heart rate variability (HRV) refers to the variation in consecutive R-R intervals on the electrocardiogram (ECG) and represents a sensitive and non-invasive measure of the autonomic nervous system (ANS) function and balance (Henry *et al*., 2010;

[1] *Babeş-Bolyai University, Faculty of Biology and Geology, Department of Molecular Biology and Biotechnology, 5-7 Clinicilor Str., Cluj-Napoca, Romania*
[2] *Regional Institute of Gastroenterology and Hepatology „Octavian Fodor", Cluj-Napoca, Romania*
3 Babeş-Bolyai University, Faculty of Physics
✉ ***Corresponding author: Ştefan-Claudiu Mirescu**, Babeş-Bolyai University, Faculty of Biology and Geology, Department of Molecular Biology and Biotechnology, 5-7 Clinicilor Str., Cluj-Napoca*
E-mail: claudiu.mirescu@ubbcluj.ro

Tsai *et al.*, 2014). HRV is usually evaluated by visual methods (the R-R intervals tachogram and the Poincaré plot) and, more accurate, by time-domain, frequency-domain and nonlinear parameters (Acharya *et al.*, 2006).

Long-term cigarette smoking is reported as a major and independent risk factor for cardiovascular morbidity and mortality, especially by sympathetic mediation (Karakaya *et al.*, 2007). However, only a few studies focused their investigations on short-term (acute) effects of smoking, i.e. the effects of smoking on fast-changing parameters. HRV is a method of assessing acute effects of cigarette smoking on the ANS balance (Gondim *et al.*, 2015).

The aim of the study was to evaluate the acute effects of electronic cigarette smoking on HRV.

Materials and methods

The study included 15 healthy habitual smokers (20-22 years old, 8 females, all smokers for at least 2 years, both electronic and regular cigarettes), and HRV was continuously recorded for 25 minutes: 5 minutes without smoking (the control period), 5 minutes the subject was asked to smoke from an electronic cigarette and 15 more minutes after the smoking of the cigarette; this last period was divided into three 5-minutes periods, for statistical purposes. The first 5-minutes period was considered control and Student *t* test was used for value comparison. A $p < 0.05$ was considered statistically significant.

ECG traces used for HRV analysis were performed using a Neurosoft Poly-Spectrum-8 device, which interfaces a personal computer *via* an USB port. Leads were placed on both arms and legs and the ECG was performed in 6 derivations (DI, DII, DIII, aVL, aVR and aVF). R-R intervals were calculated using Poly-Spectrum integrated software and HRV parameters were computed using Kubios HRV, a freeware academic tool for HRV analysis developed by University of Kuopio, Finland.

Table 1.

HRV parameters included in the study

Time-domain parameters		
Mean R-R interval	ms	Average of all R-R intervals
Standard deviation of RR intervals	ms	Standard deviation of all R-R intervals
Mean heart rate	beats/min	Average heart rate
Standard deviation of heart rates	beats/min	Standard deviation of all momentarily heart rates
Frequency-domain parameters		
LF/HF		Ratio between low frequency and high frequency power in the fast Fourier transform
Nonlinear parameters (descriptors of the Poincaré diagram)		
SD1	ms	The short radius of the Poincaré plot
SD2	ms	The long radius of the Poincaré plot

Results and discussion

Time-domain parameters expressed discordant values. Heart rate increased during smoking and in the first 5-minutes interval after smoking ($p < 0.05$), with a decrease in the following 5-minutes interval. On the other hand, SDRR increased during smoking compared to the control interval ($p < 0.05$) and returned or the control values in the next 15 minutes (Fig. 4).

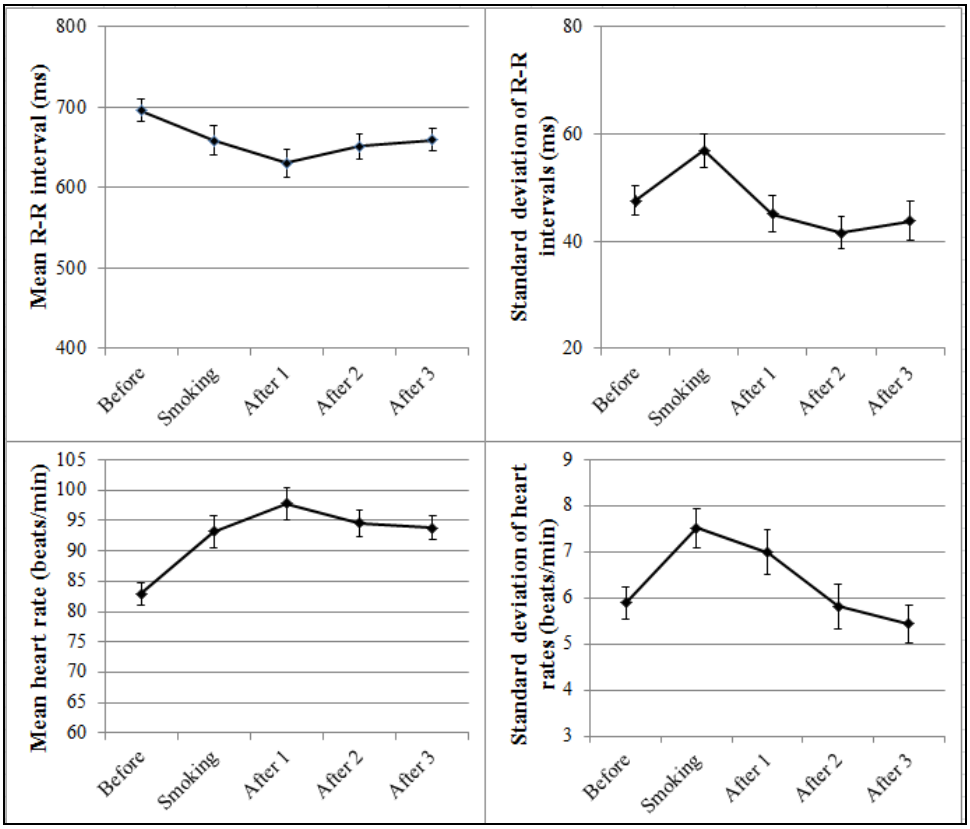


Figure 4. Time-domain parameters in subjects smoking electronic cigarettes.

LF/HF had a similar behavior to the regular cigarette users, returning to the control values in the next 15 minutes ($p < 0.05$ when comparing the smoking interval to the control interval and the three 5-minutes intervals with the smoking interval) (Fig. 5-Left).

The Poincaré plot statistical descriptors are in accordance with the variation of SDRR: both SD1 and SD2 expressed an increase during smoking and a decrease in the next 15 minutes (Fig. 5-Right).

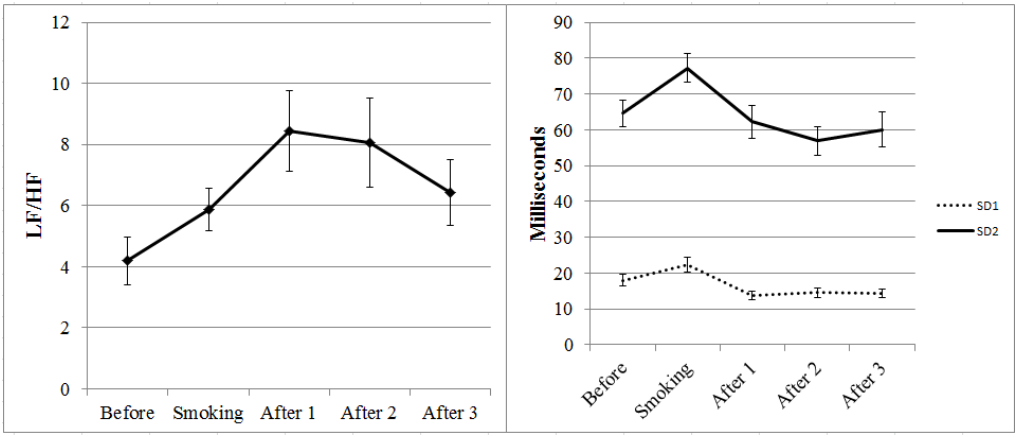


Figure 5. Left – LF/HF and Right – statistical descriptors of the Poincaré diagram in subjects smoking electronic cigarettes.

Conclusions

While the vast majority of the studies assayed the effects of long-term smoking on the heart and autonomic balance, our study premierly compared the acute effects of electrical cigarette on the autonomic balance, by the means of HRV. Despite the reduced number of volunteers, we clearly demonstrated that smoking has a direct influence on HRV parameters.

While HRV expressed an overall decrease in regular cigarette smoking, suggesting a sympathetic prevalence during smoking and 5 minutes after, the situation concerning electronic cigarette was different. The differences between the two types of smoking habits were pronounced in those parameters that are considered independent descriptors of HRV (SDRR, SDHR, SD1 and SD2), which were increased in electronic cigarette smokers.

Very few studies have been performed on the acute effects of smoking on HRV and none concerning the effects of electronic cigarette smoking. Nevertheless, our results are consistent with the literature, HRV parameteres decreasing during smoking (Karakaya *et al.*, 2007). Other studies (Middlekauff *et al.*, 2014; Gondim *et al.*, 2015 focused on the comparison between HRV parameters in smokers and non-smokers, not taking into consideration the acute effects.

The decrease in HRV described by regular cigarette smokers was expected, considering the fact that nicotine and carbon monoxide triggers adrenalin and noradrenalin release, which increase the activity of the sympathetic nervous system (Karakaya *et al.*, 2007; Henry *et al.*, 2010; Middlekauff *et al.*, 2014; Gondim *et al.*, 2015).

Unexpected results were found concerning HRV parameters in electronic cigarete smokers. They were found to have increased values during smoking, which is unexplainable on the knowledge of current data about the effect of nicotine (even in pure solution) on the nervous system.

The limitations of the current study are the small number of subjects and their young age.

REFERENCES

Acharya, U., Rajendra, K., Joseph, P., Kannathal, N., Min Lim, C., Jasit, S. (2006) Heart Rate Variability: A Review, *Med. Biol. Eng. Comput.*, **44**:1031-1051

Gondim, R. M., Farah, B. Q., Santos, F., Ritti-Dias, R. M. (2015) Are Smoking and Passive Smoking Related with Heart Rate Variability in Male Adolescents?, *Einstein (Sao Paolo)*, **13**:27-33

Henry, B. L., Minassian, A., Paulus, M. P., Geyer, M. A. (2010) Heart Rate Variability in Bipolar Mania and Schizophrenia, *J. Psychiatr. Res.*, **44**:168-176

Karakaya, O., Barutcu, I., Kaya, D., Essen, A. M., Saglam, M., Melek, M., Onrat, E., Turkmen, M., Esen, O. B., Kaymaz, C. (2007) Acute Effect of Cigarette Smoking on Heart Rate Variability, *Angiology*, **58**:620-624

Middlekauff, H. R., Park, J., Moheimani, R. S. (2014) Adverse Effects of Cigarette and Noncigarette Smoke Exposure on the Autnomic Nervous System: Mechanisms and Implications for Cardiovascular Risk, *J. Am. Coll. Cardiol.*, **64**:1740-1750

Tsai, J. F., WenChun, C., Jou, S. J., Lin, C. M. (2014) Heart Rate Variability and Meditation with Breath Suspension, *Biomed. Res-India*, **25**:6-10