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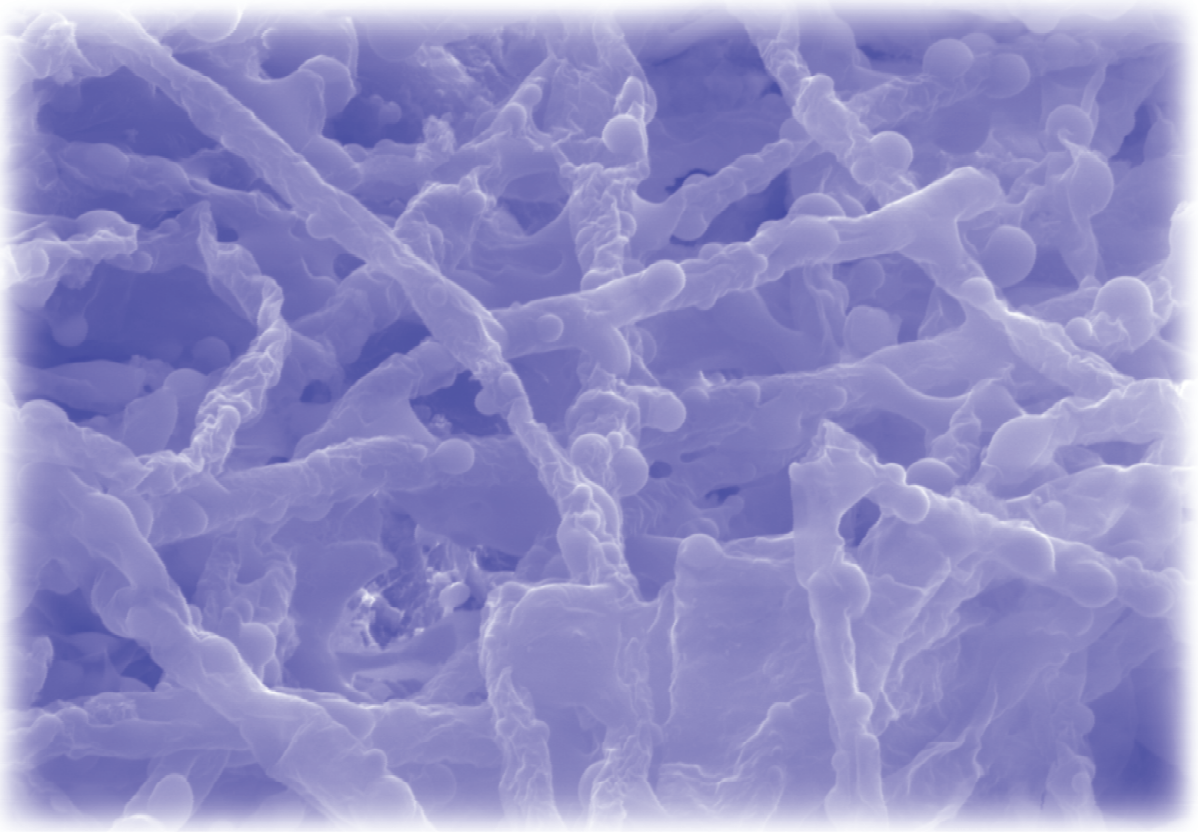

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STUDIA UBB EDITORIAL OFFICE: B.P. Hasdeu no. 51, 400371 Cluj-Napoca, Romania, Phone + 40 264 405352, www.studia.ubbcluj.ro

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IN MEMORIAM

REGULAR ARTICLES

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

Original pictures on front cover: Hyphae and conidiophores of *Trichoderma reesei* in SEM

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=== IN MEMORIAM ===

Dan Munteanu, PhD,
Corresponding member of the Romanian Academy

(2 June 1937 - 25 February 2017)

Some people don't show their age. They are constantly in motion, dynamic, take initiatives, have never-ending ideas, are cheerful but firm, wise but flexible, optimistic, sometimes caustic and dedicated warriors for noble causes connected to their areas of activity. This is how Dr. Dan Munteanu was, a dreamer, a great connoisseur and a lover of nature.

He was born on June 2nd, 1937, in a family of intellectuals with ancient Transylvanian roots. Father, Didi Munteanu, paediatrician, associate professor at the Medical University and director of the Cluj orphanage. The mother, Maria Cupcea, was an emeritus artist of the National Theater in Cluj, the descendant of an erudite family of teachers and priests from Maramureş.

Young Dan attended the General School "I. Bob", then "Emil Racoviţă" High School in Cluj. Although the parents wanted him to study medicine, the passion for nature and especially for birds led him to study biology at the Faculty of Natural Sciences, Department of Biology and Zoology at the "Victor Babeş" University of Cluj. Since childhood, he captured birds with glue from oak mistletoe, kept them in captivity in winter and studied their song and their changes according to the food they fed on. All these experiments were the roots of his further research.

During his student years, the ornithological adventures with Mircea Mătieş and Alexandru Filipaşcu succeeded uninterruptedly. The "Flobert" pistol and sling were handled skilfully, but in secret. All experiences from his school years and student hood are later reflected in his competence and inventivity as a mature researcher.

After his faculty graduation, he held a position as a trainee researcher at the "Stejarul" Research Center in Pângăraţi, Neamţ County. In 1965 he enrolled in a PhD program at the University of Bucharest addressing a theme referring to the mountain basin of the Moldavian Bistriţa, under the supervision of Professor Alexandru V. Grossu. After graduating and obtaining his PhD degree in biology - ornithology, at the University of Bucharest (1969), he became the director of the "Stejarul" Research Station, from where, in 1973, he moved to the Biological Research Institute in Cluj. Over the years he was promoted to Senior Scientific Researcher in the Biological Research Center in Cluj-Napoca. Since January 29th, 1999, he has been a correspondent member of the Romanian Academy. In 2004 he changed positions from the Institute of Biological Research in Cluj to the Romanian Academy, where he has been chairman of the Commission for the Protection of Natural Monuments since 2000. Because of his loyalty to Cluj, Dr. Dan Munteanu keeps his office at the Institute for Biological Research on 48 Republicii Str., but he was also present in Bucharest whenever it was required to solve the many nature-related problems in Romania. He also obtains the right to supervise doctorates, but he prefers to run this noble activity at the Babeş-Bolyai University in Cluj-Napoca.

Dan Munteanu spent a lifetime studying the birds in Romania. The studies were of a faunistic, distributional, systematic, ecological and biological nature, but also about human-bird interrelations, with emphasis on protection and conservation measures. Alone or together with the most well-known ornithologists in the country, Dan Munteanu initiates and coordinates the most important avifaunistic research projects in Romania (Werner Klemm, Kohl Stefan, Valeriu Puşcariu, Korodi Gál, Dimitrie Radu, Mircea Mătieş, Ion Cătuneanu, Matei Tălpeanu, Sergiu Paşcovschi, Aurel Papadopol, Alexandru Filipaşcu, Kalabér László, Peter Weber, Victor Ciochia, Dan Stănescu, Eugen Petrescu, Beres Josif, Cătălin Rang, Iordache Ion, Nicolae Valenciuc, Maria Paspaleva, Peter-Klaus Zsivanovits, Kiss János Botond, Kelemen Attila, Szabo Josef, Kiss Andrei etc.). We are grateful to them for the high level reached by ornithology and ornithological volunteering in Romania today.

There is no established Romanian ornithologist, who wasn't at least once with Dan Munteanu somewhere in the field.

Dan Munteanu participated in all important ornithological events in Romania since 1960. He, together with a handful of ornithologists, founded in the spring of 1990 the Romanian Ornithological Society (S.O.R.) and wrote its first publications, being considered the "father" and **the elected president-founder** of this NGO, that is so appreciated today. **Therefore, the president-founder of S.O.R. was implied in the elaboration of several European projects on the bird knowledge and protection in South-Eastern Europe.** Later on, many important works followed, opening new roads in the knowledge of the bird fauna from Romania, e.g. the provisional atlas of the nesting birds in Romania etc. (see bibliography).

In order to boost the knowledge of bird fauna in Romania, obtaining the right to translate the well-known "Illustrated bird guide of Europe", adapted to the birds in Romania, was probably a crossroads-point. Dan Munteanu was also behind the initiative and adaptations for Romania of this guide.

But Dan Munteanu was also, together with Acad. Nicolae Botnariuc, Acad. Nicolae Boşcaiu, Prof. Valeriu Puşcariu and others, a permanent militant for the protection and conservation of nature in Romania. Before, but especially after having served as chairman of the Commission for Natural Monuments Protection within the Romanian Academy, Dan Munteanu visited and researched almost all the protected areas in Romania. He supported the initiatives to create many new protected areas. Under his signature, the number and surface of protected areas in Romania has increased significantly.

During the years, he has participated at numerous and prestigious international conferences, where he started collaborations and lasting friendships with the world's most well-known ornithologists. Many of them later visited the country, and were impressed by the nature and ornithological fauna of Romania.

He has trained a number of young ornithologists, students and PhD students. He responded to any request with excitement and a word of advice, with patience and goodwill. Through his knowledge, publishing activity, his presence at scientific events and field trips, Dr. Dan Munteanu made the transition from the classical 1960's to the modern-day ornithology.

Over the years his work has been appreciated and admired. He obtained more than 20 diplomas and medals, including "Academic Merit" in 2013, "Senior Citizen" in Cluj-Napoca, 2009, the "Emil Racovita" Award of the Romanian Academy in 1978.

He published more than 100 scientific articles in Romania and abroad, 10 books as main author, he wrote 13 book chapters, of which 8 abroad, wrote over 140 popular-science articles. He was editor of major scientific journals in Romania and a member of other editorial committees. I would like to mention here also the numerous ornithological and other contributions he wrote in the Romanian journal "The Hunter and the Fisherman".

Dan Munteanu was married to Monica Coprean, a medical doctor, who gave up her medical career to follow her husband to Pângărați, where she dedicated herself to the biology and pathology of fish. After settling in Cluj-Napoca, Dr. Monica Munteanu led the fish pathology laboratory, with emphasis on salmonids at the Forest Research Centre and Forestry Institute. They had two children, Claudia and Victor. Claudia followed his father's example, studied biology and became a biology teacher in Cluj-Napoca, and Victor is an economist at an important Cluj company.

Personally, I met Dan Munteanu during my study years, but only got to know him better after 1985, when we became colleagues at the Institute of Biological Research in Cluj. His friendly, communicative nature combined with a fine sense of ironic humour, and especially the joint research projects, made us close friends. Perhaps the most interesting common activities were the nocturnal ones, Dan recording tapes of the songs of night birds, trying to find out their composition and abundance, and myself, with baits and various types of lights, to study moth communities. That's how I spent memorable nights in Tișiței Gorge, the Retezat, the Cozia, the Călimani, the Cozia, the Ceahlău, the Rarău Mountains etc. But perhaps the most beautiful trip, and the last one for us, was the one in the Rodna Mountains National Park, in 2014, when we were accompanied by three other members of the Park's Scientific Board. We climbed into the alpine zone to see the Black grouse. We slept under the open sky, at 1900m altitude, next to an uninhabited sheepfold. At the melody of the fire, the cracking of the coals and the symphony of the sky, we shared memories and Dan was the one who told us about things only he knew. In the morning, at sunrise we were on the top of the mountain and we were able to see four Black grouses. For me they were the firsts, for Dan they were the lasts.

He manifested obvious signs of disease since 2013. Like many of us, he made the mistake of ignoring them, continuing to work with dedication and self-sacrifice. He managed to finish and publish the comprehensive book vol. XV, fasc. 2 from ***Aves***, in Romanian.

But the disease is unforgiving, not slowing down, not withdrawing, giving him no respite. He worked hard and especially never regretted that he chose biology, nature, birds, and not medicine.

On February the 25th, 2017, at dawn, the academician Dan Munteanu's soul rose to the heavens carried by a flock of mournful birds.

We thank you Dan, for everything you did and what you left behind!

Rest in peace!

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Professor László Rákosy, PhD

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

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Revised checklist and new faunistic data of the Romanian Culicidae (Insecta, Diptera)

Edina Török[1,2,✉], Beáta-Lujza Ujvárosi[3],
Levente-Péter Kolcsár[1] and Lujza Keresztes[1]

SUMMARY. We present here an update checklist increases the number of Culicidae species in Romania from 50 to 60, split into 7 genera: *Aedes* (29 species), *Anopheles* (10 species), *Coquillettidia* (2 species), *Culex* (9 species), *Culiseta* (8 species), *Orthopodomyia* (1 species) and *Uranotaenia* (1 species). Additionally, 20 new faunistic records to different regions of Romania, mostly from Transylvania.

Keywords: faunistic data, checklist, mosquito, Transylvania

Introduction

Culicidae is a well-known worldwide distributed family of Diptera, present in different ecosystems from natural permanent waters to many ephemeral or artificial waters. The present number of taxa is 3601 (species and subspecies) included in 110 different genera (http://mosquito-taxonomic-inventory.info/). A number of 100 species belonging to nine genera have been recorded on the continental Europe (Harbach, 2013). In Romania, first faunistic surveys of Culicidae were initiated by Zotta (1927, 1932), along with the first monitoring of malaria cases from the country. A comprehensive revision of the Culicidae fauna of Romania was published in 1995 by Nicolescu, with a first checklist containing 50 species. Further, six other species with uncertain records were presented together with a synthesis of the literature data and an updated checklist for different major regions of Romania.

Nowadays, the Romanian mosquito surveys are mostly focused on the role of different Culicidae species as vectors for different pathogens, rather than faunistic surveys. In 1998, Nicolescu, while working on the distribution of the West Nile

[1] *Babeş-Bolyai University, Hungarian Department of Biology and Ecology, Center of Systems Biology, Biodiversity and Bioresources, 400006 Cluj-Napoca, Clinicilor 5-7, Romania..*
[2] *Romanian Academy Institute of Biology, Splaiul Independenţei 296, 060031 Bucureşti, Romania.*
[3] *University of Debrecen, Egyetem tér 1., 4032 Debrecen, Hungary.*
✉ ***Corresponding author: Edina Török**, Babeş-Bolyai University, Hungarian Department of Biology and Ecology, Center of Systems Biology, Biodiversity and Bioresources, E-mail: edinatorok7@gmail.com*

virus, published important new faunistic data on the distribution of mosquitoes from the southern part of Romania, followed by contributions from other 14 different ecological regions (Nicolescu *et al.,* 2002, 2003a, 2003b, 2003c). Further on, a new species, *Anopheles daciae* Linton, Nicolescu and Harbach, 2004 was detected and described using two molecular markers (nuclear rDNA ITS2 and mitochondrial COI) and morphological characters of the eggs (Nicolescu *et al.,* 2004). In the same publication, another Culicidae species, *An. melanoon* Hackett, 1934 was mentioned for the first time to the fauna. Additionally, recent data on further 10 *Anopheles* species from Romania were published by different authors (Ciolpan *et al.,* 1998; Gunay *et al.,* 2017; Vincent *et al.,* 2011; Marí and Peydró, 2012).

Preliminary data on the mosquito fauna from the Danube Delta were published by Pârvu (2005, 2008) and Prioteasa *et al.* (2007). The *Anopheles maculipennis* complex from the Danube Delta - was investigated by Fălcuță *et al.* (2008, 2010). *Ochlerotatus zammitii* (Theobald, 1903) was also recorded for the first time from here, followed by a comprehensive checklist of the area, containing 31 different mosquito species (Prioteasa and Fălcuță 2010). Distribution data of different *Anopheles* species from Bucharest and the surrounding area were published by Fălcuță *et al.* (2011). Moreover, the invasive *Aedes albopictus* was detected for the first time in Bucharest (Prioteasa *et al.,* 2015). Moreover, data on distribution of mosquito species responsible for West Nile virus circulation added Sîrbu *et al.* (2011); Dinu *et al.* (2015); Cotar *et al.* (2016). A comprehensive monitoring survey on the Culicidae fauna from the Danube Delta area was initiated by the authors in 2014, focusing on the most representative ecosystems from the area. Here, we present the results of our integrative approach for species identification (morphological characters) and first records of *Aedes hungaricus* (Mihályi, 1955) and *Anopheles algeriensis* Theobald, 1903 from Romania (Török *et al.,* 2016).

Despite the generally well-known Culicidae fauna of Romania, faunistic investigations of different regions are biased. Most of the literature data comes from southern Romania (Nicolescu *et al.,* 2003b, 2003c), and the Transylvanian mosquitoes have been sporadically explored.

The present paper is a synthesis of our integrative surveys of the last few years (1995-2018), focused mostly on some less studied areas in Romania, such as Transylvania. These data were completed with the most recent faunistic literature on the Romanian Culicidae and an updated checklist is presented here.

Materials and methods

In this study, we used the systematic classification proposed by Harbach 2018 (Mosquito Taxonomic Inventory, www.mosquito-taxonomic-inventory.info Updated 17 October 2018). Specimens were collected between 2015 and 2018 using sweep nets and Malaise traps. The material was stored in 70% ethanol or pinned. All material is

deposited in the Diptera Collection of the Faculty of Biology and Geology, Babeş-Bolyai University, Cluj-Napoca, Romania (DCBBU). The Culicidae material was identified based on morphological characteristics of males and females using identification keys (Becker *et al.,* 2010, Kenyeres and Tóth, 2008) as well as different software programs (Schaffner *et al.,* 2001). All our faunistic data are available on the TransDiptera Online Database (Kolcsár et al., 2018, http://transdiptera.ro, doi: 10.18426/OBM.5sskmlll3ip0). Our collecting sites are presented in Figure 1.

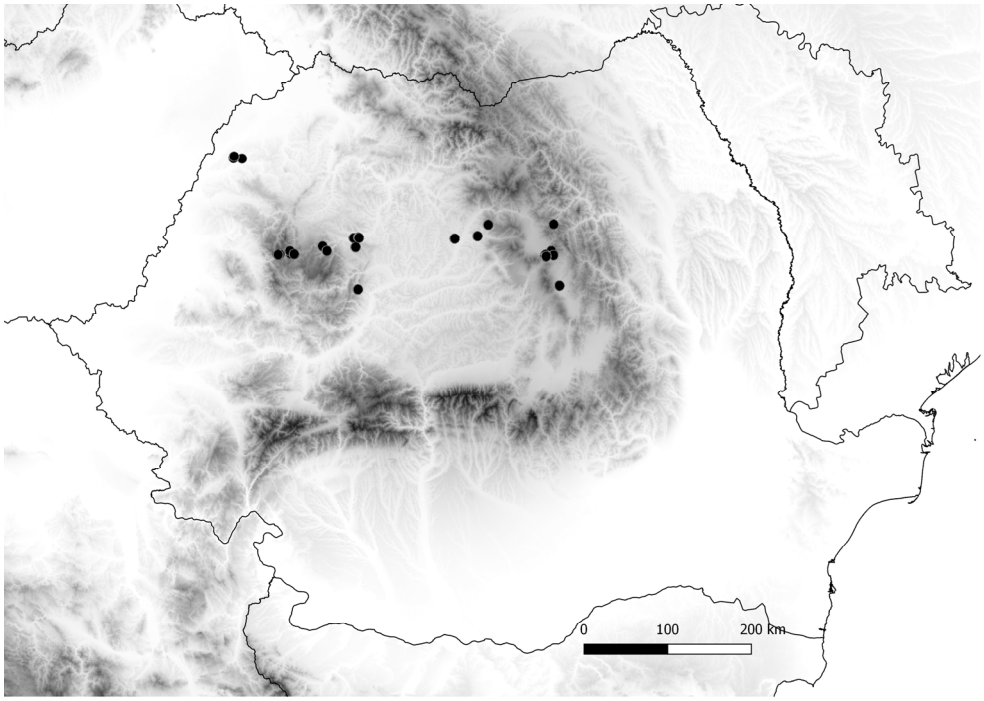

Figure 1. Culicidae collection sites.

Results and discussion

We present here new faunistic data of 20 different Culicidae species from Transylvania, based on 86 males and 268 females. Furthermore, an updated checklist of the Culicidae fauna of Romania is presented below.

Chelcklist of the Romanian Culicidae fauna
Subfamily Anophelinae
Genus *Anopheles* (Meigen)
Subgenus *Anopheles* (Meigen)

1. *algeriensis* Theobald, 1903 **Note 1**
2. *atroparvus* van Thiel, 1927
3. *claviger* (Meigen, 1804) **Note 2**
4. *daciae* Linton, Nicolescu and Harbach, 2004
5. *hyrcanus* (Pallas, 1771)
6. *maculipennis* Meigen, 1818 **Note 3**
7. *melanoon* Hackett, 1934
8. *messeae* Falleroni, 1926
9. *plumbeus* Stephens, 1828
10. *sacharovi* Favre, 1903

Subfamily Culicinae
Tribe Aedini
Genus *Aedes* (Meigen)
Subgenus *Acartomyia* Theobald

11. *zammitii* (Theobald, 1903)

Subgenus *Aedes* (Meigen)

12. *cinereus* Meigen,1818
13. *geminus* Peus, 1970 **Note 4**
14. *rossicus* (Dolbeskin, Gorickaja and Mitrofanova, 1930) **Note 5**

Subgenus *Stegomyia* Theobald

15. *albopictus* (Skuse 1895)

Subgenus *Aedimorphus* (Theobald)

16. *vexans* (Meigen, 1830) **Note 6**

Subgenus *Dahliana* Reinert, Harbach and Kitching

17. *geniculata* (Olivier, 1791) **Note 7**

Subgenus *Woodius* Reinert, Harbach and Kitching

18. *intrudens* (Dyar, 1919)

Subgenus *Ochlerotatus* Lynch Arribalzaga

19. *annulipes* (Meigen, 1830) **Note 8**
20. *behningi* (Martini, 1926) (Schaffner *et al.,*2001, Gunay *et al.,*2017) **Note 9**
21. *cantans* (Meigen, 1818) **Note 10**
22. *caspius* (Pallas, 1771) **Note 11**
23. *cataphylla* (Dyar, 1916) **Note 12**
24. *communis* (de Geer, 1776) **Note 13**
25. *detritus* (Haliday, 1833)
26. *dorsalis* (Meigen, 1830)

27. *duplex* (Martini, 1926)
28. *excrucians* (Walker, 1856)
29. *flavescens* (Muller, 1764)
30. *hungaricus* (Mihályi, 1955)
31. *intrudens* (Dyar, 1919)
32. *leucomelas* (Meigen, 1804)
33. *nigrinus* (Eckstein, 1918) **Note 14**
34. *pulcritarsis* (Rondani, 1872)
35. *pullatus* (Coquillett, 1904) **Note 15**
36. *punctor* (Kirby, 1837) **Note 16**
37. *riparius* (Dyar and Knab, 1907) **Note 17**

Subgenus *Rusticoidus* Shevchenko and Prudkina, 1973
38. *refiki* (Medschid, 1928)

Tribe Culicini
Genus *Culex* Linnaeus
Subgenus *Barraudius* (Edwards)
39. *modestus* Ficalbi, 1890 **Note 18**

Subgenus *Culex* (Linnaeus)
40. [*impudicus* (Ficalbi, 1890)] **(QR) Note 19**
41. *laticinctus* Edwards, 1913
42. *mimeticus* (Noe, 1899) **Note 20**
43. *pipiens* Linnaeus, 1758 **Note 21**
44. *theileri* Theobald, 1903
45. *torrentium* Martini, 1925 **Note 22**

Subgenus *Neoculex* (Dyar)
46. *martinii* Medschid, 1930
47. *territans* Walker, 1856

Subgenus *Maillotia* Theobald, 1907
48. *hortensis* Ficalbi, 1889 **Note 23**

Tribe Culisetini
Genus *Culiseta* Felt
Subgenus *Allotheobaldia* (Broelemann)
49. *longiareolata* (Macquart, 1938)
Subgenus *Culiseta* (Felt)
50. *alaskaensis* (Ludlow, 1906)
51. *annulata* (Schrank, 1776) **Note 24**

52. *fumipennis* (Stephens, 1825)
53. *glaphyroptera* (Schiner, 1864) **Note 25**
54. *morsitans* (Theobald, 1901)
55. *subochrea* (Edwards, 1921)

Subgenus *Culicella* Felt
56. *ochroptera* (Peus, 1935)

Tribe Mansoniini (Dyar)
Genus *Coquillettidia* (Dyar)
57. *buxtoni* (Edwards, 1923)
58. *richiardii* (Ficalbi, 1889)

Tribus Orthopodomyiini

Genus *Orthopodomyia* Theobald, 1904
59. *pulcripalpis* (Rondani, 1872)

Tribus Uranotaeniini
Genus *Uranotaenia* (Lynch Arribalzaga)
60. *unguiculata* Edwards, 1913

Note 1: *An. algerinesis* was named after Algeria where it was collected for the first time, but the species has larger distribution in Europe (with several data from the Mediterranean area), Middle-East and North Africa. This species was recorded in Romania for the first time in the Danube Delta, close to Sulina (Török *et al.,* 2016). The specimens were collected in April and September 2014 (Török *et al.,* 2016).
Note 2: *An. claviger* is a well-known species with medical importance as it is a vector species of the malaria pathogens and some mosquito-borne viruses, such as Batai and Tahyna viruses. The species avoids lowland ecosystems (Hubalek 2008, Becker *et al.,* 2010). This species was collected by the authors in Floreşti, near the Someşul Mic River, at 354 m a.s.l, (with coordinates 46.759912° N, 23.531731° E) on 24 August 2017, 3 males, leg. Kolcsár L.-P.L.-P, Török E.
Note 3: *A*n. *maculipennis* s.l. contains a series of sibling species (female imagoes are morphologically similar) which are all important vectors of malaria pathogens and mosquito-borne viruses like Batai, Tahyna and West Nile viruses (Hubalek 2008, Becker et al., 2010)**.** We observed that the flying period of this species complex is from spring to autumn, and can be found resting in shaded places and flying below the crown of the trees.
We collected 2 females at Floreşti, near the Someşul Mic River, at 354 m a.s.l. (coordinates 46.759912° N, 23.531731° E), on 24 August 2017, leg. Kolcsár L.-P. and 1 male at Ciaracio, Ciuc Basin, near Agris brook valley, at 679 m, (coordinates 46.412255° N, 25.739988° E), on 10 August 2017, leg. Ujvárosi B.

Note 4: *Ae. geminus* is a less known species and its vector potential is unknown. During our investigation we collected 2 males, 1 female at Voşlobeni, Giurgeu Basin, Senetea, at 764 m a.s.l., (coordinates 46.625875° N, 25.597453° E) on 6 July 2017, leg. Keresztes L.; 1 male, in the same location, on 16 July 2016, leg. Kolcsár L.-P.; 1 male, in Voşlobeni, Giurgeu Basin, near Mureş River, at 754 m, (coordinated 46.636571° N, 25.59146° E), on 6 June 2017, leg. Kolcsár L.-P., Török E.; 1 female at Cluj-Napoca, Alexandru Borza Botanical Garden (Malaise trap), at 395 m, (coordinates 46.761322° N, 23.586521° E) on 19 May 2015, leg. Kolcsár L.-P.; 1 female, Ciaracio, Ciuc Basin, near Agris brook valley, at 679 m, (coordinates 46.412255° N, 25.739988° E), on 10 August 2017 leg. Ujvárosi B.; 5 females, Breaza, Breaza forest, at 392 m, (coordinates 46.755395° N, 24.616005° E) on 9 June 2017, leg. Kolcsár L.-P., Török E.; 1 female, in Gurghiu, dendrological park, at 430 m, (46.773673° N, 24.861017° E) on 5 July 2017, leg. Török E.

Note 5: *Ae. rossicus* has been recorded in neighboring countries, such as Hungary (Tóth and Kenyeres 2012). The Online Catalog of Culicidae (Schaffner *et al.,* 2001) and MosKeyTool (Gunay *et al.,* 2017) list report the species also from Romania. Nicolescu (1995) considered the species as present in Romania based on previous literature data (Mihály 1959). Based on this remarks we include the species in the present checklist.

Note 6: *Ae. vexans* is a common species in Romania. It is a multivoltin species with several generations per year. It was collected frequently in forest ecosystems. It is a very aggressive species, feeding mostly on human blood and transmitting Tahyna and West Nile viruses (Hubalek, 2008, Becker *et al.,* 2010).

Material used in the present study: Voşlobeni, Giurgeu Basin, Senetea, 764 m, 46.625875° N, 25.597453° E, 16 July 2017, 1 male, leg. Kolcsár L.-P., Török E.; same location, 20 July 2017, 2 males, leg. Kolcsár L.-P.; same location, 22 April 2016, 1 male, leg. Kolcsár L.-P., Török E.; Voşlobeni, Giurgeu Basin, Mureş River, 754 m, 46.636571° N, 25.59146° E, 6 June 2017, 3 females, leg. Kolcsár L.-P., Török E.; Breaza, Breaza forest, 392 m, 46.755395° N, 24.616005° E, 9 June 2017, 9 females, leg. Kolcsár L.-P., Török E.; Doda Pilii, Apuseni Mts., 1023 m, 46.646172° N, 22.848041° E, 1 July 2016, 1 female, leg. Kolcsár L.-P., Török E.; Gurghiu, dendrological park, 430 m, 46.773673° N, 24.861017° E, 5 July 2017, 6 males, 36 females, leg. Ujvárosi B., Török E.; Crestur, Csatári forest, 164 m, 47.359438° N, 22.245955° E, 17 July 2017, 9 females, leg. Török E.; Cluj-Napoca, Alexandru Borza Botanical Garden (Malaise trap), 395 m, 46.761322° N, 23.586521° E, 11 June 2015, 2 males, leg. Kolcsár L.-P.; same location, 22 May 2017, 2 males, leg. Kolcsár L.-P.; Floreşti, Someşul Mic River, 354 m, 46.759912° N, 23.531731° E, 24 August 2017, 1 male, leg. Kolcsár L.-P.; Ciaracio, Ciuc Basin, Agris brook valley, 679 m, 46.412255° N, 25.739988° E, 10 August 2017, 4 females, leg. Ujvárosi B.

Note 7: *Ae. geniculata* is a typical forest species, breeds in tree-holes. Females prefer to feed on humans. They have one or two generations per year. The species was detected positive for West Nile virus (Hubalek 2008, Becker *et al.,* 2010). Material: Voşlobeni, Giurgeu Basin, Mureş River, 754 m, 46.636571° N, 25.59146° E, 6 June 2017,

3 females, leg. Kolcsár L.-P., Török E.; Voşlobeni, Giurgeu Basin, Senetea, 764 m, 46.625875° N, 25.597453° E, 6 July 2017, 1 female, leg. Keresztes L.; same location, 20 July 2017, 1 male, leg. Keresztes L.; Gurghiu, dendrological park, 430 m, 46.773673° N, 24.861017° E, 5 July 2017, 2 males, 2 females, leg. Ujvárosi B., Török E.; Glajarie, Gurghiu Mts., 885 m, 46.857766° N, 24.974917° E, 6 July 2017, 1 female, leg. Kolcsár L.-P., Török E.; Cluj-Napoca, Mikó Garden, 337 m, 46.763588° N, 23.580218° E, 10 August 2015, 2 females, leg. Kolcsár L.-P.; Cluj-Napoca, Feleacu hills, Sáros-bükk marsh, 459 m, 46.69262° N, 23.55124° E, 15 April 2017, 2 females, leg. Keresztes L., Ujvárosi B.; Măguri - Răcătău, Gilău Mts., Someşul Rece River, 585 m, 46.665605° N, 23.242337° E, 11 August 2017, 1 male, leg. Kolcsár L.-P.

Note 8: *Ae. (Oc.) annulipes* is a widely distributed species, occurs mostly in forest ecosystems, it is univoltin. It is the suspected vector of Tahyna virus (Hubalek 2008, Becker *et al.,* 2010).

Material: Voşlobeni, Giurgeu Basin, Mureş River, 754 m, 46.636571° N, 25.59146° E, 6 June 2017, 3 males, 7 females, leg. Kolcsár L.-P., Török E.; Voşlobeni, Giurgeu Basin, Senetea, 764 m, 46.625875° N, 25.597453° E, 20 July 2017, 2 females, leg. Kolcsár L.-P., Török E.; Gurghiu, dendrological park, 430 m, 46.773673° N, 24.861017° E, 5 July 2017, 16 females, leg. Ujvárosi B., Török E.; Glajarie, Gurghiu Mts., 885 m, 46.857766° N, 24.974917° E, 6 July 2017, 2 females, leg. Kolcsár L.-P., Török E.; same location, 29 June 2017, 2 females, leg. Kolcsár L.-P., Török E.; Ciaracio, Ciuc Basin, Agris brook valley, 679 m, 46.412255° N, 25.739988° E, 10 August 2017, 1 female, leg. Ujvárosi B.; Crestur, Csatári forest, 164 m, 47.359438° N, 22.245955° E, 17 July 2017, 6 females, leg. Török E.

Note 9: *Ae. (Oc.) behningi* has been recorded from Moldova (Sulesco *et al.,*2013). The Online Catalog of Culicidae (Schaffner *et al.,* 2001) and MosKeyTool (Gunay *et al.,* 2017) list the species from Romania. Nicolescu (1986) suggested the presence in the Romanian fauna based on former literature data (Zotta, 1932, Giurca, 1982).

Note 10: *Ae. (Oc.) cantans* has a long lasting active period, distributed mostly in forest ecosystems. It is a univoltin species; it has one generation per year. West Nile virus could be transmitted by this species (Hubalek 2008, Becker *et al.,*2010).

Material: Gurghiu, dendrological park, 430 m, 46.773673° N, 24.861017° E, 5 July 2017, 5 females, leg. Kolcsár L.-P., Török E.; Voşlobeni, Giurgeu Basin, Mureş River, 754 m, 46.636571° N, 25.59146° E, 6 June 2017, 1 male, 7 females, leg. Kolcsár L.-P., Török E.; Cluj-Napoca, Feleacu hills, Sáros-bükk marsh, 459 m, 46.69262° N, 23.55124° E, 15 April 2017, 1 female, leg. Keresztes L., Ujvárosi B.; Cluj-Napoca, Mikó garden, 337 m, 46.763588° N, 23.580218° E, 16 May 2016, 1 male, 1 female, leg. Kolcsár L.-P.; Ciaracio, Ciuc Basin, Agris brook valley, 679 m, 46.412255° N, 25.739988° E, 10 August 2017, 2 females, leg. Ujvárosi B.; Gurghiu, dendrological park, 430 m, 46.773673° N, 24.861017° E, 5 July 2017, leg. Ujvárosi B., Török E.

Note 11: *Ae. (Oc.) caspius* is a multivoltin vector species. It prefers saline habitat, but a small number of individuals could be frequently detected from forest hillsides. West Nile virus and Tahyna virus could be transmitted by this species (Hubalek 2008, Becker *et al.,* 2010).
Material: Gurghiu, dendrological park, 430 m, 46.773673° N, 24.861017° E, 5 July 2017, 1 female, leg. Ujvárosi B., Török E.
Note 12: *Ae. (Oc.) cataphylla* is an univoltin species. It feeds mostly on human blood (Hubalek, 2008, Becker *et al.,* 2010). Peak of its biting activity is during dusk, also at strongly shaded places and can be troublesome before rains even during daytime. The species is active mostly in summer.
Material: Gurghiu, dendrological park, 430 m, 46.773673° N, 24.861017° E, 5 July 2017, 6 females, leg. Ujvárosi B., Török E.; Ciaracio, Ciuc Basin, Agris brook valley, 679 m, 46.412255° N, 25.739988° E, 10 August 2017, 1 female, leg. Ujvárosi B.; Voşlobeni, Giurgeu Basin, Senetea bog, 760 m, 46.625875° N, 25.597453° E, 16 July 2016, 1 female, leg. Kolcsár L.-P.; same location 6 July 2017, 1 female, leg. Keresztes L.; Doda Pilii, Bihor Mts., Cheile Someşului Cald, 1200 m, 46.637443° N, 22.718924° E, 1 July 2016, 2 females, leg. Kolcsár L.-P., Török E.; Smida, Gilău Mts., Belis dam lake, 1000 m, 46.641554° N, 22.889426° E, 30 June 2016, 2 females, leg. Kolcsár L.-P., Török E.
Note 13: Biology of *Ae. (Oc.) communis* is not well known. We were able to collect only a small number of individuals.
Material: Voşlobeni, Giurgeu Basin, Mureş River, 754 m, 46.636571° N, 25.59146° E, 6 June 2017, 2 females, leg. Kolcsár L.-P., Török E.
Note 14: *Ae. (Oc.) nigrinus* was recorded in neighboring countries (Hungary, Tóth and Kenyeres 2012). The Online Catalog of Culicidae (Schaffner *et al.,* 2001) and MosKeyTool (Gunay *et al.,* 2017) list the species from Romania. Nicolescu (1995) listed this species from Romania based on former literature data (Mihály 1959). We agree with the presence of the species in Romania.
Note 15: *Ae. (Oc.) pullatus* has only one generation per year. It has not yet been detected as a vector species for any pathogens. The females are active all day.
Material: Smida, Gilău Mts., Belis dam lake, 1000 m, 46.641554° N, 22.889426° E, 30 June 2016, 5 females, leg. Kolcsár L.-P., Török E.; Doda Pilii, Bihor Mts., Cheile Someşului Cald, 1200 m, 46.637443° N, 22.718924° E, 1 July 2016, 2 males, 2 females, leg. Kolcsár L.-P., Török E.; Padiş, Bihor Mts., Cheile Someşului Cald, 1159 m, 46.64° N, 22.736036° E, 1 July 2016, 1 male, leg. Kolcsár L.-P., Török E.; Gurghiu, dendrological park, 430 m, 46.773673° N, 24.861017° E, 5 July 2017, 2 females, leg. Ujvárosi B., Török E.
Note 16: *Ae. (Oc.) punctor* is a "snow-melt" mosquito, having only one generation per year. It is a vector species, which could transmit West Nile and Tahyna viruses (Hubalek 2008, Becker *et al.,* 2010). The females are active all day long.

Material: Cluj-Napoca, Alexandru Borza Botanical Garden (Malaise trap), 395 m, 46.761322° N, 23.586521° E, 22 May 2017, 1 male, leg. Kolcsár L.-P.; Voşlobeni, Giurgeu Basin, Senetea bog, 760 m, 46.625875° N, 25.597453° E, 22 April 2016, 6 males, 1 female, leg. Kolcsár L.-P.; Voşlobeni, Giurgeu Basin, Mureş River, 754 m, 46.636571° N, 25.59146° E, 6 June 2017, 9 females, leg. Kolcsár L.-P., Török E.; Doda Pilii, Bihor Mts., Cheile Someşului Cald, 1200 m, 46.637443° N, 22.718924° E, 1 July 2016, 4 females, leg. Kolcsár L.-P., Török E.; Voşlobeni, Giurgeu Basin, Senetea bog, 760 m, 46.625875° N, 25.597453° E, 20 July 2017, 1 female, leg. Keresztes L.; Gurghiu, dendrological park, 430 m, 46.773673° N, 24.861017° E, 5 July 2017, 1 female, leg. Ujvárosi B., Török E.; Smida, Gilău Mts., Belis dam lake, 1000 m, 46.641554° N, 22.889426° E, 30 June 2016, 1 female, leg. Kolcsár L.-P., Török E.

Note 17: *Ae. (Oc.) sticticus* prefers water-pits in forests. The species has several generations per year, and migrates long distances from breeding-sites to available habitats.

Material: Breaza, Breaza forest, 392 m, 46.755395° N, 24.616005° E, 9 June 2017, 1 female, leg. Kolcsár L.-P., Török E.; Gurghiu, dendrological park, 430m, 46.773673° N, 24.861017° E, 5 July 2017, 3 females, leg. Ujvárosi B., Török E.; Voşlobeni, Giurgeu Basin, Mureş River, 754 m, 46.636571° N, 25.59146° E, 6 June 2017, 3 females, leg. Kolcsár L.-P., Török E.; Voşlobeni, Giurgeu Basin, Senetea bog, 760 m, 46.625875° N, 25.597453° E, 20 July 2017, 1 male, 3 females, leg. Keresztes L.

Note 18: *Cx. modestus* is a widely distributed, multivoltin species. Females bite humans and are considered very aggressive . This species could be found mostly around the larval breeding sites. It is a vector species for West Nile, Tahyna, and Sindbis viruses (Hubalek, 2008, Becker *et al.,* 2010).

Material: Voşlobeni, Giurgeu Basin, Mureş River, 754 m, 46.636571° N, 25.59146° E, 6 June 2017, 16 females, leg. Kolcsár L.-P., Török E.; Voşlobeni, Giurgeu Basin, Senetea bog, 760 m, 46.625875° N, 25.597453° E, 16 July 2016, 5 females, leg. Kolcsár L.-P.; same location, 20 July 2017, 1 male, leg. Keresztes L.; Voşlobeni, Giurgeu Mts., Súgó brook valley, 905 m, 46.665022° N, 25.652262° E, 8 Julne 2017, 1 female, leg. Keresztes L.; Cluj-Napoca, Alexandru Borza Botanical Garden (Malaise trap), 395 m, 46.761322° N, 23.586521° E, 20 July 2017, 1 female, leg. Kolcsár L.-P.; Doda Pilii, Bihor Mts., Cheile Someşului Cald, 1200 m, 46.637443° N, 22.718924° E, 1 July 2016, 1 male, leg. Kolcsár L.-P., Török E.; Ciaracio, Ciuc Basin, Agris brook valley, 679 m, 46.412255° N, 25.739988° E, 10 August 2017, 1 female, leg. Ujvárosi B.

Note 19: Presence of *Cx. impudicus* in Romania is questionable. We did not fiund any reliably published record about the species in Romania and it has not been recorded in the neighboring countries either. However, the Online Catalog of Culicidae (Schaffner *et al.,* 2001) and MosKeyTool (Gunay *et al.,* 2017) list the species from Romania. Based on this remarks we include it in the present list.

Note 20: *Cx. mimeticus* occurs in Hungary (Tóth and Kenyeres 2012) and it is mentioned in the Online Catalog of Culicidae as member of the Romanian fauna, as well (Schaffner *et al.,* 2001). Based on former literature data (Sicart *et al.,* 1961, Motaş *et al.,* 1962, Nicolescu (1995) the potential presence of the species in Romania is acceptable.

Note 21: *Cx. pipiens* s.l. species group contains important vector species, which can transmit many zoonotic pathogens including human diseases. The complex consists of several species, subspecies, forms, races, physiological variants, or biotypes according to various authors (Farajollahi *et al.,*2011). Up to the present, no taxonomic investigation on the *Cx. pipiens* complex from Romania has been initiated and further cryptic species may be expected. The subspecies *Cx. pipiens molestus* has no reliable faunistic data from the country.
Material: Valişoara, Trascău Mts., Valişoarei Cayon, 520 m, 46.384371° N, 23.575915° E, 14 November 2017, 1 female, leg. Kolcsár L.-P.; Doda Pilii, Apuseni Mts., Firei valley, 1045 m, 46.664635° N, 22.8439377° E, 2 January 2018, 1 male, leg. Keresztes L.; Cluj-Napoca, Mikó garden, 337 m, 46.763588° N, 23.580218° E, 10 August 2015, 1 female, leg. Kolcsár L.-P.; Cluj-Napoca, Alexandru Borza Botanical Garden (Malaise trap), 395 m, 46.761322° N, 23.586521° E, 14 July 2015, 1 female, leg. Kolcsár L.-P.; same location, 22 May 2017, 1 female, leg. Kolcsár L.-P.; same location, 15 October 2015, 3 males, leg. Kolcsár L.-P.; same location, 2 November 2017, 2 males, leg. Kolcsár L.-P.; 26 September 2017, 1 male, 1 female, leg. Kolcsár L.-P.; Cluj-Napoca, Feleacu hills, Sáros-bükk marsh, 495 m, 46.69262° N, 23.55124° E, 15 April 2017, 1 female, leg. Keresztes L., Ujvárosi B.; Cluj-Napoca Sáros-bükk marsh, 459 m, 46.69262° N, 23.55124° E, 29 November 2017, 4 males, leg. Keresztes L., Ujvárosi B.; Voşlobeni, Giurgeu Basin, Senetea, 764 m, 46.625875° N, 25.597453° E, 16 July 2016, 1 female, leg. Kolcsár L.-P.; same location, 7 June 2017, 1 male, leg. Keresztes L.; same location, 20 July 2017, 4 males, 1 female, leg. Keresztes L.; Ciaracio, Ciuc Basin, Agris brook valley, 679 m, 46.412255° N, 25.739988° E, 10 August 2017, 3 females, leg. Ujvárosi B.; Breaza, Breaza forest, 392 m, 46.755395° N, 24.616005° E, 9 June 2017, 1 female, leg. Kolcsár L.-P., Török E.; Floreşti, Someşul Mic River, 354 m, 46.759912° N, 23.531731° E, 24 August 2017, 16 males, leg. Kolcsár L.-P.; Marghita, 135 m, 47.343892° N, 22.331358° E, 21 December 2017, 12 females, leg. Török E.; same location, 8 February 2018, 6 females, leg. Török E.; Gurghiu, dendrological park, 430 m, 46.773673° N, 24.861017° E, 5 July 2017, 9 females, leg. Ujvárosi B., Török E.; Marişel, Gilău Mts., Someşul Cald River, 577 m, 46.699827° N, 23.195381° E, 28 August 2016, 1 male, leg. Keresztes L.
Note 22: *Cx. torrentium* is a multivoltin species. It avoids feeding on humans, preferring birds instead (Becker *et al.,* 2010).
Material: Izvoru Mureşului, Giurgeu mts., Mureş River spring, 925 m, 46.633739° N, 25.67577° E, 16 July 2016, 2 males, leg. Kolcsár L.-P.; Gurghiu, dendrological park, 430 m, 46.773673° N, 24.861017° E, 5 July 2017, 1 male, leg. Ujvárosi B., Török E.; Voşlobeni, Giurgeu Basin, Senetea bog, 760 m, 46.625875° N, 25.597453° E, 16 July 2016, 1 male, leg. Kolcsár L.-P.
Note 23: *Cx hortensis* feeds mostly on amphibians and reptiles, having numerous generations per year (Becker *et al.,* 2010).

Material: Vălișoara, Trascău Mts., Valișoarei Cayon, 520 m, 46.384371° N, 23.575915° E, 14 November 2017, 1 male, leg. Kolcsár L.-P.; Breaza, Breaza forest, 392 m, 46.755395° N, 24.616005° E, 9 June 2017, 1 female, leg. Kolcsár L.-P., Török E.

Note 24: *Cs. annulata* has more than one generation per year. It is active all year round, and females are overwintering as imagoes. We found the species on walls inside of buildings and caves during winter.
Material: Cluj-Napoca, Alexandru Borza Botanical Garden (Malaise trap), 395 m, 46.761322° N, 23.586521° E, 26 February 2017, 1 male, leg. Kolcsár L.-P.; Breaza, Breaza forest, 392 m, 46.755395° N, 24.616005° E, 9 June2017, 1 female, leg. Kolcsár L.-P., Török E.; Cluj-Napoca, Mikó garden, 337 m, 46.763588° N, 23.580218° E, 20 July 2016, 1 female, leg. Török E.; same location, 26 November 2017, 1 female, leg. Török E.; Vălișoara, Trascău Mts., Vălișoarei Cayon, 520 m, 46.384371° N, 23.575915° E, 14 November 2017, 2 females, leg. Kolcsár L.-P.; Crestur, 160 m, 47.346511° N, 22.239962° E, 20 July 2017, 7 females, leg. Török E.
Note 25: *Cs. glaphyroptera* is a cold relict species. It lives in forest habitats. The species feeding preferences are related to different species of birds (Hubalek, 2008, Becker *et al.,* 2010).
Material: Hagota, Giurgeu Mts., Tisasul valley, 860 m, 46.861794° N, 25.677228° E, 11 February 2016, 2 males, leg. Kolcsár L.-P.; Ciaracio, Ciuc Basin, Agris brook valley, 679 m, 46.412255° N, 25.739988° E, 10 August 2017, 1 female, leg. Ujvárosi B.

Conclusions

The first checklist of the Culicidae fauna of Romania was published in 1995 by Nicolescu. He presented 50 Culicidae. In the present study, we updated this checklist and increased the number of species to 60. Our faunistic results of the last few years focused mainly on some less studied areas in Romania such as Transylvania, from where new faunistic data on 20 different Culicidae species were recorded.

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Multiple impact assessment and water quality based on diatom, benthic invertebrate and fish communities in the Arieș River catchment area (Transylvania, Romania)

Karina Paula Battes[1], Mirela Cîmpean[1,✉], Laura Momeu[1],
Anca Avram[2], Klaus Werner Battes[3] and Ionuț Viorel Stoica[3]

SUMMARY. The present paper represents an assessment of human impacts affecting the Arieș River catchment area, a region heavily affected by the mining industry documented in the middle river course (Roșia Montană, Abrud, Roșia Poieni) since the Roman period. Other important impacts in the study area were: eutrophication / organic pollution due to discharges of untreated domestic wastes of villages and towns from the region; river regularization works, wood exploitation and processing facilities and industrial wastes downstream Turda and Câmpia Turzii localities. Water quality evaluation was carried out using river biotic communities recommended by the European legislation (Water Framework Directive, WFD): diatoms, benthic invertebrates and fish. Twenty-three sampling sites were considered along the Arieș River main course and its main tributaries, and standardized methods were employed for sampling and processing of biological data. Benthic invertebrates prooved to be the most sensitive community, indicating disturbed ecological status downstream the mining-affected region mainly due to high contamination of river sediments. While ichthyofauna responses were moderate (with water quality classes usually ranging from high to moderate), diatoms reflected better the effects of eutrophication / organic pollution caused by human settlements.

Keywords: biotic indices, physico-chemical parameters, trade-off analysis, Water Framework Directive.

[1] *Babeș-Bolyai University, Faculty of Biology and Geology, Department of Taxonomy and Ecology, 5-7 Clinicilor Str., 400006, Cluj-Napoca, Romania.*
[2] *Romanian Waters National Administration, Someș-Tisa Branch, 17 Vânătorului Str., Cluj-Napoca Romania.*
[3] *Vasile Alecsandri University of Bacău, Faculty of Sciences, Department of Biology, 157 Calea Mărășești Str., 600115, Bacău, Romania.*
✉ ***Corresponding author: Mirela Cîmpean,*** *Babeș-Bolyai University, Faculty of Biology and Geology, Department of Taxonomy and Ecology, Cluj-Napoca,*
E-mail: mirela.cimpean@ubbcluj.ro

Introduction

Compared to terrestrial systems, freshwaters are more susceptible to degradation, due to synergic effects of multiple human pressures: pollution from agriculture and industrial areas, domestic wastes, hydromorphological alterations, overexploitation of resources etc. (Allan and Castillo, 2007). All these activities severely affect freshwater biota, which is currently facing a biodiversity crisis (Revenga and Kura, 2003). Streams are among the most affected ecosystems, due mainly to changes in water chemistry (organic pollution, nutrients, acidifiation), habitat alteration and distruction, and species removal or additon (Malmqvist and Rundle, 2002).

In this context, the development of impact assessment methodologies and techniques are imperative (Anjaneyulu and Manickam, 2007). Monitoring programs for river water quality should include hydrological, hydromorphological, physico-chemical and biological parameters (Chapman (ed.), 1996). Current European water legislation, the Water Framework Directive WFD (Directive 2000/60/EC) stressed the importance of bioassessment in surface water quality assessment and monitoring, using indicator communities like algae, macroinvertebrates and fish, sensitive to habitat degradation, land-use effects or toxicity (Heiskanen *et al*., 2004; Hering *et al*., 2006; Solimini *et al*. (eds.), 2006).

Benthic algae, especially diatoms, are considered to be the most important primary producers in streams, because they are found in nearly all running waters and fluvial food webs (Allan and Castillo, 2007). Numerous algal biological indices are based on diatoms, due to their high ecological diversity, short life cycles, or promt answer to short and long term changes of water (Dokulil, 2003; Wang *et al*., 2014; Bellinger and Sigee, 2015). Since benthic organisms have limited mobility, their presence or absence is most likely to be associated with alterations in their environmental conditions (Chapman (ed.), 1996; Malmqvist, 2002). This is the reason why benthic invertebrates are commonly used in water quality assessment studies. They exhibit a wide diversity of form, tolerance to habitat parameters and adaptation to survival in different conditions (Resh and Rosenberg (eds.), 1993; Kenney *et al*., 2009). Fish on the other hand are associated with certain river habitats or areas, and they are very sensitive to changes in water physical, chemical and biological quality, so they are extensively used in water quality assessment studies (Chapman (ed.), 1996; Hering *et al*., 2006; Trautwein *et al*., 2013).

The Arieş River catchment area was considered for the present study for two reasons. Firstly, it represents the largest right tributary of the Mureş River, with a total course length of 167 km and a catchment area of almost 3000 sqkm (Ujvari, 1972; Bătinaş, 2010). Secondly, various human activities cause a wide range of impacts in different reaches of the river: human settlements and hydromorphological alterations are present from the headwaters to mouth, similar to industrial operations like mining or wood processing, that have severe consequences on the environment (Bătinaş, 2010).

This is why the impacts of mining industry in the middle reach of the Arieș River represented the topic of numerous previous studies conducted in the area, focusing on the influence of polluted right tributaries draining the Roșia Montană - Roșia Poieni regions (Forray, 2002; Senila *et al*., 2007; Whitehead *et al*., 2009; Bătinaș, 2010; Levei *et al*., 2013; Voica *et al.,* 2013; Levei *et al*., 2014). Pathogenic germs (Bodoczi, 2009) and algae (Péterfi and Momeu, 1984, 1985; Momeu and Péterfi, 2007; Butiuc-Keul *et al.,* 2012; Olenici *et al*., 2017) were also considered. Water quality was only assessed from the hyporheic zone (Moldovan *et al*., 2011; 2013), or using the saprobian system (data from the Romanian Waters National Administration, cited in Bătinaș, 2010). Diatom, invertebrate and fish communities considered for the present study were described in Momeu *et al.* (2007) and Momeu *et al*. (2009).

In this context, the aim of the present paper was to assess the major impacts from the Arieș River and its main tributaries, related to the water quality based on three biotic communities indicated by the WFD. Diatoms, benthic invertebrates and fish communities yielded comparable water quality classes, correlated with the total impact score, but benthic invertebrates were more susceptible to degradation.

Materials and methods

A number of 23 sampling sites was considered for diatoms and benthic invertebrates: 15 on the main river course (AR1-The Arieș source; AR2-Arieşeni: ski track; AR3-Arieşeni: village; AR4-Gârda; AR5-Upstream Albac; AR6-Downstream Albac; AR7-Upstream Câmpeni; AR8-Downstream junction with the Abrud; AR9-Valea Lupşii; AR10-Brăzeşti; AR11-Upstream junction with the Valea Ocoliș; AR12-Moldoveneşti; AR13-Downstream junction with the Hăşdate; AR14-Upstream junction with the Racoşa; AR15-Luncani) and 8 on several tributaries (T1-The Gârda Seacă; T2-The Albac; T3-The Arieşul Mic; T4-The Abrud; T5-The Pârâul Şesii; T6-The Valea Ocoliș; T7-The Hăşdate; T8-The Racoşa). Fish communities were analyzed in 15 sites (Fig. 1). Standardized methods were used in sampling and analyzing biotic communities (Momeu *et al*., 2009).

Water quality was assessed using data sets collected in 2007, with the exception of diatoms (where 2006 samples were also included), and fish (where samples from 2005 for AR4 and 2006 for T7 were used, from Ureche *et al*., 2007 and Pricope *et al*., 2009). Several biotic indices were considered for water quality assessment (Table 1). Water quality was ranked using classes defined in the WFD: 1(high), 2(good), 3(moderate), 4(poor), 5(bad). Class 6 was added (no organisms found at site) (Fig. 1).

Table 1.

List of biotic indices used for water quality assessment in the present study

Index	Description
DBI: Diatom Biological Index (IBD)	- based on diatoms; references: Lenoir and Coste (1996); Prygiel and Coste (eds.) (2000); - quantitative, counts > 400 individuals; - identifications to species level; - output: water quality classes from 1 (high) to 5 (bad).
SI: Saprobity Index	- based on diatoms; references: Zelinka and Marvan (1961); - semi-quantitative; frequency ranging from 1 (not frequent) to 5 (dominant species); - identifications to species level; - output: water quality classes based on saprobity: from xenosaprobic (very clean) to polysaprobic waters (heavily polluted, very high loads of organic matter).
BMWP: Biological Monitoring Working Party	- based on benthic invertebrates; references: Hawkes (1998) for UK; Dumnicka *et al*. (2006) for BMWP-PL (adapted for Poland); - identifications to family level for all taxa; - output: water quality classes from 1 (high) to 5 (bad).
ASPT: Average Scor Per Taxon	- based on benthic invertebrates; references: Armitage *et al*. (1983); - identifications to family level for all taxa; - calculated as BMWP divided by the total number of families per sample; - output: water quality classes from 1 (high) to 5 (bad).
EBI: Extensive Biotic Index (IBE)	- based on benthic invertebrates; references: Ghetti (1997); - identifications to family level for all taxa, except for Plecoptera, Ephemeroptera, Turbellaria and Hirudinea, identified to genus level; - output: water quality classes from 1 (high) to 5 (bad).
NGBI: Normalized Global Biotic Index (IBGN)	- based on benthic invertebrates; references: AFNOR (1992); - identifications to family level for all taxa; - output: water quality classes from 1 (high) to 5 (bad).
IBI: Index of Biological Integrity	- based on fish; references: Karr (1981); - parameters used: species composition and richness, trophic structure, fish stock and biomass; - output: integrity classes from 1 (unchanged gene pool of native ichthyofauna) to 5 (originally 9) (fish population disappeared entirely, mostly due to long-term alterations).
EFI+: European Fish Index	- based on fish; references: EFI+ Consortium (2009); - parameters used: species richness and number of individuals; species guilds with respect to habitat and oxygen depletion; - salmonid and cyprindid river types, according to percentage of intolerant species belonging to salmonid dominated fish communities; - output: water quality classes from 1 (high) to 5 (bad).

An adaptation of the *trade-off analysis* was employed to calculate impact scores (Anjaneyulu and Manickam, 2007). Six impact categories were identified in the Arieș catchment area, ranked according to their importance on a scale from 0 (minimum) to 5 (maximum): mining industry: extraction and processing (5); human settlements: local houses and touristic facilities (4); agriculture (3); river regularization (3); wood processing points (3); and industry, other than wood processing and mining (3). A total impact score was then calculated for every sampling site (see Table 2), by multiplying impact importance with impact intensity, also ranked from 0 to 5. The following classes were used: 0=no impact, 1=very low intensity, 2=low intensity, 3=moderate intensity, 4=strong impact, 5=severe impact. For ranking mining industry impacts, only sites located < 10 km downstream from extraction or processing facilities were considered, while for human settlements the following threshold values were used: <5000 inhabitants; 5000-10000 inhabitants; and >10000 inhabitants.

Principal Component Analysis (PCA) was used to observe aggregation trends in the sampling sites based on abiotic characteristics, impact score, number of taxa and water quality classes indicated by biotic communities. Xlstat software 2018.6 was used (Addinsoft, 2018).

Results and discussion

Sampling sites: impact characteristics

Variations of physico-chemical parameters usually reflect the negative effects of human activities in/near rivers. In the Arieș catchment area, temperature and dissolved oxygen values carried little information in this respect, since they recorded annual means that followed an expected pattern (Momeu *et al*., 2009). The mean temperature increased from headwaters to mouth, as described for most temperate rivers (Lampert and Sommer, 2007), while dissolved oxygen recorded constant mean values from headwaters to mouth.

Water conductivity and pH, however, were more sensitive to human pressures. Low pH values were recorded in T4 and T5, two right tributaries that collect waters from Roșia Montană - Roșia Poieni mining area (Momeu *et al*., 2009). Acid waters (pH <5.5, acidity generated through oxidation of Fe-rich sulfides, Lottermoser, 2007) have significant impacts on river systems, because mine effluents and acid rock drainage are associated with high concentrations of metals (Whitehead *et al*., 2009). Heavy metals (Cu, Pb, Zn etc.) and cyanides were reported in the literature, often in concentrations exceeding the legal limits stipulated in M.O. 161/2006 (Table 2).

Table 2.

List and characteristics of impacts for the 23 sampling sites from the Arieș catchment area; S/R - sources/references: *a* - Bătinaș (2010), *b* - Bird *et al*. (2005), *c* - Bodoczi (2009), *d* - Butiuc-Keul *et al*. (2012), *e* - Costan (2010), *f* - Forray (2002), *g* - Levei *et al*. (2013), *h* - Levei *et al*. (2014), *i* - Luca *et al*. (2006), *j* - Senila *et al*. (2007), *k* - Voica *et al*. (2013), *l* - *in situ* observations; for site codes: see text.

Site	Impact categories and characteristics [total impact score in square brackets]	S/R
AR1	1) domestic wastes (local houses and guest houses) [12] 2) wood processing facilities [3]	*l*
AR2	1) domestic wastes (local houses and guest houses) [12] 2) wood processing facilities [6]	*l*
AR3	1) domestic wastes (local houses and guest houses) [12] 2) wood processing facilities [9]	*a, i, l*
T1	1) domestic wastes (local houses and guest houses) [8] 2) wood processing facilities [9]	*l*
AR4	1) domestic wastes (local houses and guest houses) [12] 2) wood processing facilities [9]	*a, i, l*
AR5	1) domestic wastes (local houses and guest houses) [8] 2) wood processing facilities [9]	*a, i, l*
T2	1) domestic wastes (local houses and guest houses) [8] 2) wood processing facilities [9] 3) regularization (banks) [6]	*a, i, l*
AR6	1) domestic wastes (local houses and guest houses) [8] 2) wood processing facilities [9]	*a, i, l*
T3	1) domestic wastes (local houses) [8] 2) wood processing facilities [9]	*l*
AR7	1) domestic wastes (no treatment plant); water eutrophication (high PO_4 values) [16] 2) wood processing facilities [9] 3) regularization (banks) [9] 4) industrial facilities [12]	*a, d, l*
T4	1) domestic wastes (no treatment plant) [12] 2) mining industry (Roșia Montană mining exploitation for Au and Ag, closed in 2006: Cetate quarry, 2 ore dumps, 2 tailing ponds, preparation site in Gura Roșiei): acid waters; high concentrations of Fe, Cu, Zn, Cd, Mn (values exceeding legal limits according to M.O. 161/2006), but also As (arsenic); severe risk of Pb and As contamination from the Gura Roşiei tailing pond; high contamination with Zn in the sediments of the Abrud River [25]	*a, b, f, g, h, i, k, l*
AR8	1) domestic wastes (no treatment plant); water eutrophication (high PO_4 values) [16] 2) mining industry (Roșia Montană mining exploitation, closed in 2006): acid waters; high concentrations of Fe, Cu, Zn, Cd, Mn (values exceeding legal limits according to M.O. 161/2006) [25]	*a, b, d, i, k, l*
AR9	1) domestic wastes; faecal pollution (faecal coliforms / faecal enterococi germs), predominantly animal [12] 2) mining industry (Roșia Montană mining exploitation, closed in 2006) [20] 3) cultivated lands [3]	*c, k, l*
T5	1) domestic wastes [8] 2) mining industry (Abrud mining exploitation for Cu: Roșia Poieni quarry, 3 ore dumps, 3 tailing ponds, preparation site in Roșia Poieni): acid waters; high concentrations of sulfates, Fe, Mn, Cu, Cd (values exceeding legal limits according	*a, b, g, h, i, j, l*

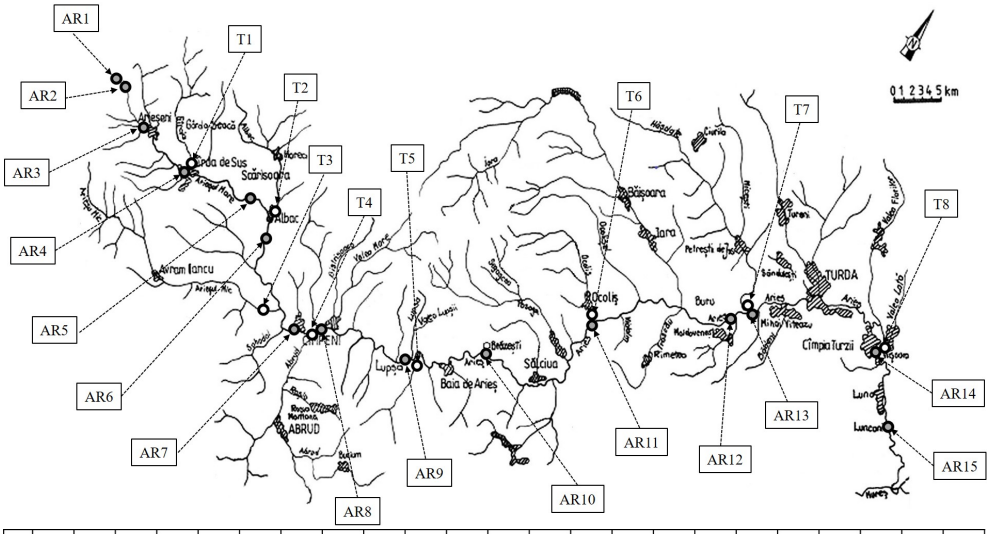
Site	Impact categories and characteristics [total impact score in square brackets]	S/R
	to M.O. 161/2006); severe risk of Cu contamination from the Șesii tailing pond; high contamination with Cu and As in the sediments of the Pârâul Șesii Rivulet [25]	
AR10	1) domestic wastes (no treatment plant); faecal pollution (faecal coliforms / faecal enterococi germs), predominantly animal; water eutrophication (high PO_4 values) [12] 2) mining industry (Baia de Arieș mining exploitation for Au, Ag, sulfides, closed in 2006: subterranean extraction, ore dumps, 4 tailing ponds, preparation plant): acid waters; high concentrations of cyanides, Fe, Cu, Pb, Zn (values exceeding legal limits according to M.O. 161/2006); severe risk of Pb and As (and also Ba) contamination from the Brăzești tailing pond [25]	*a, b, c, d, g, i, k, l*
AR11	1) domestic wastes [8] 2) regularization (exploitation of construction materials in/near the riverbed) [15]	*i, l*
T6	1) domestic wastes [8]	*l*
AR12	1) domestic wastes [12] 2) mining industry (Iara mining exploitation for Fe: mine, tailing pond, waste dump; closed in 2006) [5] 3) cultivated lands [9] 4) regularization (exploitation of construction materials in/near the riverbed) [9]	*b, e, h, l*
T7	1) domestic wastes [12] 2) cultivated lands [3] 3) regularization (exploitation of construction materials in/near the riverbed) [6]	*l*
AR13	1) domestic wastes; water eutrophication (high PO_4 values) [12] 2) cultivated lands [9] 3) regularization (exploitation of construction materials in/near the riverbed) [6]	*d, l*
AR14	1) domestic wastes; high biochemical oxygen demand (BOD) values in effluents coming from the RAGCL Câmpia Turzii treatment plant [20] 2) cultivated lands [12] 3) regularization (exploitation of construction materials in/near the riverbed) [9] 4) industrial facilities (Turda industrial plants: S.C. Holcim S.A., S.C. Sticla S.A., S.C. Electroceramica, S.C. Uzina Chimică Turda, some closed): high concentrations of chlorides, sulfates, Fe, Cu, Pb, Zn, Cd etc. [15]	*a, b, i, l*
T8	1) domestic wastes [20] 2) cultivated lands [12] 3) regularization (exploitation of construction materials in/near the riverbed) [9] 4) industrial facilities (Câmpia Turzii wire production plant, Industria Sârmei Câmpia Turzii): high concentrations of chlorides, sulfates, Fe, Cu, Pb, Zn, Cd etc. [15]	*a, i, l*
AR15	1) domestic wastes; faecal pollution (faecal coliforms / faecal enterococi germs), predominantly human [16] 2) cultivated lands [15] 3) regularization (exploitation of construction materials in/near the riverbed) [9] 4) industrial facilities: high concentrations of Cu and Cd [12]	*b, c, l*

The upper reach of the Arieș River, from AR1 to T3 was mainly impacted by the presence of human settlements without wastewater treatment plants and by wood processing facilities like sawmills, different sawing machines, sawdust deposits etc., but total impact scores were low (< 25) in all sites, except for AR7, where river regularization and industrial impacts also occurred. High impact scores (30-40) were assigned to sites from the middle river stretch (T4 - AR10), due to severe effects of past and present mining activities in the area: discharges and

seepages of acid waters from mines, ore dumps and tailing ponds (both active and inactive), enriched in metals (Fe, Cr, Ni, Pb, Zn, Cu, Cd, As) (Forray, 2002; Bird *et al*., 2005; Whitehead *et al.*, 2009; Bătinaș, 2010; Levei *et al*., 2013; Voica *et al*., 2013; Levei *et al*., 2014). The river recovered downstream this impacted area (AR11, AR12), mainly due to the input of cleaner left tributaries (T6, T7). Thus, impact scores did not exceed 30 and included human settlements, the effects of cultivated land and river regularization. The lower river course (AR14, AR15) was characterized by high impact scores (>50) that added the effects of industrial facilities located in Turda and Câmpia Turzii to the list of impact.

Water quality assessed by biotic indices

Three classes of water quality were assessed at each site, based on diatoms, benthic invertebrates and fish (when available). When biotic indices indicated different quality classes at one sampling site, the worst evaluation was used, inspired by the WFD *one-out, all-out* rule (Fig. 1).



	AR1	AR2	AR3	T1	AR4	AR5	T2	AR6	T3	AR7	T4	AR8	AR9	T5	AR10	AR11	T6	AR12	T7	AR13	AR14	T8	AR15
d	1	1	2	2	3	2	3	2	2	2	6	5	3	6	6	3	3	2	3	3	4	6	4
i	3	2	2	2	1	1	2	2	2	3	5	5	2	6	5	4	2	4	3	4	4	5	5
f			2	2	1	1	1		1	3	6		3	6	6		1	2	2				3

Figure 1. Water quality in the 23 sampling sites from the Arieș River catchment area: *d* - diatoms; *i* - benthic invertebrates; *f* -fish; *1* to *5*: water quality classes and their colour code, according to WFD (*1*- high, *2*- good, *3*- moderate, *4*- poor, *5*- bad); *6* - no organisms found at site; white squares - no fish data; for site codes: see text.

Quality classes assessed by the three biotic communities were congruent, but not identical: they showed relatively good quality in the upper river reaches (AR1-T3), the worst quality in the middle river segment affected by mining activities (T4 - AR10) and moderate to bad quality in the lower stretch of the river (AR13 - AR15) (Fig. 1).

Strong negative correlations (Pearson coefficient $r > -0.576$; $p < 0.025$) were observed between water quality classes and the number of taxa, for all biotic communities. PCA biplot (Fig. 2) depicted these relationships: the higher the number of taxa, the smaller the value of the water quality class (i.e. classes 1 - 2, meaning good ecological status). This tends to be self-evident, despite the fact that pristine ecosystems are known to harbor lower number of taxa, perfectly adapted to undisturbed conditions (the *intermediate disturbance hypothesis*, Connell, 1978). The strong positive correlation between the total impact score and the water quality reflected by benthic invertebrates was also clearly represented ($r = 0.673$; $p = 0.006$), meaning that high impact scores were found in sites with water quality classes of 4 to 6 (degraded ecological status) (Fig. 2). The PCA biplot also showed that low pH and high conductivity values also correlated with inferior water quality.

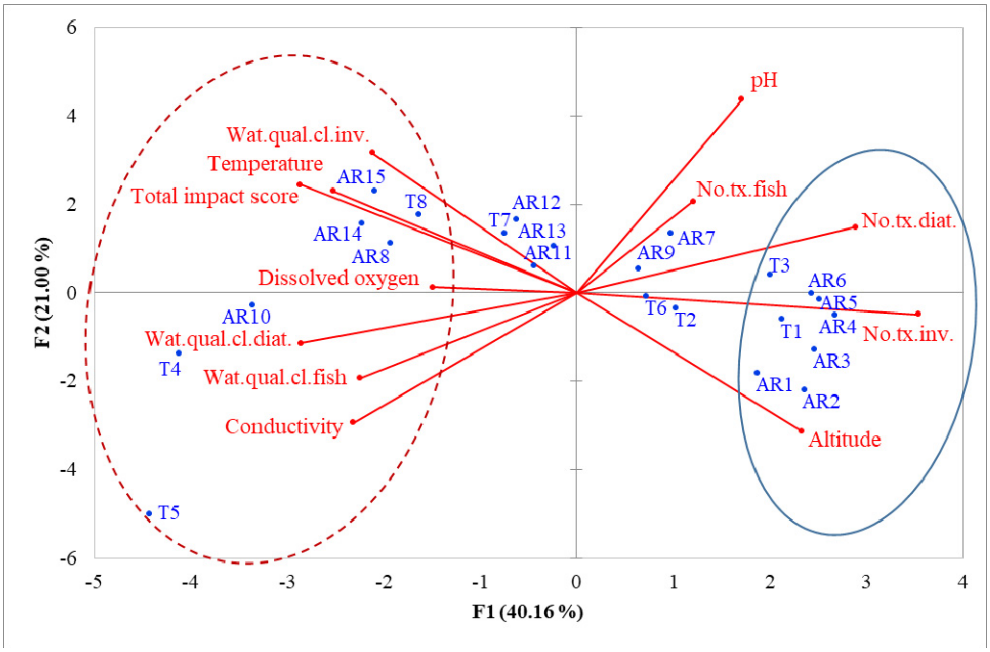


Figure 2. Principal Component Analysis PCA biplot (axes F1 and F2: 61.16 %) for the sampling sites, and their aggregation based on different biotic and abiotic parameters: altitude; pH; conductivity; temperature; dissolved oxygen; water quality classes (Wat.qual.cl.) from 1 (best) to 6 (worst) based on diatoms (diat.), benthic invertebrates (inv.) and fish; and number of taxa (No.tx.); blue circle - sites with high or good water quality; red dotted circle - sites with degraded water quality; for site codes: see text.

Benthic invertebrates were the most sensitive to various impacts, especially mining activities. Studies from the middle Arieş course reported that river sediments were highly contaminated with heavy metals like Cd, Cu or As, coming mostly from the mine-affected right-side effluents (Levei *et al*., 2014). Sediments were generally found to be more widely contaminated than surface waters (Bird *et al*., 2005). Moreover, the bioavailable fraction (i.e. percentage found in labile or dissolved forms) of elements potentially toxic in elevated concentrations (Fe, Mn, Zn, Cu etc.) was reported to be extremely high (Senila *et al*., 2015). This high contamination of sediments, habitat for benthic invertebrates, explained the severe effects shown by these communities downstream the region affected by mining activities (water quality classes were 4, 5 or 6) (Fig. 1).

The water column on the other hand, was reported by various studies to be less contaminated compared to the sediments. Bird *et al*. (2005) showed that the Arieş was much less polluted than the Abrud River, with only Cu showing concentrations above guideline values, since elevated metal levels in surface waters were confined to within approximately 10 km of point sources. The moderate influence of the polluted tributaries on the Arieş River water quality was explained by Senila *et al*. (2015) as a consequence of the tributaries low flow rate compared with that of the Arieş River. All these factors led to good water quality assessed by benthic invertebrate community at AR9. The high contamination with Cd, Pb and As coming from the Brăzeşti tailing pond, inactive at present (Levei *et al*., 2013) caused the worst water quality at AR10, since no organisms were found most of the times (Fig. 1). Benthic invertebrates showed class 5 and not 6 in T4 and AR10, due to the presence of several Chironomid individuals, probably coming from upstream.

Diatom indices used in the present study yielded comparable water quality classifications: DBI classes 1 and 2 were reflected by SI oligosaprobic and oligo-β-mesosaprobic levels, class 3 by oligo-β-mesosaprobic to β-α-mesosaprobic levels, while class 4 by α-polysaprobic levels. Similar findings were previously reported in the Arieş catchment area (Momeu and Péterfi, 2007).

Diatoms were known to be sensitive to a wide range of stressors (Wang *et al*., 2014), however diatom metrics were reported to be better correlated to eutrophication and organic pollution (Hering *et al.,* 2006). Similar trends were identified in the present study: in the upper Arieş reach, water quality at sites AR4 and T2 was ranked ”moderate” (class 3) by diatom indices, and ”high” or ”good” (classes 1 and 2) by invertebrates and fish. Since the dominant impact in the area was untreated domestic wastes coming from human settlements (Table 2), diatoms seemed to be more sensitive to eutrophication/organic pollution compared to other biotic communities.

Ichthyofauna ecological characteristics in streams are greatly influenced by hydromorphology (Solimini *et al*. (eds.), 2006), so fish communities are extensively used to assess hydromorphological degradation, especially in lowland rivers and at meso-scales (Hering *et al*., 2006). Our data however did not support these findings,

since water quality indicated by fish biotic indices ranged from high to moderate (classes 1 to 3), apart from T4, T5 and AR10, where no fish were caught (Fig. 1). The EFI+ quality classes were similar to IBI integrity classes, even though in 7 sites EFI+ was reccomended to be used with caution, because the number of fish caught was under 30.

Conclusion

The present study assessed the impacts in the Arieș River catchment area, a severely affected water course due primarly to mining industry facilities from the Roșia Montană - Roșia Poieni region, but also to domestic waste discharges, river regularization works and industrial activities, other than mining (wood processing, chemical, wire production etc.). High impact scores correlated with a decrease in water quality shown by biotic communities: diatoms, benthic invertebrates and fish. Benthic invertebrates were more sensitive to degradation, showing the poorest water quality classes in most of the cases.

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The Species of birds from the protected area ROSPA0062 – the dam basins from the Argeş River – observed during the World Championship of Kaiac-Canoe Sprint Juniors and Youth U23 (Bascov Basin, 2017)

Adrian Mestecăneanu[1,✉] and Radu Gava[2]

SUMMARY. In this paper we present the results of the researches performed on the dam basins from ROSPA0062 - The dam basins from the Argeş River during the World Championship of Kaiac-Canoe Sprint Juniors and Youth U23, held on the Bascov Basin, part of this protected area. 55 species of birds were registered. Even if in 2017 the general situation was better than in 2013, when a similar study was carried on during a period when there were no sportsmen on any basin, the situation was completely different on Bascov Basin. There was a similar number of species, albeit only 35.71% of all were common in the two sets of observations, but, regarding their abundences, these were lower than in 2013 with over 90%, the most affected being the species dependent on wetlands. The human impact is obvious as the nautical base from Bascov Basin is a permanent factor of stress for the birds from the area. The very small number of species from the Annex I of the Birds Directive observed here stregthens the previously mentioned facts.

Keywords: anthropogenic pressure, birds, dam basin, protected area.

Introduction

The avifauna of the dam basins from the Argeş River was well studied along the time, although a lot of data in regard remained unpublished (Mătieş, unpublished data). The first work on the theme appeared at the end of the ’60 after the ending of the Vidraru Basin construction (Mătieş, 1969). It was followed by references in two papers (Munteanu and Mătieş, 1983, Munteanu *et al.*, 1989) that covered the birds from several wetlands from Romania and some particular information has been published later (Gava, 1997). The studies were intensified after 2004 (Gava *et al.*, 2004a,b, 2007,

[1] *Argeş County Museum, Armand Călinescu Str., No. 44, 110047, Piteşti, Argeş, Romania.*
[2] *University of Piteşti, Târgu din Vale Str., No. 1, 110040, Piteşti, Argeş, Romania.*
✉ ***Corresponding author: Adrian Mestecăneanu**, Argeş County Museum, Armand Călinescu Str., No. 44, 110047, Piteşti, Argeş, Romania,*
E-mail: mestecaneanua@yahoo.com

2011, Mestecăneanu *et al.*, 2004, 2010, 2013, Conete *et al.*, 2006, 2011, 2012, Conete, 2011, Mestecăneanu and Gava, 2015a,b, 2016a,b,c, 2017 etc.). The avifauna of the Bascov Basin was particularly studied, in the context of the big negative influence caused by the human impact (Conete *et al.*, 2005a,b, 2008, Mestecăneanu and Gava, 2014a,b).

We consider that the present study will prove the changes of the local ornithofauna in the future.

Materials and methods

The Vâlcele (640 ha), Budeasa (643 ha), Bascov (140 ha), Piteşti (150 ha) and Goleşti (680 ha) basins appertain to the ROSPA0062 – The dam basins from the Argeş River (in Romanian, „Lacurile de acumulare de pe Argeş"), together with the Zigoneni Basin, the last one upstream among them. They are part of the Natura 2000 Network, according to the Government Decision No. 1284/2007. Previously, these basins have been declared protected wetlands at local level (conform to the Decision No. 4/1974 of the Popular Council of the Argeş County).

The Bascov Basin (initially, 162 ha) is arranged for nautical sports since 1982 (cf. http://informatiioferte.blogspot.com/2014/06/complexul-sportiv-national-bascov.html, accessed June 12, 2018). It was registered by the Argeş Natural Monuments Commission and the officials of the Argeş County Museum as ornithological reservation and proposed to validation through the Decision No. 30/26.02.2004 of the Argeş County Council. As a result, the Government Decision No. 2151/2004 (No. service of Natural Monuments Commission, Cj 93/19.03.2003) declared it as avifaunistic protection area, comprising the water surface between the Bascov and Budeasa dams (cf. Management Plan of Natura 2000 Site, ROSPA0062 Lacurile de acumulare de pe Argeş, https://lege5.ro/).

The five basins, where the research was accomplished, have been put into operation between 1970 and 1983 and were chiefly created for production of electrical energy, attenuation of the floods, and supplying with water of the objectives from the area (cf. http://www.baraje.ro).

As the name of the whole protected area mentions, these dam reservoirs are situated on the Argeş River (Fig. 1), that drains a major part of the Southern versant of the Făgăraş Mountains and the corresponding lower relief. The Argeş and the Cândeşti Hills border their left side and the Cotmeana Piedmont, the right side. Piteşti is the point where the Romanian Plain begins, so the Goleşti Basin is situated into an area that has the full plain features.

The climate is temperate-continental with hilly influence, in the North, and kinds of plain, in the South. The annual average temperature of the air is 9-10 ^{0}C. The water is a few degrees warmer than the air, so its annual average temperature fluctuates between 6.4 ^{0}C, in the Argeş Gorges and 9 ^{0}C, at Piteşti. It is usual to see an ice bridge during the harsh winters between the first half of January and the last part of February (Barco and Nedelcu, 1974).

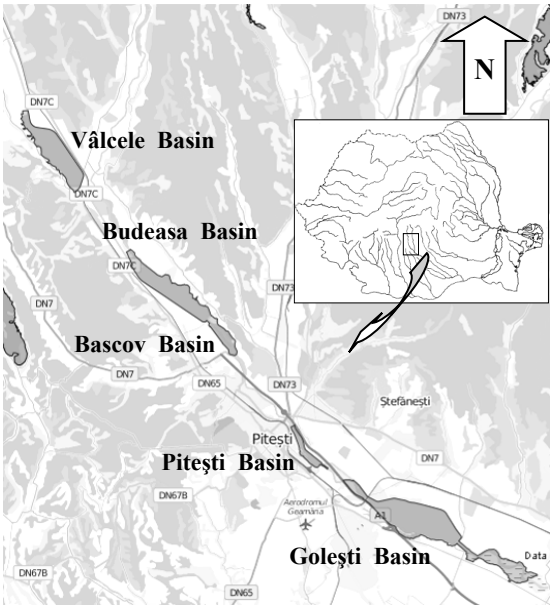

Figure 1. The map of the area (by http://biodiversitate.mmediu.ro, modified).

During both observations (2013 and 2017), the weather was sunny and warm. In 2017, there were quite big waves on the Goleşti, Bascov and Budeasa Basins, caused by the blowing wind, at gust, up to 3-4 on Beaufort scale, and, in 2013, the wind was 0, and no significant waves on the basins.

The vegetation of the basins corresponds to the diverse stages of the basins silting. It is typical for the wetlands of Romania and it grows mainly to the end of the lakes and on some parts of the banks, partially provided with bevels. There are small areas of reedbeds (*Phragmites* sp., *Typha* sp.). Other wetland plants from the genera *Myriophyllum*, *Sparganium*, *Mentha*, *Polygonum* emerge in the shallow waters. The banks are populated with species of *Carex*, *Juncus*, *Salix*, *Alnus*, *Populus* etc.

The observations were performed on July 29, 2017 during the World Championship of Kaiac-Canoe Sprint Juniors and Youth U23 from the Bascov Basin, and the obtained data was compared with the ones gathered on July 19, 2013, when the sportsmen were absent from the area. The same tracks of the itinerary method and point of observations were used during both of the days of research and the attention was focused on the aquatic species. The period of monitoring was 8:00 – 14:00. The birds were visually and auditory identified. Two binoculars (10x50) and a spotting scope (14-45x50) were used.

The avian scientific terms are the same availed in the Hamlin Guide (Bruun *et al.*, 1999).

Regarding the methods of the data processing, we used analytics ecological indicators (the density, the abundance, the constancy, the dominancy) and synthetics ecological indicators (the Dzuba index of ecological significance, the Bray–Curtis and Jaccard indices). They served for the identification of the species with the biggest weight in the ecosystem under the aspect of the energetical exchanges with the environment, of the characteristic or occasional species of the avicoenose, and of the ecological relations between the species. The Shanon–Wiener and Simpson indices (Stan, 1995, Gomoiu and Skolka, 2001) were also used, for the calculation of the diversity and the corresponding evenness and the standard procedure (Kelemen and Szombath, 1975, Gache, 2002) was applied to establish the index of relation, which shows the importance of the species or of the group of species in the respective coenose. The power function was used, too. It reflects the Theory of Island Biogeography (MacArthur and Wilson, 1967, in David, 2008) that supposes that the growth rate of the species increases as the surface increase. The correlations between parameters were explained by Zamfirescu and Zamfirescu (2006).

Results and discussion

On July 29, 2017, 55 species from 12 orders – Podicipediformes (with 2 species), Pelecaniformes (with 2 species), Ciconiiformes (with 4 species), Anseriformes (with 6 species), Falconiformes (with 1 species), Galliformes (with 1 species), Gruiformes (with 2 species), Charadriiformes (with 12 species), Columbiformes (with 2 species), Apodiformes (with 1 species), Coraciiformes (with 1 species) and Passeriformes (the richest, with 20 species) were observed on the basins of interest from the Argeş River. Among them, 30 species are dependent on wetlands and belong to 7 orders (Table 1).

Table 1.

The species of birds observed on the basins between Vâlcele and Goleşti, some ecological indexes and their protection by the Birds Directive.

No	Species	Vâlcele Basin	Budeasa Basin	Bascov Basin	Pitești Basin	Goleşti Basin	Abundance	Class of constancy	Class of dominancy	Class of Dzuba index of ecological significance	Class of dominancy*	Class of Dzuba index of ecological significance*	Birds Directive (2009/147/EC)
1	*Podiceps cristatus* (Linnaeus, 1758)*	+	+	+		+	153	C4	D3	W3	D3	W3	
2	*Podiceps nigricollis* Brehm, 1831*					+	2	C1	D1	W1	D1	W1	
3	*Phalacrocorax carbo* (Linnaeus, 1758)*	+	+		+	+	175	C4	D3	W3	D4	W3	

No	Species	Vâlcele Basin	Budeasa Basin	Bascov Basin	Pitești Basin	Goleşti Basin	Abundance	Class of constancy	Class of dominancy	Class of Dzuba index of ecological significance	Class of dominancy*	Class of Dzuba index of ecological significance*	Birds Directive (2009/147/EC)
4	*Phalacrocorax pygmeus* (Pallas, 1773)*		+		+	+	31	C3	D1	W2	D1	W2	AI
5	*Egretta garzetta* (Linnaeus, 1766)*	+	+		+	+	33	C4	D1	W2	D1	W2	AI
6	*Ardeola ralloides* (Scopoli, 1769)*				+		1	C1	D1	W1	D1	W1	AI
7	*Ardea cinerea* Linnaeus, 1758*	+	+		+		17	C3	D1	W2	D1	W2	
8	*Nycticorax nycticorax* (Linnaeus, 1758)*				+	+	12	C2	D1	W2	D1	W2	AI
9	*Cygnus olor* (Gmelin, 1789)*		+				1	C1	D1	W1	D1	W1	
10	*Anas platyrhynchos* Linnaeus, 1758*	+	+	+	+	+	208	C4	D4	W4	D4	W4	
11	*Anas querquedula* Linnaeus, 1758*	+					3	C1	D1	W1	D1	W1	
12	*Anas crecca* Linnaeus, 1758*				+		14	C1	D1	W1	D1	W1	
13	*Tadorna tadorna* (Linnaeus, 1758)*				+		6	C1	D1	W1	D1	W1	
14	*Aythya ferina* (Linnaeus, 1758)*					+	206	C1	D4	W3	D4	W3	
15	*Buteo buteo* (Linnaeus, 1758)	+					1	C1	D1	W1			
16	*Coturnix coturnix* (Linnaeus, 1758)					+	1	C1	D1	W1			
17	*Gallinula chloropus* (Linnaeus, 1758)*				+		1	C1	D1	W1	D1	W1	
18	*Fulica atra* Linnaeus, 1758*		+	+	+	+	672	C4	D5	W5	D5	W5	
19	*Vanellus vanellus* (Linnaeus, 1758)*				+		28	C1	D1	W2	D1	W2	
20	*Actitis hypoleucos* (Linnaeus, 1758)*					+	5	C1	D1	W1	D1	W1	
21	*Tringa ochropus* Linnaeus, 1758*				+	+	3	C2	D1	W1	D1	W1	
22	*Tringa glareola* Linnaeus, 1758*				+		2	C1	D1	W1	D1	W1	AI
23	*Tringa nebularia* (Gunnerus, 1767)*				+		1	C1	D1	W1	D1	W1	
24	*Himantopus himantopus* (Linnaeus, 1758)*				+		17	C1	D1	W1	D1	W2	AI
25	*Larus michahellis* Naumann, 1840*	+	+		+	+	205	C4	D4	W3	D4	W3	
26	*Larus ridibundus* Linnaeus, 1766*	+	+	+	+	+	1,521	C4	D5	W5	D5	W5	
27	*Larus minutus* Pallas, 1776*	+					1	C1	D1	W1	D1	W1	AI
28	*Chlidonias niger* (Linnaeus, 1758)*					+	1	C1	D1	W1	D1	W1	AI
29	*Chlidonias hybridus* (Pallas, 1811)*			+	+		3	C2	D1	W1	D1	W1	AI
30	*Sterna hirundo* Linnaeus, 1758*		+	+		+	6	C3	D1	W2	D1	W2	AI

No	Species	Vâlcele Basin	Budeasa Basin	Bascov Basin	Pitești Basin	Goleşti Basin	Abundance	Class of constancy	Class of dominancy	Class of Dzuba index of ecological significance	Class of dominancy*	Class of Dzuba index of ecological significance*	Birds Directive (2009/147/EC)
31	*Columba palumbus* Linnaeus, 1758	+					2	C1	D1	W1			
32	*Streptopelia turtur* (Linnaeus, 1758)	+					2	C1	D1	W1			
33	*Apus apus* (Linnaeus, 1758)					+	8	C1	D1	W1			
34	*Alcedo atthis* (Linnaeus, 1758)*					+	1	C1	D1	W1	D1	W1	AI
35	*Upupa epops* Linnaeus, 1758					+	2	C1	D1	W1			
36	*Riparia riparia* (Linnaeus, 1758)		+			+	10	C2	D1	W2			
37	*Hirundo rustica* Linnaeus, 1758	+	+	+		+	28	C4	D1	W2			
38	*Delichon urbica* (Linnaeus, 1758)	+	+	+		+	8	C4	D1	W2			
39	*Motacilla flava* Linnaeus, 1758	+	+			+	5	C3	D1	W1			
40	*Motacilla alba* Linnaeus, 1758	+	+	+		+	7	C4	D1	W2			
41	*Lanius collurio* Linnaeus, 1758	+				+	3	C2	D1	W1			AI
42	*Pica pica* (Linnaeus, 1758)	+			+	+	7	C3	D1	W2			
43	*Corvus monedula* Linnaeus, 1758					+	40	C1	D2	W2			
44	*Corvus frugilegus* Linnaeus, 1758					+	20	C1	D1	W2			
45	*Acrocephalus palustris* Bechstein, 1798*				+	+	2	C2	D1	W1	D1	W1	
46	*Sylvia curruca* (Linnaeus, 1758)	+					1	C1	D1	W1			
47	*Phylloscopus collybita* Vieillot, 1817	+				+	3	C2	D1	W1			
48	*Parus caeruleus* Linnaeus, 1758	+			+		7	C2	D1	W1			
49	*Parus major* Linnaeus, 1758		+		+		2	C2	D1	W1			
50	*Aegithalos caudatus* (Linnaeus, 1758)				+		6	C1	D1	W1			
51	*Passer domesticus* (Linnaeus, 1758)	+				+	10	C2	D1	W2			
52	*Passer montanus* (Linnaeus, 1758)	+		+		+	40	C3	D2	W2			
53	*Fringilla coelebs* Linnaeus, 1758				+	+	4	C2	D1	W1			
54	*Carduelis chloris* (Linnaeus, 1758)					+	2	C1	D1	W1			
55	*Carduelis carduelis* (Linnaeus, 1758)				+		1	C1	D1	W1			

Legend: * - species dependent on wetlands; + - presence; C1 – occasional species, C2 – accessory species, C3 – constant species, C4 – euconstant species; D1, W1 – subrecedent species, D2, W2 – recedent species, D3, W3 – subdominant species, D4, W4 – dominant species, D5, W5 – eudominant species; AI – Annex I.

The species richness observed in 2017 on the basins was bigger than in 2013 (55 versus 48 species) and, also, the number of individuals (3,551, versus 2,859); the months when the observations were made, both in 2013 and 2017 were warmer and drier than normal (http://www.ier.ro/webfm_send/5189, http://www.stirimeteo.com/, http://www.asas.ro/wcmqs/). The situation was similar in the case of the species dependent on wetlands. Their number was bigger in 2017 (30) than in 2013 (27) and, also, their strength (3,331 and 2,594 individuals respectively). Some factors like the time of starting of the migration, that varies each year depending on the weather conditions, the increasing silting of the basins, which in the first phase leads to an enhancement of the species diversity, or the direct human derange, which can be considered less evident in 2017, than in 2013, except the Bascov Basin, may be involved here. So, in 2017, additional to the athletic competition from the Bascov Basin, there were 11 fishermen (with a car) on Goleşti Basin, 9 fishermen and a boat on Piteşti Basin, and 1 kiteboarding man on Budeasa Basin. In 2013, there were 25 fishermen on Goleşti Basin, 6 fishermen on Piteşti Basin, 3 fishermen on Bascov Basin, 20 fishermen on Budeasa Basin and 6 fishermen on Vâlcele Basin.

The most of the species were registered on the Piteşti, respectively Goleşti Basin (Fig. 2). Also, the Piteşti Basin had the most of the individuals (including the ones of the dependent on wetlands species), while the Bascov Basin was the last in all situations. By comparison, in 2013, the Bascov Basin was the last regarding the number of species, including the dependent on wetlands ones. Regarding the number of individuals, at that time it was overpassed by the one of the Vâlcele Basin that, despite its larger size, had the lowest values. The Vâlcele Basin is placed upstream and its vegetation is very poor and situated toward its end. It must also be mentioned that the overall antropogenic pressure, in 2013, was the lowest on the Goleşti and Piteşti Basins, and the highest on the Budeasa Basin (Mestecăneanu and Gava, 2016c).

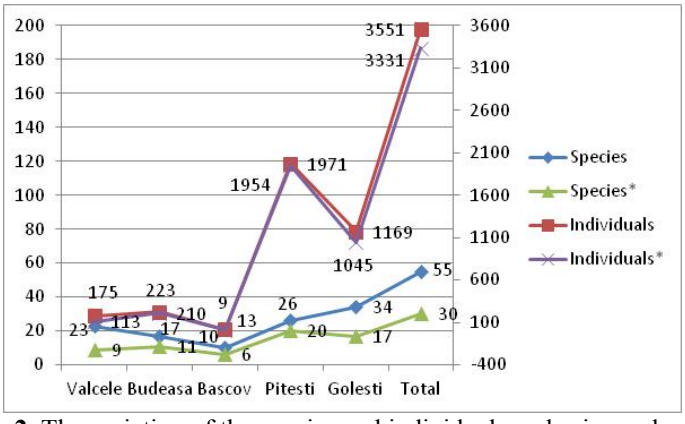

Figure 2. The variation of the species and individuals on basins and per total (* - values for the species dependent on wetlands).

As a result, the density of species and density of individuals were the biggest on the Piteşti Basin. The lowest density of species was registered on Budeasa, respectively Vâlcele Basins and the lowest densities of individuals (both for all species and for the species dependent on wetlands) were registered on the Bascov Basin, where the density of species is comparable to the one from 2013; here, the density of individuals is bigger in 2013 when the athletes were absent, that in 2017 when they were present (Table 2).

The correlation between the surface of the basins and the number of all species was 0.44 (positive and moderate correlation), the correlation between the surface of the basins and the number of species dependent of wetlands was 0.04 (positive and very weak correlation), the correlation between the surface of the basins and the number of individuals was –0.26, and the correlation between the surface of the basins and the number of individuals of species dependent of wetlands was –0.31 (negative and weak correlations). These mean that to some extent the number of species grew as the surface of the basins increased and the number of individuals decreased as the surface of the basins increased. That shows that, at the moment of research, there were other factors more important than the area of the basins that influenced the dynamics of the birds on the basins.

Table 2.

The density of the species and individuals on every basin and per total.

Basin	**Vâlcele (2017)**	**Budeasa (2017)**	**Bascov (2017)**	**Bascov (2013)**	**Piteşti (2017)**	**Goleşti (2017)**	**Total (2017)**
No. species/ha	0.04	0.03	0.07	0.06	0.17	0.05	0.02
No individuals/ha	0.27	0.35	0.09	0.99	13.14	1.72	1.58
No. species/ha*	0.01	0.02	0.04	0.03	0.13	0.03	0.01
No individuals/ha*	0.18	0.33	0.06	0.88	13.03	1.54	1.48

Legend: * - values for the species dependent on wetlands.

Applying the power regression on the relation between the surface of the every dam reservoir and their species richness or their total number of individuals, we detect a positive and quite small (in the case of species) and a moderate (in the case of individuals) slope, but, also, we notice a low (23.47%) or a very low (6.78 %) correlation between the variables (Fig. 3, Fig. 4). The slopes are quite similar in the case of the species and individuals dependent on wetlands, but the correlation between the variable is even lower (3.71%, respectively 5.83%). These suggest that the species rate of accumulation grows slowly as the reservoirs surface increases and the individuals' rate of accumulation grows moderately as the reservoirs surface increases. Consequently, the basins can sustain more species and more

individuals than those that were observed. Because the predictions of the Theory of Island Biogeography can be applied only to the groups of the creatures whose existence is strictly conditioned by a particular type of habitat (Usher, 1987), in this case it should best be applied to the breeding species dependent of wetlands, although at the end of July it is impossible to tell them apart without any doubts.

From the point of view of the similarities between the avifauna of the basins, in 2017, we stated that the smallest similarity was between the Bascov and Piteşti avicoenosis, even if the basins have an almost identical area. It must be noted, however, that the real aquatic surface of the Bascov Basin is lower than the initial one because of the islands formed inside. On the other hand, the actual area of the Piteşti Basin decreased because of silting, too. Also, even if the Piteşti Basin is situated near the Piteşti town, the overall anthropogenetical pressure is lower here than on the Bascov Basin, as it has been observed in 2013 (Mestecăneanu and Gava, 2014b). The similarity was the biggest between Budeasa and Vâlcele, two big and consecutive reservoirs (each ca. 640 ha area and 6 km distance among them), except the Jaccard index for all species, when the biggest one was established between Bascov and Budeasa; the two lakes are united, but very different in terms of as area. Otherwise, the similarity between the Bascov Basin and the other basins was small or medium (Tables 3, 4). It must highlighted that the Bray–Curtis index is based on the presence/absence of the species in the samples and on their number of individuals and the Jaccard index is based only on the presence/absence of the respective species in the samples (Gomoiu and Skolka, 2001).

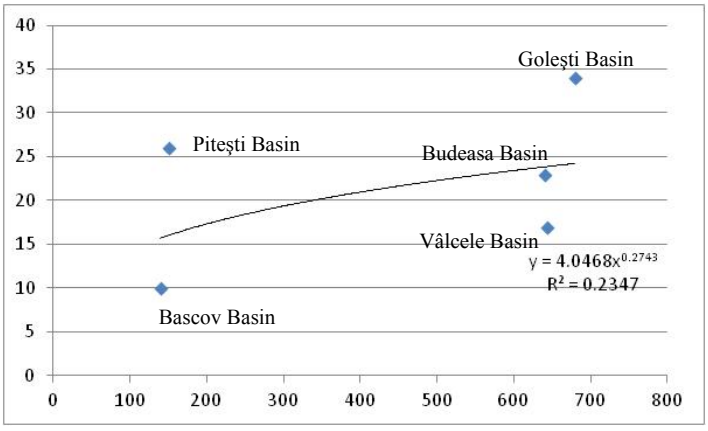

Figure 3. The relation between the surface of the basins and their species richness.

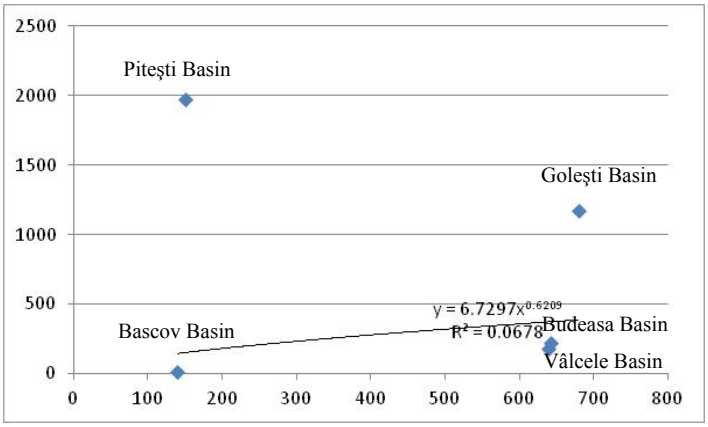

Figure 4. The relation between the surface of the basins and the strengths of all species.

Table 3.

The similarity matrix (by Bray–Curtis) between the avicoenosis (cenosis of species dependent on wetlands) of the dam reservoirs.

Similarity	Vâlcele	Budeasa	Bascov	Piteşti	Goleşti
Vâlcele	-	45.7 (52.6)	9.5 (8.1)	2.8 (2.7)	19.3 (17.7)
Budeasa	-	-	9.3 (7.3)	9.6 (9.7)	24.1 (25.1)
Bascov	-	-	-	0.5 (0.5)	1.8 (1.3)
Piteşti	-	-	-	-	24.3 (25.2)
Goleşti	-	-	-	-	-

Table 4.

The similarity matrix (by Jaccard) between the avicoenosis (cenosis of species dependent on wetlands) of the dam reservoirs.

Similarity	Vâlcele	Budeasa	Bascov	Piteşti	Goleşti
Vâlcele	-	37.9 (53.8)	26.9 (25.0)	19.5 (26.0)	35.7 (30.0)
Budeasa	-	-	42.1 (41.6)	26.7 (34.7)	37.8 (47.3)
Bascov	-	-	-	12.5 (18.1)	25.7 (27.7)
Piteşti	-	-	-	-	25.0 (37.0)
Goleşti	-	-	-	-	-

Comparing the similarities between the samples on every basin from July 19, 2013 and July 29, 2017, by Bray-Curtis, it results that the lowest similarity was undoubtedly for the Bascov Basin, regardless if all species or only the species dependent on wetlands were taken into account. By Jaccard, the similarities for the Bascov Basin were in the range of the other similarities of the basins, which varied between 33.33% and 53.84%. That means a relatively big heterogeneity of the species between the years of observations, generally with big differences of strengths

and with less than 50% regular species between samples. It is the consequence of the period of passage for many species, mainly of shores and waders, and probable it should be attenuated through more sessions of monthly observations. The effect of the athletes on the number of individuals was observable in this case, too (Table 5).

In 2017, the Shanon–Wiener ecological diversity was between 1.09 (1.03, in the case of the species of wetland) for Piteşti and 2.25 for Vâlcele and Bascov (1.81, in the case of the species of wetland for Goleşti) and the Simpson ecological diversity was between 1.85 (1.82, in the case of the species of wetland) for Piteşti and 26.00 (12.00, in the case of the species of wetland) for Bascov. From the evenness point of view, the smallest evennesses were on the Piteşti dam basin: 0.33 (0.34), for the Shanon–Wiener index, and 0.07 (0.09) for the Simpson index. The biggest ones were on the Bascov dam basin: 0.98 (0.97) for the Shanon–Wiener index and 0.65 (0.75) for the Simpson index (Table 6). The natural and the artificial conditions from each basin are reflected here. The values of diversity from the Bascov Basin catch the attention, but these are not the result of a big number of species represented by a big number of individuals, but inversely, these express a relatively low number of species, each with few individuals, as the evenness shows. The low values from Piteşti prove that here there was a small number of species that summed the majority of the individuals. It must be said that the Shannon–Wiener index takes into account both the number of species and the number of individuals of each species and the Simpson index takes into account the number of individuals of the species in relationship with the number of individuals of all observed species.

Table 5.

The similarities between the avicoenosis on every dam reservoirs and per total.

Similarity	Bray Curtis	Jaccard
Bascov 2013 – Bascov 2017	9.21	35.71
Bascov 2013* – Bascov 2017*	7.51	37.5
Budeasa 2013 – Budeasa 2017	38.68	41.66
Budeasa 2013* – Budeasa 2017*	39.78	53.84
Golesti 2013 – Golesti 2017	40.42	39.53
Golesti 2013* – Golesti 2017*	39.58	45.83
Pitesti 2013 – Pitesti 2017	39.52	34.88
Pitesti 2013* – Pitesti 2017*	40.35	40.74
Valcele 2013 – Valcele 2017	45.27	35.71
Valcele 2013* – Valcele 2017*	50.26	33.33
Total 2013 – Total 2017	51.74	53.73
Total 2013* – Total 2017*	51.60	51.35

Legend: * - values for the species dependent on wetlands.

According to the constancy we remark that the occasional species were the most numerous (28 species, 50.91%, respectively 16 species dependent on wetlands, 53.33%), while the constant species were the less numerous (6 species, 10.91%, respectively 3 species dependent on wetlands, 10%). About the dominance, the most

numerous were the subrecedent species (46 species, 83.64%, respectively 23 species dependent on wetlands, 76.67%), the other groups being poorly represented. In the case of Dzuba ecological index of ecological significance, also the subrecedent species were the most (33 species, 60.00%, respectively 16 species dependent on wetlands, 53%). The dominant species were the least represented (1 species, 1.82 %, respectively 1 species dependent on wetlands, 3.33%), (Table 1, Figs. 5, 6, 7).

Table 6.

The ecological diversity and the evenness of the avifauna from the dam basins.

Basin	Shanon Wiener index	Hsmax	Shanon Wiener eveness	Simpson index (1/λ)	S	Simpson eveness
Vâlcele	2.25	3.14	0.72	6.33	26.32	0.24
Vâlcele*	1.51	2.20	0.69	3.45	9.69	0.36
Budeasa	1.98	2.83	0.70	5.07	18.32	0.28
Budeasa*	1.78	2.40	0.74	4.52	11.55	0.39
Bascov	2.25	2.30	0.98	26.00	40	0.65
Bascov*	1.74	1.79	0.97	12.00	16	0.75
Piteşti	1.09	3.26	0.33	1.85	26.33	0.07
Piteşti*	1.03	3.00	0.34	1.82	20.19	0.09
Goleşti	2.19	3.53	0.62	5.16	34.98	0.15
Goleşti*	1.81	2.83	0.64	4.16	17.26	0.24
Overall	2.09	4.01	0.52	4.27	55.84	0.08
Overall*	1.80	3.40	0.53	3.76	30.26	0.12

Legend: * - values for the species dependent on wetlands.

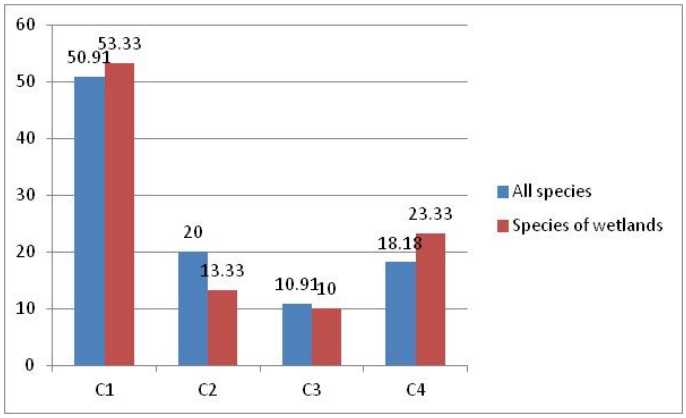

Figure 5. The distribution of the species by categories of constancy
(C1 – occasional species, C2 – accessory species, C3 – constant species, C4 – euconstant species).

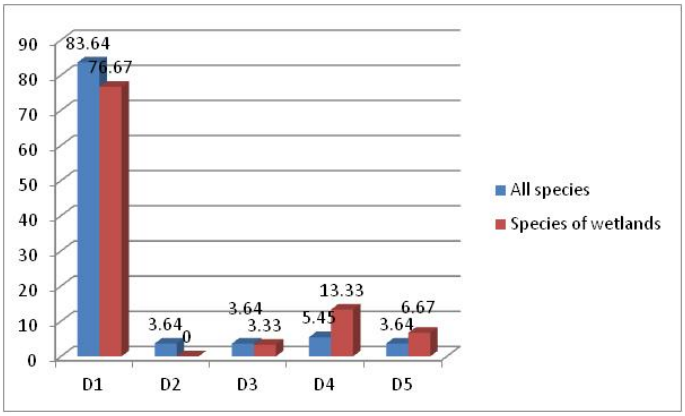

Figure 6. The distribution of the species by categories of dominance (D1 – subrecedent species, D2 – recedent species, D3 – subdominant species, D4 – dominant species, D5 – eudominant species).

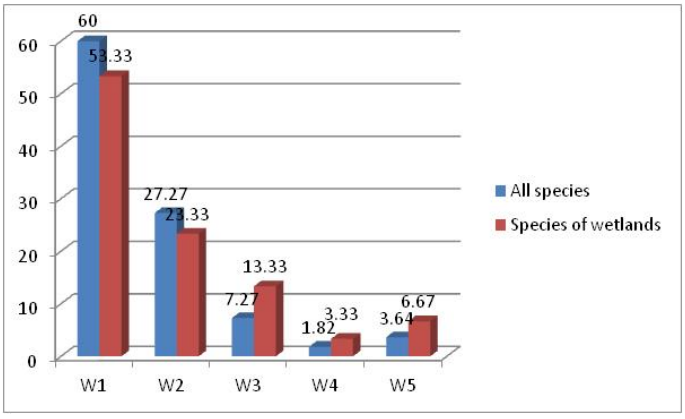

Figure 7. The distribution of the species by categories of Dzuba index of ecological significance (W1 – subrecedent species, W2 – recedent species, W3 – subdominant species, W4 – dominant species, W5 – eudominant species).

The only eudominant species were *Larus ridibundus* and *Fulica atra* (3.64% of all species, respectively 6.67% of the species dependent on wetlands). The Piteşti Basin was preferred by *Larus ridibundus*, while the Goleşti Basin was preferred by *Fulica atra* (Fig. 8). The depth of the water, the food and the lower degree of anthropogenic pressure contributed to this.

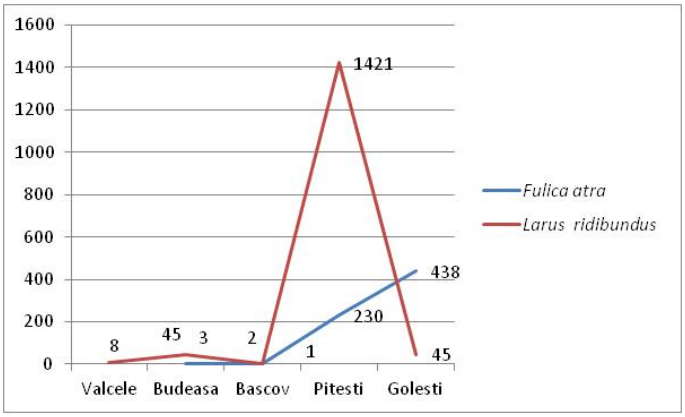

Figure 8. The variation of strengths of the eudominant species on every dam reservoir.

As a result of the facts previously shown, by the index of relation between the orders, Charadriiformes (with 1,993 individuals) and Gruiformes (with 673 individuals) were the overdominant orders, Anseriformes (with 438 individuals) was the dominant order and the other orders were complementary (Fig. 9). At the level of the species dependent on wetlands, Charadriiformes remained the only overdominant order. Gruiformes and Anseriformes were the dominant orders, the others being complementary (Fig. 10).

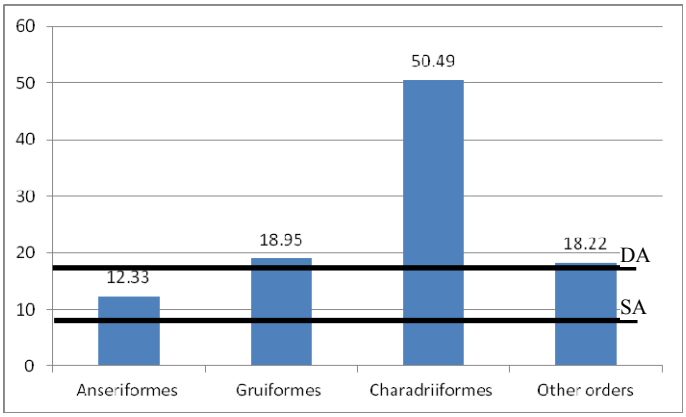

Figure 9. The participation of the orders to the formation of the avicoenose (SA – the static axis, DA – the dominance axis).

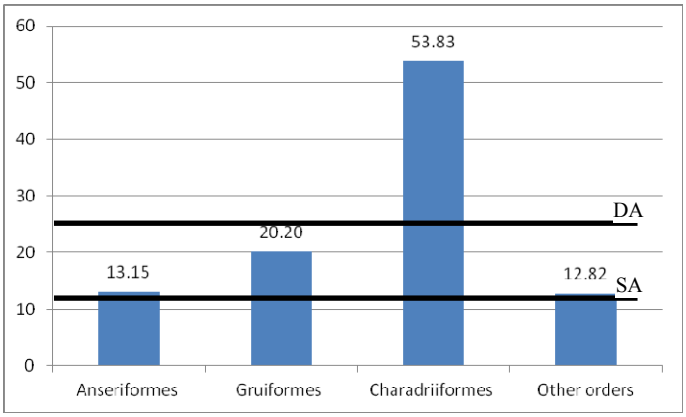

Figure 10. The participation of the orders to the formation of the coenose of species dependent on wetlands (SA – the static axis, DA – the dominance axis).

Inside the Charadriiformes, *Larus ridibundus* was the overdominant species, with 84.83% of the individuals *Larus michahellis* was the dominant species, with 11.43% of the individuals, the others (3.74% of the individuals) being complementary. Inside the Gruiformes order, formed only by two species, *Fulica atra* was the dominant species, with 99.85% of individuals, and *Gallinula chloropus* was the complementary species, with 0.15%.

Some species were observed in 2017 with chicks or independent juveniles: *Podiceps nigricollis* (with 2 well developed juveniles on the Goleşti Basin), *Fulica atra* (with 3 medium juveniles on the Piteşti Basin), *Larus michahellis* (with many pairs that breed in the city of Piteşti), *Larus ridibundus*, *Tringa glareola*, *Phalacrocorax pygmaeus*, *Phalacrocorax carbo*, *Egretta garzetta*, *Ardea cinerea*, *Vanellus vanellus*, etc. but a few did not breed in the area. It is generally known that the artificial basins are places less attractive for the birds in the breeding season than the natural ones (Munteanu and Mătieş, 1983).

12 species (21.81% of all, (*Phalacrocorax pygmeus*, *Egretta garzetta*, *Ardeola ralloides*, *Nycticorax nycticorax*, *Tringa glareola*, *Himantopus himantopus*, *Larus minutus*, *Chlidonias niger*, *Chlidonias hybridus*, *Sterna hirundo*, *Alcedo atthis* and *Lanius collurio*) are in the AI of the Birds Directive (2009/147/EC) and 2 of them (16.66%, *Chlidonias hybridus* and *Sterna hirundo*) were observed on the Bascov Basin. Measures for the habitat protection intended to make sure their survival and reproduction in their area of distribution must be taken (http://ec.europa.eu/environment/nature/legislation/birdsdirective/index_en.htm). *Tringa glareola* and *Larus minutus* were surely in passage; the specimens of the others seen either in migration or had a certain status of breeding in the area, being observed in the characteristic habitat.

Other aspects of the avicoenose from the Bascov Basin

As we have seen, the birds' fauna of the Bascov Basin was very poor, so that a total of only 14 species were recorded here during both dates of research (Table 7). 9 species were recorded in the sample from 2013 (when the athletes were absent), and, 10, in the one from 2017 (when they were present). Instead, the number of individuals was clearly bigger in 2013 (139, versus 13), when *Fulica atra* numbered almost 100 individuals and *Larus ridibundus* almost 20 (Mestecăneanu and Gava, 2014a). In 2017, these counted only 1, respectively 2 individuals and this was obviously a consequence of the athletes' competition that determined the birds to hide or to move somewhere else. In 2017, the number of individuals was smaller than in 2013 (by 90.64%) and, also, the number of individuals of the species dependent on wetlands (by 92.74%); similarly, the strength of *Fulica atra* was smaller (by 98.95%) in 2017 than in 2013 and, also, the strength of *Larus ridibundus* (by 89.47%).

Table 7.

The avicoenose of the Bascov Basin.

No.	Species	19.07.2013 (without athletes)	29.07.2017 (with athletes)
1.	*Podiceps cristatus**	0	2
2.	*Egretta garzetta**	1	0
3.	*Ciconia ciconia**	1	0
4.	*Anas platyrhynchos**	0	1
5.	*Fulica atra**	96	1
6.	*Larus ridibundus**	19	2
7.	*Chlidonias hybridus**	0	1
8.	*Sterna hirundo**	7	2
9.	*Hirundo rustica*	0	1
10.	*Delichon urbica*	4	1
11.	*Motacilla alba*	2	1
12.	*Pica pica*	3	0
13.	*Passer domesticus*	6	0
14.	*Passer montanus*	0	1
	Number of species	9	10
	Number of individuals	139	13
	Number of species*	5	6
	Number of individuals*	124	9

Legend: * - species dependent on wetlands.

As resulted from the Jaccard similarity, too, 5 species (35.71% of all, *Fulica atra*, *Larus ridibundus*, *Sterna hirundo*, *Delichon urbica*, and *Motacilla alba*) were common in the two days of observations. In the absence of the athletes, 4 additional species (28.57% of all, *Egretta garzetta*, *Ciconia ciconia*, *Pica pica* and *Passer domesticus*) were seen and when the athletes were present, 5 species (35.71% of

all, *Podiceps cristatus, Anas platyrhynchos*, *Chlidonias hybridus*, *Hirundo rustica* and *Passer montanus*). 8 species (57.14 of all) were dependent on wetlands and only 3 of them (37.5%) were observed both in 2013 and 2017; 5 species (62.5%) have been registered only in 2013 and 6 (75%) only in 2017. The few individuals of the latter seemed to be less sensitive to the stress provoked by people, but, because the rest of the basin was occupied by the boats, they were located toward the dam, and, here, it is noticeable the presence of a pair of *Podiceps cristatus* in nest. This was the only confirmed breeding species in the area, fact that vouches the idea that the presence of the athletes on the water obstructs mainly the formation of a rich aquatic breeding avifauna, a topic that was discussed on other occasions, too (Mestecăneanu and Gava, 2014a,b, 2016c etc.).

Conclusions

The avifauna of the basins between Vâlcele and Goleşti from ROSPA0062 – The dam basins from the Argeş River, observed in the breeding season (or passage season for some species) was relatively poor: 55 species (30 dependent on wetlands), from 12 orders. It reflects both the natural and anthropogenic conditions from the date of study, seasonal or permanent. Even if on the Bascov Basin a sportsmen competition was in progress, the general qualitative and quantitative situation of the birds from all basins was better in 2017 than in 2013, when a similar study was effected in the same month, when the athletes were not present in the area.

The majority of the species was formed of occasional or subrecedent species. *Anas platyrhynchos* and *Larus ridibundus* were the only species observed on all basins, and *Larus ridibundus* and *Fulica atra* had the most individuals. Therefore, they determined the hierarchy of the orders, where the Charadriiformes and the Gruiformes were the most important.

Because of the strongly anthropogenic characters of the dam basins, a few species with chicks were observed.

Among the 55 observed species, only 12 species belong to the Annex I of the Birds Directive, some of them being in passage.

Like on other occasions, independently of the fact that a human derange occurred or not at the time of the observations, the avifauna of the Bascov Basin proved to be the most modest of all. The number of species or their strengths, the density of species and individuals, the similarity between the basins, the index of diversity and the evenness sustain this assertion. This is an effect of the anthropogenic pressure, mainly because of the frequent trainings and competitions of the athletes on water and, also, the presence of the permanent stands and installations that mark the corridors of navigation. All of these intensively influence the breeding, too, only a few pairs of aquatic species having success in raising chicks year by year.

Comparing the two sets of observations, the one from 2013 and the one from 2017, obtained in relatively similar climatic conditions, it results that the avifauna of the Bascov Basin was negatively influenced by the presence of the sportsmen and the spectators at the time of contest, less in terms of make up of the species, but especially in terms of strengths. Especially the birds dependent on wetlands were affected and they had to hide or to leave the area in search for less disturbed spaces. It is unlikely that part of the individuals flew to Piteşti Basin, situated at over 4 km distance, where the strengths were the biggest of all basins, and probably some of them went to the nearby Budeasa Basin.

The dam basins where the research was performed can sustain a richer avifauna than that recorded, particularly the Bascov Basin. In the circumstances in which this basin belongs to an important birds area, included in Natura 2000 Network, it is advisable, from the birds' protected point of view, to move the nautical base (although it is one of national importance) on another basin less relevant for the avifauna, outside of the conservation area.

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Optimisation control process of cyanide biodegradation from cassava mill effluent (CME) using indigenous microorganisms

Kingsley Erhons Enerijiofi [1,✉] and Chinyere Anastasia Chukwuma[1]

SUMMARY. The cyanide component of cassava mill effluent (CME) is highly toxic to man and its environment. This research was aimed at biodegrading cyanide from cassava mill effluent with various concentrations of cyanide, variable pH values, inoculum size and phenol. The heterotrophic bacterial and fungal counts were 6.32 x 10^8±0.01cfu/ml and 2.87 x 10^8±0.11cfu/ml, respectively. The microorganisms isolated and characterized were: *Staphylococcus aureus, Bacillus* sp., *Escherichia coli*, *Lactobacillus* sp., *Micrococcus* sp., *Klebsiella* sp., *Pseudomonas*, sp. *Salmonella* sp., *Corynebacterium* sp., *Aspergillus niger*, *Penicillium* sp., *Fusarium* sp. and *Saccharomyces* sp. The physicochemical parameters: pH (4.81), electrical conductivity (4860uS/cm), cyanide (17.13 mg/l), chemical oxygen demand (2041.20 mg/l), biological oxygen demand (1490.08mg/l), total dissolved solids (2478.60 mg/l), Chromium (19.44 mg/l), Manganese (136.08mg/l), Iron (340.20 mg/l) and Nickel (121.50 mg/l) were above the Federal Environmental Protection Agency standard for effluent discharge. *Pseudomonas*, *Bacillus* and *Aspergillus* species which had the highest turbidity values with enrichment medium supplemented with 1% cyanide were used for the batch biodegradation studies. *Pseudomonas* sp. had the best degradative ability of all isolates used even in the presence of phenol, an inhibitory substance. However, of all the varied substrate concentration used, 30ppm with other conditions remaining constant gave the highest degradative ability of 32.73% at a residence time of 8 days. Also, the highest biodegradation rate of 74.5% and 71.03% were achieved at pH, 6 and inoculum size of 6ml respectively at a residence time of 8days for 30ppm while other parameters were kept constant. The findings revealed that *Pseudomonas* sp., *Bacillus* sp. and *Aspergillus* sp. could be utilized for remediating cassava mill effluent contaminated environment containing cyanide.

Keywords: biodegradation, cyanide degrading microbes, environmental management, optimisation

[1] *Samuel Adegboyega University, Department of Biological Sciences, College of Basic and Applied Sciences, Km 1, Ogwa – Ehor Road, P. M. B. 001, Ogwa, Edo State, Nigeria.*

✉ ***Corresponding author: Kingsley Erhons Enerijiofi**, Department of Biological Sciences, College of Basic and Applied Sciences, Samuel Adegboyega University, Edo State, Nigeria.*

E-mail: kingsmore1luv@gmail.com; kenerijiofi@sau.edu.ng

Introduction

Cassava mill effluent (CME) from traditional grating during processing is a major cause of environmental degradation, contaminating agricultural farmlands, streams and affecting biodiversities (Enerijiofi *et al*., 2017b; Chinyere *et al*., 2018; Izah *et al*., 2018). The toxicity of cassava mill effluent discharge is basically connected to its acidic pH and cyanide content. Cyanide compounds are fast acting poison that interferes mainly with cellular respiration process, resulting in a number of illnesses such as nervous instability by lipid peroxidation, poor vision, acute intoxication and sometimes death in humans (Ifeabunike *et al*., 2017; Izah *et al*., 2018). The toxicity of cyanide to existing cells is due to these three key mechanisms: resilient chelation to metals in metallo-enzymes; reaction with keto compounds to form cyanohydrin derivatives of enzyme substrates and reaction with Schiff-base intermediates during enzymatic reactions, to form stable nitrile derivatives (Ewa *et al*., 2017). Long-term discharge of this effluent into the soil could result in a serious imbalance in the microbial population, which in turn could result in alteration of soil fertility towards a negative direction (Akpan *et al*., 2017). Heavy metals that are discharged into the surroundings during processing with the coated metal machines persist indefinitely, accumulating in living tissues through food chain and causing severe diseases to man (Enerijiofi *et al*., 2017b).

Cyanide is quite recalcitrant in the sense that it persists so long on any contaminated soil (Ewa *et al*., 2017). However, various enzymes present in microorganisms aid in the conversion of cyanide to a source of carbon and nitrogen (Ibrahim *et al*., 2015). The ability of bacteria, fungi, protozoa and other organisms to metabolize xenobiotic and harzadous compounds converting them to less toxic compound have been recognized as potentially effective means of disposal and management. (Eskander and Saleh, 2017). Microorganisms such as *Bacillus* sp., *Brevibacterium nitrophilous*, *Corynebacterium nitrophilous*, *Klebsiella oxytoca*, *Pseudomonas* sp. and *Rhodococcus* UKMP-5M have been reported to be proficient in cyanide degradation (Ibrahim *et al*., 2015; Razanamahandry *et al*., 2017; Moradkhani *et al.*, 2018).

Ebelle community of Esan Land is into subsistence farming with main focus on cassava production which are processed into Garri. The cassava mills located in these areas seldom have proper discharge channels for the effluent and upon accumulation are harmful to the environment. The aim of this research is to degrade cyanide from cassava mill effluent using *Pseudomonas* sp., *Bacillus* sp. and *Aspergillus* sp.

Materials and methods

Description of the study area. Ebelle is one of the constituent kingdoms of Igueben Local Government Area of Edo State with geographical coordinates of 6° 30' 0" North, 6° 12' 0" East. It is naturally humid and characterized by a bimodal rainfall pattern particularly in July and September. It is an agrarian setting and the

residents are mainly farmers with cassava tubers production being their leading farm output (Enerijiofi *et al*., 2017a).

Collection of Sample. Raw Cassava mill effluents samples were collected from a FADAMA (III) cassava processing mill site at Ebelle, Edo state. A sterile four (4) litre plastic container was used to collect the samples in triplicte. The samples were immediately conveyed to the laboratory in ice pack containers for physiochemical and microbiological analyses within two hours from sampling.

Determination of physicochemical parameters and heavy metals concentrations. The method of APHA, (2011) was used to determine the physicochemical parameters which included pH, electrical conductivity, total dissolved solids, total suspended solid, turbidity, alkalinity, chlorine, ammonium nitrogen, sulphate, nitrate, cyanide, phosphate, chemical oxygen demand, dissolved oxygen and biochemical oxygen demand. The cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) were determined with a Jenway flame photometer, model PFP7, while the heavy metals concentrations were determined using an atomic absorption spectrophotometer, model PG 550 (Enerijiofi *et al*., 2017b).

Microbiological Analysis. Aliquot 1ml of appropriate ten - fold serial dilution (10^{-3}, 10^{-6} and 10^{-9}) of the cassava mill effluent sample was inoculated into Nutrient agar plates containing fuscin and potato dextrose agar plates containing streptomycin in triplicate using pour plate method for bacterial and fungal enumeration, respectively. The inoculated plates were incubated at 37^{o}C for 24 hrs in an incubator and at room temperature of 28^{o}C, for 72hrs, for the enumeration of the total heterotrophic bacterial and fungal counts, respectively. The results were expressed in colony forming units per millilitre of the sample. (Cheesbrough, 2006; Enerijiofi *et al.,* 2017a).

Isolation of cyanide degrading microbes. Cyanide - degrading microorganisms were isolated from cassava mill effluent samples and purified by repeatedly transferring the cells to enrichment medium. For enrichment of microorganisms Nutrient broth was used and the sample cultivated in a 500ml Erlenmeyer flask containing 100ml Nutrient broth, with 1% cyanide concentration. To screen for cyanide degrading bacteria, 6ml of culture broth from 1.5×10^8 cfu/ml was transferred into 500ml Erlenmeyer flask containing 100ml of buffer medium (K_2HPO_4 4.35g, NaOH 4g and 10 ml of trace salts solution; $FeSO_4 \cdot 7H_2O$ 300 mg, $MgSO_4 \cdot 7H_2O$ 180 mg, $CoCl_2$ 130 mg, $CaCl_2$ 40 mg, $MnCl_2 \cdot 4H_2O$ 40 mg and MoO_3 20 mg in 1 litre deionized water) and 0.1% yeast extract containing 1% of cyanide was incubated at 30°C, 150rpm. This process was repeated three times by inoculating into fresh medium with 10% (v/v) of the previously grown culture. Cyanide-degrading microbes were isolated on a 2-day interval for 8 days by smearing on nutrient agar medium. Colonies that differed mainly in morphology were selected and pure isolates were obtained by continuous sub-culturing. The bacteria isolated were verified for their Gram staining reactions and biochemical tests (Mirizadeh *et al*., 2014).

Cyanide degradation experiment. Bacterial and fungal isolates with distinct morphology were inoculated into nutrient broth and potato dextrose agar, respectively, for 24 hours. This was used for the purpose of isolates comparison study. Removal of cyanide was determined every 48 hours for 8 days. At regular intervals of 48 hours samples were drawn and tested for cyanide reduction. Non-inoculated medium served as control. The effect of substrate concentration, pH, inoculum size and phenol were determined (Arutchelvan *et al.*, and Mirizadeh *et al*., 2014). Cell growth was examined by determining the optical density (O.D) of 1 ml culture at 660 nm through Spectrophotometry (model GENESYS 10 UV-Vis Scanning, Thermo Scientific) and expressed as OD660 nm (Kandasamy *et al.*, 2015).

Optimisation conditions

Effect of Initial Substrate concentration on cyanide degradation. Five different substrate concentrations, 30ppm, 60ppm, 90ppm, 120ppm and 150ppm, were used in the study, while all other given parameters remained constant. To each flask, 100ml of NFG medium was added and the pH was adjusted to 6 before sterilization. To each sterilized and cooled flask 6ml of 1.5×10^8 cfu/ml inoculum was added.

Effect of pH on cyanide degradation. The effect of pH on degradation of cyanide was determined by maintaining pH ranges. One hundred milliliters (100ml) of NFG medium was prepared at a cyanide concentration of 30ppm in different flasks labelled with pH of 4, 5, 6, 7 and 8. All flasks were subjected to sterilization before adding 6ml of 1.5×10^8 cfu/ml inoculum.

Effect of Inoculum Size on cyanide degradation. In other to determine the effect of inoculum variation on cyanide degradation, another set of well labelled five flasks with different inoculum loadings: 2ml, 3ml, 4ml, 5ml and 6ml, containing the same species and regulated to a final volume of 100ml of the same medium. The solution was maintained at pH 6 and the initial concentration of cyanide was 30ppm.

Effect of Phenol on cyanide degradation. The NFG medium with pH 6 was dispensed in each of the 5 flasks. Subsequently, after sterilization and cooling, the content was inoculated with 6ml of 1.5×10^8 cfu/ml culture and added with 0.30%, 0.50%, 0.70%, 0.90% and 1.10% of phenol concentration. Cyanide concentration at 30ppm was maintained and experimentations were carried out.

Degradation Efficiency. The degradation efficiency (DE) of cyanide degrading bacterial and fungal isolates were calculated using the formula below:

$$DE(\%) = \frac{Ic - Rc}{Ic} x100$$

where DE = Degradation efficiency, Ic = Initial concentration of cyanide (mg/l) and Rc = Residual concentration of cyanide (mg/l).

Results and discussion

The concentrations of the different physicochemical parameters of the raw cassava mill effluent analysed are shown in Table 1. The cyanide content was 17.13mg/l. The pH of 4.81 reported was highly acidic. Other major pollutants reported were heavy metals, except lead (0.29mg/l). The results of physicochemical quality of the cassava mill effluent revealed high level of pollution, particularly Cyanide (17.13 mg/l) which was far above the 0.2mg/l limit recommended by FEPA (1991). The pH reported was acidic (4.81) which could have resulted from the high cyanide content reported.

Table 1.

Physiochemical properties of cassava mill effluent

Physicochemical parameters	**Units**	**Concentration**	**FEPA Effluent Limitation Guideline (1991) mg/l**
pH		4.81±0.21	6-9
Electrical Conductivity	uS/cm	4860±0.02	1000
Chlorine	mg/l	34.02±0.11	600
Alkalinity	mg/l	27.65±0.01	NA
Total Suspended Solid	mg/l	29.65±0.11	30
Total Dissolved Solid	mg/l	2478.60±0.10	2000
Turbidity	NTU	166.74±0.11	300
Chemical oxygen demand	mg/l	2041.20±0.01	40
Dissolved oxygen	mg/l	0.63±0.11	40
Biochemical oxygen demand	mg/l	1490.08±0.14	10
Cyanide	mg/l	17.13±0.01	0.2
Sulphate	mg/l	257.58±0.10	50
Nitrate	mg/l	140.94±0.11	1.0
Phosphate	mg/l	102.06±0.00	5.0
Ammonium nitrogen	mg/l	0.97±0.10	NA
Calcium	mg/l	156.98±0.23	100
Magnesium	mg/l	58.32±0.18	100
Sodium	mg/l	680.40±0.01	200
Potassium	mg/l	1506.60±0.01	NA
Zinc	mg/l	58.32±0.32	1.0
Copper	mg/l	72.90±0.11	1.5
Chromium	mg/l	19.44±0.20	0.5
Lead	mg/l	0.29±0.26	0.5
Manganese	mg/l	136.08±0.22	0.5
Iron	mg/l	340.20±0.01	20
Nickel	mg/l	121.50±0.20	1.0

Legend: NA - Not Available; Values are in Mean ± standard error of triplicate samples

Other physicochemical parameters, particularly heavy metals recorded apart from Lead (0.29 mg/l), were higher than the FEPA (1991) limit for effluent discharge. These high heavy metals concentrations could be traced to the anthropogenic inputs, such as corrosion of the metal parts of equipment used in harvesting and milling into the environment, as reported by earlier authors (Ebukiba, 2010).

The results in Table 2 shows the bacterial and fungal counts. The total heterotrophic bacterial count ($6.32 \times 10^8 \pm 0.01$cfu/ml) was higher than the total heterotrophic fungal count of ($2.87 \times 10^8 \pm 0.11$cfu/ml). The isolation of high numbers of cassava mill effluent utilising bacterial and fungal isolates from cassava mill effluent was an indication these organisms are active cassava mill effluent degraders in the environment (Enerijiofi *et al.,* 2017a).

Table 2.

Mean bacterial and fungal counts ($\times 10^8$ cfu/ml)

	THBC (cfu/ml)	**THFC (cfu/ml)**
Cassava mill effluent	6.32±0.01	2.87±0.11

Legend: THBC – Total Heterotrophic Bacterial Count; THFC – Total Heterotrophic Fungal Count; Values are in Mean ± standard error of triplicate samples

Figure 1 shows the bacterial and fungal taxa that could utilise cyanide as substrate for growth. However, *Bacillus* and *Pseudomonas* species had turbidity values of 0.543mg/l and 0.31mg/l among the bacterial isolates while *Aspergillus niger* had the highest value of 0.41mg/l.

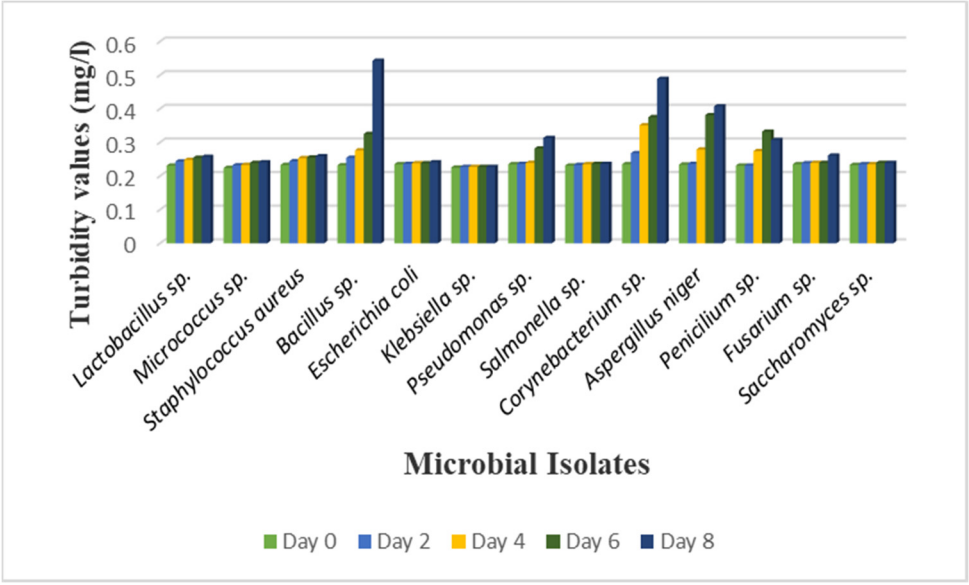

Figure 1. Isolation of cyanide degrading microbes (bacteria and fungi) with mineral salt medium containing 1% cyanide

The microorganisms surviving in such environment are those that have developed enzymatic and physiological responses that allow them to use cyanide as substrate for growth and subsequent proliferation. This agreed with the earlier findings (Izah *et al*., 2018; Orji and Ayogu, 2018). A similar observation was made by Enerijiofi *et al*. (2017a) on biodegradation potentials of cassava mill effluents using indigenous microorganisms.

It was established that *Pseudomonas* sp. performed best because it was able to reduce the substrate concentration of 30ppm and 60ppm by 32.73% and 17.62%, respectively, at a residence time of 8 days while *Bacillus* sp. gave 16.93% and 15.29% reduction for 120ppm and 150ppm, respectively, at residence time of 8 days. In summary, Table 3 revealed that the smaller the concentration, the better the degradation efficiency.

Table 3.

Effect of substrate concentration of cyanide; Inoculum size = 6ml of 1.5 x 10^8cfu/ml, pH =6

Day 0	**30ppm**	**60ppm**	**90ppm**	**120ppm**	**150ppm**
Bacillus sp.	29.98	60.14	90.07	120.03	149.98
Pseudomonas sp.	29.96	60.07	89.96	120.05	149.95
Aspergillus niger	29.99	60.09	89.99	120.02	150.04
Control	29.97	60.02	90.03	120.04	149.99
Day 2	30ppm	60ppm	90ppm	120ppm	150ppm
Bacillus sp.	27.65	58.76	90.01	120.01	149.98
Pseudomonas sp.	26.78	58.32	89.92	120	149.95
Aspergillus niger	28.94	59.89	89.98	120.02	150.04
Control	30.01	60.01	90.03	120.04	149.99
Day 4	30ppm	60ppm	90ppm	120ppm	150ppm
Bacillus sp.	25.58	58.46	89.11	112.81	146.98
Pseudomonas sp.	22.13	57.79	86.32	117.60	148.45
Aspergillus niger	26.98	59.56	86.38	114.02	147.04
Control	29.97	59.03	90.01	120.02	150.01
Day 6	30ppm	60ppm	90ppm	120ppm	150ppm
Bacillus sp.	24.05	57.29	87.33	106.04	139.63
Pseudomonas sp.	20.80	54.32	83.73	115.25	146.97
Aspergillus niger	26.17	57.18	85.52	107.18	142.63
Control	29.97	59.03	90.01	120.02	150.01
Day 8	30ppm	60ppm	90ppm	120ppm	150ppm
Bacillus sp.	22.60	55.57	86.45	99.68	127.06
Pseudomonas sp.	20.18	49.43	80.38	112.94	145.50
Aspergillus niger	24.60	56.03	82.10	101.82	138.35
Control	29.98	59.01	89.99	120.01	150.02

The result revealed that the smaller the substrate concentration, the better the degradation efficiency. *Pseudomonas* sp. degraded cyanide best to the tune of 32.73% at a residence period of 8 days. From this, it can be concluded that increased

substrate will inhibit the degradation process as reported by earlier researchers (Kandasamy *et al*., 2015; Mekuto *et al*., 2013; Mirizadeh *et al*., 2014).

Cyanide serves as a nutrient for bacterial growth, acting as nitrogen source. Some bacteria make use of cyanide compounds both as carbon and nitrogen source. Therefore, external supply of carbon is no longer necessary for these bacteria. In the presence of cyanide other bacteria required glucose as carbon source for their survival (Bouari, 2012).

Table 4 revealed the ability of *Pseudomonas* sp., *Bacillus* sp. and *Aspergillus niger* to degrade cyanide at varying pH, while other parameters remained constant. pH 6 gave the highest reduction in cyanide concentration by *Pseudomonas* sp. at 63.17%, 73.70%, 73.97% and 74.50% at day 2, 4, 6 and 8 respectively. The pH concentration also plays a major role in the biological activity of the degradation process. *Pseudomonas* sp. gave the maximum of 74.50% degradation at pH 6 at a residence time of 8 days. From this study, it can be concluded that the bacterial and fungal isolates performed best in the slightly acidic condition of pH 6.

Table 4.

Effect of pH on cyanide degradation; Cyanide concentration = 30ppm, Cell suspension=6 ml of 1.5 x 10^8cfu/ml

Day 0	**pH 4**	**pH 5**	**pH 6**	**pH 7**	**pH 8**
Bacillus sp.	15.34	15.42	15.33	15.37	15.44
Pseudomonas sp.	15.28	15.36	15.41	15.38	15.42
Aspergillus niger	15.43	15.31	15.35	15.42	15.38
Control	15.33	15.41	15.42	15.37	15.41
Day 2					
Bacillus sp.	12.54	11.89	11.55	13.33	15.11
Pseudomonas sp.	14.11	11.43	11.05	13.97	15.25
Aspergillus niger	14.68	13.75	12.76	14.78	15.31
Control	15.14	15.38	15.19	15.36	15.4
Day 4					
Bacillus sp.	10.89	10.35	8.63	12.78	14.98
Pseudomonas sp.	13.77	10.85	7.89	12.43	15.06
Aspergillus niger	14.11	12.98	12.04	14.31	15.34
Control	15.05	15.33	15.14	15.31	15.39
Day 6					
Bacillus sp.	10.67	9.83	8.46	12.27	14.68
Pseudomonas sp.	13.63	10.63	7.81	11.81	14.76
Aspergillus niger	13.83	12.59	11.68	14.17	15.03
Control	14.90	15.02	14.84	15.00	15.08
Day 8					
Bacillus sp.	10.46	9.64	8.29	12.02	14.39
Pseudomonas sp.	13.36	10.42	7.65	11.57	14.46
Aspergillus niger	13.55	12.34	11.45	13.88	14.73
Control	14.89	15.01	14.83	14.89	15.07

However, Kwon *et al*. (2002) and Ibrahim *et al*. (2015) reported that microbial isolates did perform best at acidic, slightly acidic and neutral conditions.

The findings in Table 5 show that inoculum size of 6ml and 5ml, *Pseudomonas* sp. gave a degradation efficiency of 71.03% and 68.73% at day 8. It was observed that with increased incubation time, degradation efficieny increases. It was evident that the degradation percentage increased with increased biomass concentration. At inoculum size of 6ml, *Pseudomonas* sp. gave the optimum degradation capacity of 71.03% at a residence time of 8 days.

Table 5.

Effect of Inoculum size on cyanide degradation; pH = 6, Cyanide Concentration = 30ppm

Day 0	**2ml**	**3ml**	**4ml**	**5ml**	**6ml**
Bacillus sp.	15.34	15.42	15.33	15.37	15.44
Pseudomonas sp.	15.28	15.36	15.41	15.38	15.42
Aspergillus niger	15.43	15.31	15.35	15.42	15.38
Control	15.33	15.41	15.42	15.37	15.41
Day 2					
Bacillus sp.	13.76	13.24	12.88	11.69	11.05
Pseudomonas sp.	12.93	12.55	10.48	9.97	9.36
Aspergillus niger	14.97	14.16	13.97	13.26	12.98
Control	15.33	15.39	15.4	15.37	15.41
Day 4					
Bacillus sp.	12.93	12.84	12.75	10.99	10.06
Pseudomonas sp.	12.54	11.42	10.06	9.77	9.27
Aspergillus niger	14.07	13.88	13.41	12.60	12.59
Control	15.31	15.39	15.4	15.35	15.39
Day 6					
Bacillus sp.	12.16	12.59	12.11	10.33	9.55
Pseudomonas sp.	11.79	10.74	9.66	9.58	9.17
Aspergillus niger	13.65	13.32	13.28	11.84	12.21
Control	15.31	15.34	15.38	15.34	15.37
Day 8					
Bacillus sp.	11.43	12.21	11.99	9.71	9.08
Pseudomonas sp.	11.44	9.77	9.27	9.38	8.69
Aspergillus niger	12.83	13.06	12.75	11.25	11.85
Control	15.31	15.39	15.38	15.33	15.35

This result also revealed that with increased inoculum size and residence time, degradation efficiency also increased, which agreed with previous report (Arutchelvan *et al.*, 2005).

Table 6 records the cyanide degradation efficiency of the bacterial and fungal isolates at varied concentrations of phenol with other parameters kept constant. *Pseudomonas* sp. gave the best result, of 12.04, at 0.30% phenol concentration, *Bacillus* sp. at 0.50% and 0.70% gave 13.68 and 14.46, respectively, while at 0.90% and 1.10% *Pseudomonas* sp. gave a reduced value of 14.18 and 14.43, respectively.

Table 6.

Effect of inhibitory substance (phenol); Inoculum size = 6ml of 1.5 x 10^8cfu/ml, pH =6, Cyanide Concentration = 30ppm

Day 0	**0.30%**	**0.50%**	**0.70%**	**0.90%**	**1.10%**
Bacillus sp.	15.34	15.42	15.33	15.37	15.44
Pseudomonas sp.	15.28	15.36	15.41	15.38	15.42
Aspergillus niger	15.43	15.31	15.35	15.42	15.38
Control	15.33	15.41	15.42	15.37	15.41
Day 2					
Bacillus sp.	13.88	15.01	15.27	15.31	15.43
Pseudomonas sp.	13.25	14.95	15.25	15.29	15.4
Aspergillus niger	14.96	15.13	15.33	15.41	15.38
Control	15.33	15.41	15.42	15.37	15.41
Day 4					
Bacillus sp.	12.78	14.69	15.06	15.28	15.41
Pseudomonas sp.	12.41	14.25	15.19	15.23	15.03
Aspergillus niger	13.99	15.21	15.28	15.39	15.36
Control	15.32	15.39	15.41	15.36	15.41
Day 6					
Bacillus sp.	12.52	13.96	14.76	14.67	15.10
Pseudomonas sp.	12.29	13.97	15.04	14.47	14.73
Aspergillus niger	13.71	14.75	14.82	15.24	15.05
Control	15.32	15.37	15.42	15.35	15.41
Day 8					
Bacillus sp.	12.27	13.69	14.46	14.38	14.80
Pseudomonas sp.	12.04	13.68	14.74	14.18	14.43
Aspergillus niger	13.44	14.46	14.53	14.93	14.75

It was recorded that, with increase of phenol concentrations, the degradation of cyanide reduced due to the inhibitory action of phenol and the residence time increased with interference of phenol. *Pseudomonas* sp., *Bacillus* sp. and *Aspergillus niger* were more active in degrading the phenol rather degrading the cyanide, which corroborated with earlier findings (Neetu and Chandrajit, 2016; Singh *et al*., 2018).

Conclusions and recommendations

Pseudomonas sp.*, Bacillus* sp. and *Aspergillus* sp. were the most dominant microbial isolates which shown the highest ability of improving cassava mill effluent by reducing the cyanide content; under precise cultural conditions. However, *Pseudomonas* sp. had the best degradative ability. This study has unveiled the potentials of biodegradation of cyanide from cassava mill effluent.

It is recommended that further leaps be taken in a bid to exploring newer, more effective, less costly and better satisfactory methods of cyanide management from cassava mill effluent prior to eventual discharge into the environment.

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Expression of adhesins by some *Bordetella pertussis* strains isolated in Romania in different time periods

Georgeta Cristina Oprea[1,2,✉] and Veronica Lazăr[2]

SUMMARY. *Bordetella pertussis* is the etiological agent of whooping cough or pertussis, a respiratory infection in humans which can be prevented by vaccination. *B. pertussis* is characterized by a set of virulence factors involved in bacterial adherence to host-cells and consecutive colonization of the respiratory epithelium and in immunity too. In this study, we compared the expression of the specific adhesins with antigenic properties in some *B. pertussis* strains, including collection strains, vaccine strains and recently-isolated strains. The specific adhesins / surface antigens of *B. pertussis* are: a surface protein, named FHA (filamentous hemagglutinin) and fimbria Fim2 and Fim3. These antigens were evidenced using an indirect ELISA method, based on specific monoclonal antibodies binding to specific epitopes. The results showed that fimbrial antigens Fim3 are expressed by all new-isolated strains, compared to the older isolates, which expressed Fim2 or both Fim2, 3.

Keywords: adherence, fimbriae, pertussis vaccine, virulence factors.

Introduction

Bordetella pertussis is a Gram-negative bacterium which infects the respiratory tract in humans and represents an important cause of worldwide deaths in children (Tsang *et al.*, 2004). It is a 3-5 years cyclically reported infection, even though whooping cough is a vaccine preventable disease (Bouchez *et al.*, 2015). Pertussis vaccination was introduced in Romania in 1961, using the whole-cell vaccine produced by Cantacuzino Institute and after that the number of infections and epidemics has drastically dropped. In 2008, the whole-cell pertussis vaccine was replaced by the acellular one and whooping cough incidence decreased from 2.8 $‰_{000}$ in 2008 to 0.5 $‰_{000}$ in 2017. However, the number of cases is underestimated, because there are reported only the confirmed cases, included in the surveillance programs. In our country, the vaccination coverage in 2017 was lower than 90%, the same situation being reported during the previous years (Popovici, 2017).

[1] *Cantacuzino National Institute of Medico-Military Research - Development, 103 Splaiul Independentei, sector 5, Bucharest, 050096.*
[2] *University of Bucharest, Faculty of Biology.*
✉ ***Corresponding author: Georgeta Cristina Oprea,*** *103 Splaiul Independentei, Bucharest, Romania. E-mail:crisstina_bio@yahoo.com*

B. pertussis produces a surface protein, respectively the filamentous hemagglutinin (FHA) and fimbrial proteins Fim2 and Fim3, encoded by *fim2* and *fim 3* genes (Livey *et al.*, 1987) (Willems *et al.*, 1990), which confer to the bacterial cells the capacity to adhere to host cells (Tsang *et al*., 2004). Fimbriae adhere to the ciliated epithelium and altogether with FHA ensure a prolonged bacterial colonization of the respiratory tract (Matoo *et al*., 2000) (Scheller *et al*., 2015). Fim2 and Fim3 are agglutinogens responsible for serotype determination and FHA is a non-fimbrial hemagglutinating molecule (Ashworth *et al*., 2006). *B. pertussis* binds to sulfated sugars at Fim2 receptors level, which contain 2 regions similar to heparin-binding site of fibronectin (Geuijen *et al*., 1996). These heparin-binding sites of Fim2 are parts of epitopes recognized by specific antibodies from infected patient's sera. It is thought that Fim3 has the same binding specificity and the bacteria evades host-immune responses by switching the expression of their encoding genes (Geuijen *et al*., 1998). FHA, Fim2 and Fim3 antigens were included in both whole-cell vaccines and currently used acellular pertussis vaccines, together with purified pertussis toxoid (PT) and an outer membrane protein, named pertactin (PRN).

The selective pressure observed for the circulating *B. pertussis* strains is a consequence of the introduction of pertussis vaccination, using initially the whole-cell vaccine, replaced by the acellular vaccine in 1990s (Bouchez *et al*., 2015). This pressure led to non-expression of pertactin especially in high-income countries, but the prevalence of the isolates lacking the expression of this antigen and their emergence is difficult to be interpreted (Bouchez *et al*., 2018).

The aim of this study was to estimate the expression of adhesins/antigens involved in bacterial adherence of *B. pertussis* strains and compare the expression profiles of FHA, Fim2 and Fim3 antigens of collection strains with the vaccine strains and new-isolated *B. pertussis* strains.

Materials and methods

Patients and strains. A number of 20 *Bordetella pertussis* strains were analyzed using ELISA technique in order to estimate the expression of FHA, Fim2 and Fim3 adhesins with antigenic properties: 11 *B. pertussis* collection strains, 5 new isolated strains and 4 vaccine strains, used for in house preparation of whole-cell pertussis vaccine. The tested collection strains have been isolated between 1954-1976, 3 of them being collected before the introduction of pertussis vaccine in our country (the whole-cell vaccine). The 5 new strains were isolated in 2014-2017 from unvaccinated or incompletely vaccinated children, aged from 6 months to 4 years old, 3 of them being treated with antibiotics, other than macrolides in 2 cases (a beta-lactam antibiotic and a third generation cephem) and 1 dose of azithromycin for the other patient.

Bacterial growth and identification of new-isolated strains. *B. pertussis* was isolated on selective Bordet-Gengou agar (containing 40µg/ml cephalexin) supplemented with 15% sheep blood and 1% glycerol and incubated for 3-5 days at 37^0C. Then the colonies were plated on Bordet-Gengou medium without antibiotic and molecular species identification was performed, detecting the gene encoding for pertussis toxin promoter (*ptxP*).

Expression of antigens performing indirect ELISA method. The indirect ELISA was performed using Heikkinen *et al.* adapted method (2008), modified as follows: the blocking buffer and the conjugate used in the reaction were replaced by other reagents. *B. pertussis* ATCC 9797 (American Type Culture Collection) was used as a positive control for the expression of adhesins and sterile phosphate-buffered saline-PBS (Amresco, USA-cat no E504) as negative control.

Bordetella pertussis colonies were harvested on Bordet-Gengou specific medium and incubated for 3-5 days at 37^0C. Bacterial suspensions were prepared for each tested strain and the positive control strain in PBS 1X at an optical density of 0.1. The suspensions were inactivated at 56^0C for 1h, then 96-well microtiter plates were coated with 100 µL of *B. pertussis* inactivated suspension of each tested strain and incubated over night at room temperature. On the second day, the plates were washed as described by Heikkinen *et al.* For blocking of the plates, 1% normal sheep serum (Sigma-Aldrich, USA-cat no S3772) in PBS was added for 1 h; after the washing, the microplates were incubated with a 1:1000 dilution of monoclonal antibodies specific to FHA, FIM2 and FIM3 (NIBSC, United Kingdom) antigens. Secondary antibody (goat anti-mouse IgG antibody conjugated with alkaline phosphatase-Sigma-Aldrich, cat no A3562, USA) was incubated with the microplates for 2 h, then alkaline phosphatase substrate was added (Sigma-Aldrich, USA - cat no S0942). NaOH solution (Thermo Fischer, Germany) was used to stop the reaction and the optical densities were measured at 405 nm using a Stat Fax spectrophotometer (Awareness Technology) with a reference wavelength of 630 nm.

DNA extraction. In order to identify the genes encoding for fimbrial proteins Fim2 and Fim3 by conventional PCR, genomic DNA was extracted from 200 µL of *B. pertussis* suspension using High Pure PCR Template Preparation Kit (Roche - Mannheim, Germany), according to manufacturer's instructions.

Polymerase chain reaction. PCR was performed for the identification of *fim2* and *fim3* genes, encoding for fimbriae serotype 2 and 3 (Fig.3). An in-house protocol was used to detect *fim2* gene, as follows: the reaction mixture contained 5 µL PCR buffer, 1.5mM MgCl2, 0.2 mM dNTP mix, 0.4 pmol/µL of forward and reverse primers/reaction, 1.25U Taq polymerase and 14.8 µL DNase free water. Then 1 µL of DNA was added in the reaction mixture, in a final working volume equal to 25 µL. The forward and reverse primer sequences used for PCR were designed as described by Zhang *et al.* (Table 1) and the amplification program is described in table 2.

Table 1.

Primers used to amplify *fim2* and *fim3* genes (Zhang *et al.*, 2010)

Identified gene	Forward primer	Reverse primer
fim2	ACCCATGCAAATCCCTTTCCAACGC	GGGGGTTGGCGATTTCCAGTTTCTC
fim3	ATGTCCAAGTTTTCATACCCTGCCT	TTCGTCTCCTGACGCCGCGTAGCGG

Table 2.

Thermal profile used for the amplification of *fim2* gene

Step	Temperature	Time (minutes)
Initial denaturation of DNA double strand	95^0C	2
Amplification (x 30 cycles)	Denaturation at 95^0C	0.5
	Primer annealing at 59^0C	1
	Polymerization at 72^0C	1
Final elongation of DNA double strand	72^0C	5

Results and discussion

The results indicated that the binding of specific monoclonal antibody to FHA epitope was positive for all tested strains; for Fim2, only 2 of the tested strains showed specific binding and in case of Fim3 epitope, the specific binding to the monoclonal antibody used in the reaction was positive for 50% (n=10) of the tested strains (Fig. 1).

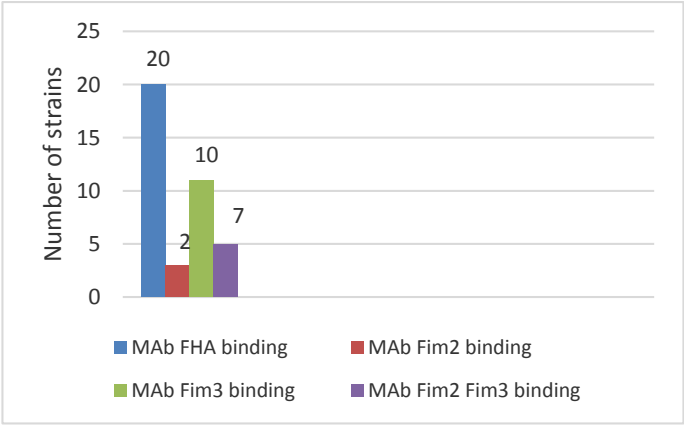

Figure 1. Monoclonal antibodies specific binding to expressed antigens

For the vaccine strains, the results showed low expression of Fim3 antigen in case of 2 strains, one of them expressed Fim2,3 and 1 strain had no expression of Fim2 or Fim3 antigen using this method.

For the older isolated strains, 2 out of 11 expressed Fim2 antigen, 4 expressed Fim3 and 5 strains expressed Fim2,3.

In case of the new-isolated strains, 4 expressed Fim3 antigen and 1 strain expressed Fim2,3 (Fig.2), indicating the same results described by other authors, that since the introduction of vaccination the main fimbriae serotype expressed in the vaccinated populations with pertussis vaccine is Fim3 (Heikkinen *et al*., 2008) (Alexander *et al*., 2012).

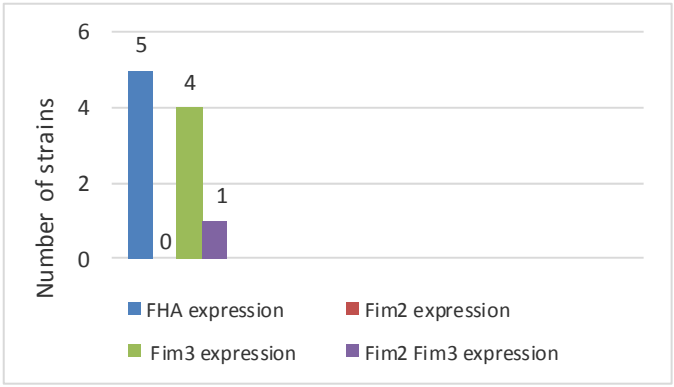

Figure 2. Antigen expression for the new-isolated strains (2014-2017)

The identification of a 700 bp amplicon indicating *fim 2* gene and of the 697 bp amplicon for *fim3* (Fig.3) gene was positive for all tested strains. Sequencing of the amplicons was not performed in this study.

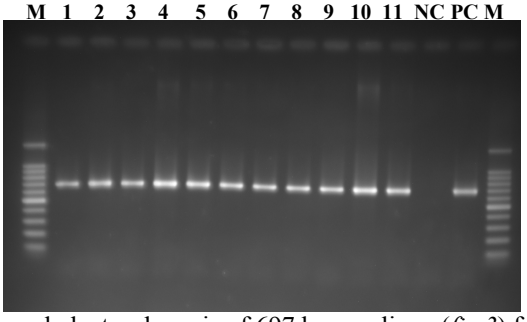

Figure 3. Agarose gel electrophoresis of 697 bp amplicon (*fim3*) for 11 *B. pertussis* collection strains; **M- molecular weight marker (100 bp DNA ladder); 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11- *B. pertussis* DNA extracted from strains; NC- negative control (DNase free water); PC- positive control (ATCC 9797).**

In our study, even though the expression of *B. pertussis* fimbriae was not tested for a large number of strains, it is important to notice that in case of all new-isolated strains, Fim3 expression was positive. This evidence is concordant with other studies in Europe, which describe the identification of Fim3 as the major fimbriae serotype, characteristic for vaccinated populations (Alexander *et al*., 2012). Fim3 prevalence is explained by different authors as a shift from Fim2 expression, as a consequence of the introduction of pertussis vaccine (Gorringe *et al*., 2014). The new strains were isolated from unvaccinated or incompletely vaccinated children and the Fim3 expression for all of them demonstrates that most of the circulating B. *pertussis* strains included in this study belong to Fim3 serotype.

This could be explained by the fact that serotype 2 antigen contained in whole-cell vaccine is more immunogenic and produces a stronger immune response than the serotype 3 antigen, as explained by other authors (Tsang *et al*., 2004; Gorringe *et al*., 2014) and the type Fim3 strains would have been selected in the population as a result of vaccine pressure. Fim3 prevalence in the population could also be a consequence of the fact that the partially immunized children are mainly infected by serotype 3 strains (Preston *et al*., 1985). Our study confirms this hypothesis, considering the fact that the tested strains were isolated from partially immunized or non-vaccinated children.

In Finland, Fim2 serotype is the most common, in spite of high vaccine coverage and Fim3 expressing strains emerge in case of epidemics (Heikkinen *et al*., 2008). In contrast to that, in other countries it was observed that for the areas with lower vaccination coverage, the prevalence of Fim2 strains increases (Gorringe *et al*., 2014). In previous studies it was revealed that in case of serotype 2 strains, *fim3* gene was not expressed and the selective pressure was not to be taken into consideration (Tsang *et al*., 2004).

For the collection strains, Fim2 and Fim2, 3 serotypes were found in case of more than half of the tested strains, compared to the recently isolated ones, with Fim3 expression positive for all the strains. It seems that Fim2 antigen contained in the whole-cell vaccine was more immunogenic and produced a better immune response than Fim3, considering the fact that in time, Fim3 type strains have not been eliminated from the circulation.

The strains included in the vaccine expressed Fim3 and Fim2,3 antigens, indicating that Fim3 serotype was not the prevalent serotype of the isolated strains, because the strains included in the vaccine expressed Fim3. In this case, the selective pressure of the vaccine could explain the prevalence of serotype 2. Other authors agreed that the methods used for serotyping may only report the predominant serotype of the infecting strain while during an infection, both antigens are expressed (Vaughan *et al*., 2014).

Expression of adhesins in *B. pertussis* circulating strains is important because the virulence of the strains is related to the antigenic profile. Whooping cough is a respiratory infectious disease, with severe symptomatology in case of non-vaccinated children. On

the other hand, the efficiency of the acellular vaccine is important to be evaluated, and the circulating strains should be isolated and tested to improve better vaccine formulas.

B. pertussis adhesins are encoded by genes regulated by the *BvgAS* operon and its expression occurs *in vivo* in the virulent phase, which is enough to produce a respiratory infection (Melvin *et al.*, 2014). The nucleotide substitutions in fimbriae genes help the bacteria to adapt to the immune pressure and fimbrial antigens are selected in the population as induced by the used vaccine or as a consequence of specific antibodies production following infection. Even though the sequencing of fim2 and fim 3 genes was not performed in this study, it would be necessary further investigation of the *B. pertussis* strains to elucidate the molecular mechanisms involved in fimbriae gene expression.

Conclusions

In our study, same as indicated by many other studies, Fim3 was found to be the main fimbrial serotype isolated from unvaccinated or incompletely vaccinated children. Further studies on adhesins expression should be performed on a larger number of strains, in order to compare the serotypes of the strains isolated before the vaccine was introduced in the population, with the recently circulating ones. This study supports the isolation and characterization of new *B. pertussis* strains, in order to estimate the social and economic impact of pertussis vaccination.

FHA and Fim2/Fim3 expression is important for the colonization and persistence of the bacterial cells in the respiratory tract and as a consequence, different mechanisms were developed by the bacteria to maintain their virulence potential.

The hypothesis related to vaccine pressure affecting the expression of pertactin, the shift from Fim2 expression to Fim3 in some countries since the introduction of the vaccine or the waning immunity to the antigens included in pertussis vaccines could explain the changes in antigen expression of *Bordetella pertussis* strains in time.

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Microbiological quality assessment and proximate analysis of fish and shrimps sold in open markets and grocery stores in Benin city, Nigeria

Faith Iguodala Akinnibosun[1,✉] and Natural Ezewele[1]

SUMMARY: The aim of this research was to determine the microbiological quality and proximate composition of fish and shrimps sold in open markets and grocery stores in Benin City, Nigeria. Samples of fish and shrimps were analyzed microbiologically using pour plate isolation method. The total bacterial count/coliform count were7.80 ± 0.12 x 10^5/1.20 ± 0.13 x 10^5 and 5.44 ± 0.23 x 10^5/1.50 ± 0.11 x 10^5 for fish and shrimp samples respectively in the open market whilst for the grocery stores they were:3.61 ± 0.32 x 10^5/4.15 ± 0.33 x $10^5$and 1.42 ± 0.24 x 10^5/1.36 ± 0.13 x $10^5$for fish and shrimp samples respectively. The mean fungal count for fish and shrimp samples was highest in open market shrimps (2.11 ± 0.20 x 10^2) and lowest in grocery stores shrimp (1.33 ± 0.12 x 10^2). Bacterial species isolated were *Pseudomonas aeruginosa, Enterobacter aerogenes, Staphylococcus aureus, Micrococcus luteus* and *Staphylococcus epidermidis* while the fungi isolated were: *Aspergillus niger*, *Penicillium* sp and *Mucor* sp. Proximate analysis showed that the samples were mainly made up of protein, carbohydrate and lipids whilst moisture, fibre and ash were also present in all the samples at varying concentrations. Results revealed no significant difference in the proximate composition of the open market and grocery stores fish and shrimps. From the foregoing, these high sources of nutrients should be added to our daily meal, while proper measures, such as public enlightenment, washing and cooking of the samples with potable water to ensure their microbiological safety are recommended.

Keywords: coliform, fish, microbiological safety, proximate analysis, shrimps

Introduction

Seafoods is an important part of a healthy diet and one of the most important sources of animal protein source and other element for the maintenance of health body, however they are highly perishable food, with its quick perishability being

[1] *University of Benin, Faculty of Life Sciences, Department of Microbiology, P.M.B 1154, Benin City, Edo State, Nigeria.*
✉ ***Corresponding author: Faith Iguodala Akinnibosun,*** *Department of Microbiology, Faculty of Life Sciences, University of Benin, P.M.B 1154, Benin City, Edo State, Nigeria.*
E-mail: faith.akinnibosun@uniben.edu

the main problem during its preservation (Khan, 2001; Musa *et al*., 2010; Okoro *et al*., 2010; Dewi *et al.*, 2011; Renvichandran *et al.,* 2012). In handling and storage of fish, its quality deterioration rapidly occurs and truncates the shelf-life (Alemu, 2013). Fish contains proteins, minerals, vitamins as important sources of nutrients. However fish meat spoils more quickly than muscle foods, particularly via natural bacterial spoilage; about 30% of landed fish are lost through microbial degradation only (Ghaly *et al.,* 2010). Fish spoilage with microorganisms shows environmental pollution (Adeyemo, 2003). Therefore, the microbial biota of fish is a reflection of its aqueous environment. If the fish surrounding environment is polluted with bacteria, their consumption will be risky to human health (Arafat, 2013). Many researches of microbial flora in the body and internal organs of fish have been carried out (Al-Harbi and Uddin, 2004; Yagoub, 2009; Okoro *et al*., 2010; Das Trakroo and Agarwal, 2011; Adebayo-Tayo *et al.,* 2012a, b). These studies have revealed variation in the bacterial flora of fish species collected in different places and in different countries. Bacteria such as *Pseudomonas fluorescence*, *Aeromonas hydrophila*, *Edwardsiella tarda*, *Vibrio* sp. and Myxobacteria are common in the aquatic environment (Gilmour *et al*., 1976; Allen *et al.,* 1983). However, disease-causing bacteria are mainly introduced into water bodies through faeces from humans or animals (Arafat, 2013).Shrimp is one of the most delicious sea food and is part of the almost every nation's traditional meal (Ehigiator *et al.,* 2014)

Shrimps are found worldwide and they include commercially significant species such as the White leg shrimp, Atlantic white shrimp*,* Indian prawn and Tiger prawn. The shrimps constitute a large group of crustaceans varying in size from microscopic to about 35 cm long. The body is almost always laterally compressed, the rostrum usually compressed and toothed, and the abdomen long, longer than the carapace or head (Adedeji and Ibrahim, 2011).Shrimps can be pink, green, brown, blue, white or yellow before cooking but turn pink with white meat after cooking. Shrimps have suitable moisture contents (73.14% to 73.91%). The protein was found as the major constituent, indicating that shrimp muscle can be a good source of amino acids. Crude protein levels showed a tendency to increase in wild shrimp. The ash content in this study was a little higher than that obtained by Sriket *et al*. (2007).

Seafood refers to all fresh or salt water organisms such as shellfish, fin fish, mollusks, crustaceans and other forms of aquatic animal life. Nigeria has a large number of frozen seafood processing plants and retail marketsdistributed along the country, where considerate amount of people buy their frozen seafood product daily. The source of pathogenic bacteria may be from environmental cotamination or unhygienic handling of shrimp by the workers. When processed frozen sea foods are consumed raw, there is likelihood of endangering the health of consumers especially when the microorganisms present include pathogenic ones. (Okonko, 2008). The microbiological safety of food is achieved by ensuring the absence of

pathogenic microorganisms and by all means preventing microbial multiplication (Edema *et al*., 2005). Control and prevention of contamination in shrimps, through good water source, improved hygienic handling of shrimp , proper sewage disposal, proper storage of shrimp lead to reduction in shrimp (seafood) hazard and danger to public health (Ehigiator *et al*., 2014). Unsafe water used in processing seafood products pose a global public health threat, placing consumers at risk for a host of diarrhea and other diseases (Hughes and Koplan, 2005). Handling of raw materials influences the bacteriological quality of frozen shrimps. Insufficiently iced and improper storage of shrimp at higher temperature enhance the growth of microorganisms responsible for microbiological quality changes (Reilly *et al*., 1986).

Most of the methods that have been used to estimate the quality of fresh fish measure or evaluate parameters that are formed, varied or modified during deterioration of fish. These methods are either microbiological or chemical (Huss, 1995). Some of the microbiological methods used to assess fish freshness are total plate count, total coliform and fecal coliform. Total plate count is a good indicator of the sensory quality or expected shelf life of the product (Olafsdottir *et al*., 2006; Koutsoumanis and Nychas, 2000). A good knowledge of the microbial loads of raw processed seafood such as fish and shrimp is necessary so as to guide the unsuitability for consumption. Thus, regular microbiological analysis of seafood products at source or processing plant must be carried out to check for the effectiveness of the processes of processing and packaging. The study, therefore aims to determine the microbial load and the proximate analysis of fish and shrimps sold in the open markets and grocery stores in Benin City, Nigeria.

Materials and methods

Sample collection

Fresh fishes and shrimps were purchased in Oba market and grocery stores in Benin City. The samples were immediately transported to the laboratory for microbiological analysis.

Sample Preparation

Ten grammes of each sample *Clarias gariepinius* and *Caridina* sp.was of cut from the head, middle and tail regionswere weighed and mashed in a sterile laboratory mortar and pestle and aseptically introduced into 90 mL of sterile distilled water, properly shaken before a 10-fold serial dilution was prepared.

Preparation of culture media

All media were prepared accordingly to manufacturer's instruction. The media used in this study were Nutrient agar (used for heterotrophic bacterial count), MacConkey agar (used for coliform count) and potato dextrose agar (used for fungal count).

Isolation and enumeration of microorganisms

One millilitre from 10 dilutions was plated out by pour plate method on nutrient agar, MacConkey agar and potato dextrose agar. The nutrient agar and MacConkey agar plates were amended with nystatin to prevent fungal growth and then incubated at 37^0C for 24hrs. The potato dextrose agar plates were amended with streptomycin to prevent bacterial gowth and incubated at28^0C for 72hrs. After incubation, discrete colonies of culture on nutrient agar and potato dextrose agar plates were counted and expressed in cfu/g.

Characterization and identification of isolates

Bacterial isolates were identified on the basis of cultural morphological and biochemical tests according to Jolt *et al*., 1994 and Cheesbrough, 2006. The fungal colonies were identified as described by Harrigan, 1998.

Proximate Analysis

Proximate analysis of the samplefor moisture content, crude protein, lipid, fibre, ash and carbohydrate was determined using the methods description by AOAC (1990).

Statistical Analysis

Results were expressed as means ± standard error (SE) of three replicates. Data were subjected to Analysis of Variance (ANOVA) using SPSS version 16.0 (Ogbeibu, 2015).

Results and discussion

In this study, assessment of the microbiological quality and proximate analysis of fresh fish and shrimps sold in open markets and grocery stores in Benin City was carried out. Results showed that the microbial counts of both samples (Tables 1 and 2) were high.

Table 1.

Microbial counts of samples from open markets

Counts (cfu/g)	Fish	Shrimp
Total Heterotrophic Bacterial count	$7.80 \pm 0.12 \times 10^{5a}$	$5.44 \pm 0.23 \times 10^{5a}$
Coliform count	$1.20 \pm 0.13 \times 10^{5b}$	$1.50 \pm 0.11 \times 10^{5b}$
Fungal count	$1.56 \pm 0.21 \times 10^{2c}$	$2.11 \pm 0.20 \times 10^{2c}$

Note: Values are means ± standard error; Means with the same letter are not significantly different ($P > 0.05$)

Table 2.

Microbial counts of samples from grocery stores

Counts (cfu/g)	Fish	Shrimp
Total Heterotrophic Bacterial count	$3.61 \pm 0.32 \times 10^{5a}$	$4.15 \pm 0.33 \times 10^{5a}$
Coliform count	$1.42 \pm 0.24 \times 10^{5b}$	$1.36 \pm 0.13 \times 10^{5b}$
Fungal count	$1.61 \pm 0.41 \times 10^{2c}$	$1.33 \pm 0.12 \times 10^{2c}$

Note:Values are means± standard error; Means with the same letter are not significantly different ($P > 0.05$)

The total bacterial count for fish in open market and grocery stores were $7.80 \pm 0.12 \times 10^5$ and $3.61 \pm 0.32 \times 10^5$ cfu/g respectively. Fungal counts of $1.56 \pm 0.21 \times 10^2$ cfu/g and $1.61 \pm 0.41 \times 10^2$ cfu/g were respectively observed, while total coliform counts in both samples were $1.20 \pm 0.13 \times 10^5$ and $1.42 \pm 0.24 \times 10^5$ cfu/g respectively. The total bacterial load from open market and grocery store shrimps were $5.44 \pm 0.23 \times 10^5$ cfu/g and $4.15 \pm 0.33 \times 10^5$ cfu/g respectively. Fungal counts were: $2.11 \pm 0.20 \times 10^2$ cfu/g and $1.33 \pm 0.12 \times 10^2$ cfu/g respectively, while total coliform counts were $1.50 \pm 0.11 \times 10^5$cfu/g and $1.36 \pm 0.13 \times 10^5$ cfu/g respectively. This observation agreed with those of Ehigiator *et al*. (2014) and Alemu, 2013.The high microbial count could be attributed to poor handling and storage practices adopted by the sellers.

The counts were generally high and exceeded the FAO/WHO standard limit of 1.0×10^2cfu/ml for food production and water (FAO/WHO, 2007). In both open markets and grocery stores, the bacterial and coliform counts were higher than the fungal count. This difference could have been as a result of differences in preservation methods of the different markets and grocery stores and their storage conditions. Tables 3 and 4 showed the bacterial and fungal isolates respectively, as *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Micrococcus luteus*, *Staphylococcus epidermidis* (Table 3)*,* and *Aspergilus niger, Penicillium* sp., *Mucor* sp. (Table 4).

These results were similar to those reported by Ehigiator *et al.,* 2014 and Okonko *et al*. (2008). *Pseudomonas aeruginosa*was isolatedamong patients with wounds, burns and cystic fibrosis and their presence is likely due to the action of swimmers and infected individuals in water bodies and aquatic environments which they use for recreational purposes (Ehigiator *et al*., 2014). Presence of *S. aureus*, another pathogenic bacteria might be due to possible contamination during sales and unhygienic handling of seafood products. This is in agreement with the reports of Edema *et al.* (2005). Okonko *et al*. (2008 a and b) and Oluwafemi and Simisaye, (2005).

Table 3.

Bacteria isolated from fish and shrimps

Characteristics	*1*	*2*	*3*	*4*	*5*
Cultural					
Elevation	Low	Convex	Convex	Convex	Flat
Margin	Convex	Entire	Entire	Entire	Serrated
Colour	Green	Yellow	Yellow	White	Cream
Shape	Circular	Circular	Circular	Circular	Circular
Morphological					
Gram stain	-	+	+	+	+
Cell type	Rod	Cocci	Cocci	Cocci	Rod
Cell arrangement	Single	Cluster	Single	Cluster	Single
Spore stain	-	-	-	-	-
Biochemical					
Catalase	+	+	+	+	+
Oxidase	+	-	-	-	-
Coagulase	-	-	-	-	-
Urease	-	+	+	+	-
Indole	-	-	+	+	+
Citrate	+	+	+	+	+
Glucose	+	+	+	+	+
Lactose	-	+	+	+	+
Isolates	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Micrococcus luteus*	*Staphylococcus epidermidis*	*Enterobacter aerogenes*

Table 4.

Cultural and microscopic characteristics of fungal isolates

Cultural	**Microscopic examination**	**Isolates**
Black fluffy colonies with reverse side yellow	Septate and branched hyphae and conida in chains	*Aspergillus* niger
Grey colonies that were large with white border.	Long conidiophores consisting of broom like conida in chains	*Penicillium* sp.
White flat colony with reverse side colourless	Non-septate hyphae with straight sporangiophore with many spherical spores	*Mucor* sp.

The isolation of fungi in this study is similar to result obtained by Ehigiator *et al.,* 2014 and Fagade *et al*., 2005. Fungi might have arisen due to the fact that during storage, the samples reabsorb moisture from the environment, which supported the growth of these microorganisms. Table 5 displayed the distribution of isolates in the open market and grocery store. It was observed that *S. aureus* was the most prevalent in all the samples while the least prevalent was *E. aerogenes*. The presence of coliforms indicated faecal contamination of the water for processing the frozen seafood (Adebolu and Ifesan, 2001).

Table 5.

Distribution of isolates in samples

Isolates	Open Market		Grocery store	
	Fish	Shrimp	Fish	Shrimp
Pseudomonas aeruginosa	+	+	-	+
Enterobacter aerogenes	-	+	-	-
Staphylococcus aureus	+	+	+	+
Staphylococcus epidermidis	+	+	+	-
Micrococcus luteus	+	+	-	-
Aspergillus niger	-	+	+	+
Penicillium sp.	+	-	+	+
Mucor sp.	+	+	-	-

Key**:** + =Present, - = Absent

Proximate analysis (Table 6) of the different fish and shrimps samples showed the presence of protein, carbohydrates, lipid, moisture, fibre and ash in all the samples at varying concentrations. The protein content in fish samples from open market and grocery store was found to be slightly different and were 37.22 ± 1.23 % and 41.35 ± 0.76 % respectively in agreement with the results of Olayemi *et al*. (2011). The shrimp samples were found to be lower in protein content with percentage composition of 18.90± 0.79 % and 21.54 ± 0.81 % respectively in shrimps from open market and grocery stores. Interestingly, the carbohydrate content of shrimps was found to be very high compared to that of fish. A percentage composition of 50.77 ± 1.19 % and 46.17 ± 1.01 % were observed in the respective shrimps, compared to 16.41 ± 0.05 % and 13.71± 0.89 % in the respective fish samples. This is in agreement with the work of Puga-lópez *et al*. (2013) who reported similar findings on the proximate analysis of shrimps. The ash content of any sample is a measure of the mineral content of the food (Nnamani *et al.,* 2009). The ash content were: 7.43 ± 0.08 % and 2.32 ± 0.09 % for open market and grocery store fish respectively while 4.52 ± 0.49 % and 5.31 ± 0.44 % respectively for open market and grocery store shrimps. The moisture content for open market and grocery store fish were: 31.45 ± 1.41 % and 30.63 ± 0.87 % respectively, while 21.80 ± 0.92 % and 24.98 ± 0.89 % for open market and grocery store shrimp respectively.

Table 6.

Proximate composition of samples

Nutrients (%)	Open Market		Grocery store	
	Fish	Shrimp	Fish	Shrimp
Moisture	31.45± 1.41[a]	21.80±0.92[b]	30.63±0.87[a]	24.98±0.89[b]
Crude Protein	37.22± 1.23[b]	18.90±0.79[b]	41.35±0.76[b]	21.54±0.81[b]
Lipid	5.94± 0.11[c]	1.25 ± 0.11[d]	8.72 ± 0.19[c]	1.02 ± 0.08[d]
Fibre	1.55± 0.09[d]	2.76 ± 0.09[d]	3.27 ± 0.21[d]	0.98 ± 0.10[d]
Ash	7.43± 0.08[c]	4.52 ± 0.49[c]	2.32 ± 0.09[d]	5.31 ± 0.44[c]
Carbohydrate	16.41± 0.05[c]	50.77 ± 1.19[b]	13.71 ± 0.89[c]	46.17 ± 1.01[b]

Note:Values are means± standard error; Means with the same letter are not significantly different (P > 0.05)

Open market and grocery store lipid content for fish were: 5.94 ± 0.11 % and 8.72 ± 0.19 % respectively, while 1.25 ± 0.11 % and 1.02 ± 0.08 % for open market and grocery store shrimps respectively. The fibre content for the open market and grocery store fish were: 1.55 ± 0.09 % and 3.27 ± 0.21 % respectively, while 2.76 ± 0.09 % and 0.98 ± 0.10 % for open market and grocery store shrimps respectively. Results revealed no significant difference in the proximate composition of the open market and grocery stores fish and shrimps. This finding suggests that fish and shrimps are very high in nutrient composition and should be included in our meals.

Conclusions

The microbiological and proximate analysis of fish and shrimps sold in open markets and grocery stores have been evaluated in this research. Results revealed that the samples exceeded the acceptable standard limit of contamination recommended by FAO/WHO and also microorganisms identified in this study could pose high health risk. It is recommended, therefore, that both open market and grocery store fish and shrimps be properly washed and cooked adequately before consumption. Public enlightenment and proper monitoring by food regulatory bodies are also recommended.

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Public health implication of the detection of pathogenic bacteria in beef during processing in abattoirs from Benin City, Nigeria

Faith Iguodala Akinnibosun[1,✉] and Thelma Enifome Ugbogbo[1]

SUMMARY. The aim of this study was to determine the presence of pathogenic bacteria in beef during processing in abattoirs within Benin City, Nigeria. A total of 100 samples were obtained from12 sales tables and 8 processing halls during the study period. Isolation, enumeration and characterization of bacterial isolates were carried out using standard methods. Antibiogram of the test isolates was determined using disc diffusion technique. Bacterial isolates were screened for virulence genes. The results of this study showed that the highest total bacterial count was recorded in the processing hall at abattoir 4 ($9.28 \pm 0.26 \times 10^3$ cfu/cm^2) and the least ($3.47 \pm 0.19 \times 10^3$ cfu/cm^2) was from the processing hall at abattoir 2. The identified isolates were *Escherichia coli, Staphylococcus* sp. and *Salmonella* sp. All were multi-drug resistant. In this study, 11 *Escherichia coli* isolates were screened for the *tsh* (temperature sensitive haemagglutinin) virulence gene and 63.6% were positive for the *tsh* virulence gene. The virulence-associated gene in *Staphylococcus* sp. showed that only 22.2% tested positive to *hlg* (gamma hemolysin) gene while 93.3% of *Salmonella* sp. were positive for the *inv*A (invasive protein) gene. These results revealed the presence of multi-drug resistant bacterial isolates with virulence properties in beef during processing in abattoirs. Therefore, strict hygiene measures should to be put in place to combat the proliferation of these pathogenic bacterial isolates. In addition, misuse and abuse of antibiotics should be prohibited as these pathogens are becoming more resistant to most conventional drugs, thereby making associated diseases difficult to cure.

Keywords: abattoir, antibiotics, bacteria, pathogens, virulence genes

Introduction

Food safety is a complex issue, whereby animal proteins such as meats and meat products are generally regarded as a high risk commodities, to infection and toxicity (Yousef *et al.,* 2008). Diseases arising from ingestion of bacteria, toxins and also cells produced by microorganisms present in food are referred to as food borne illnesses (Clarence *et al.,* 2009).

[1] *University of Benin, Faculty of Life Sciences, Department of Microbiology, P.M.B 1154, Benin City, Edo State, Nigeria.*

✉ ***Corresponding author: Faith Iguodala Akinnibosun,*** *Department of Microbiology, Faculty of Life Sciences, University of Benin, P.M.B 1154, Benin City, Edo State, Nigeria,*
E-mail: faith.akinnibosun@uniben.edu

Meat and meat products are sometimes contaminated with germs in the abattoirs as a result of the use of cotaminated equipment, during handling and power shortage during storage as power outage results in the reduction of animal products' shelflife (Stagniitta *et al.,* 2006). Hygiene conditions are poor when foods are produced in non-industrial establishments, mainly due to insufficient monitoring or improper conditionsduring processing. These contaminated food ends up infecting or intoxicating children, elderly and immuno-suppressed individuals who are highly susceptible (Stagnitta *et al*., 2006). Raw beef and beef products could inevitably contain pathogenic microorganisms (Nichlos and de Louvous, 1995). Various Gram-negative *(Escherichia* sp, *Enterobacter* sp, *Yersinia* sp, *Pseudomonas* sp.and *Salmonella* sp.) and Gram-positive bacteria (Bacillus sp., Micrococcus sp. and Lactobacillus sp.) are frequently isolated from the meat surface (Polster and Hartiova, 1985). Gracey (1981) reported that, the organisms responsible for food poisoning by infection were *Salmonellae, Escherichia coli* and *Vibrio parahaemolyticus*. Those responsible for poisoning by toxin production included *Staphylococcus aureus, Clostridium perfringens, Clostridium botulinum, Bacillus cereus* and Streptococci. Other bacteria which may cause occasionally outbreaks of food poisoning included: *Streptococci, Proteus, Pseudomonas, Providencia, Citrobacter, Aeromanas Hydrophila, Yersinia enteracolitica, Campylobacter,* and *Shigella flexineri* and are the most commonly implicated organisms in food-borne illnesses (Meadand Dietz, 1999).

Many of the slaughter houses/abattoirs are more than 50 years old without adequate basic amenities viz. proper flooring, ventilation, water supply, lairage, transport etc. In addition to these deficiencies, slaughter houses/abattoirs suffer from very low hygiene standard posing a major public health and environmental hazards due to discrete disposal of waste and highly polluted effluent discharge. Bacterial infections/diseases are widely treated with a variety of antibiotics in both animals and humans (Erb *et al*., 2007). However, misuse of antibiotics in clinical *and veterinary settings has resulted in the emergence of multidrug-resistant* microbes (Schierack *et al*., 2006; Wang *et al*., 2011). Researchers have characterized that *antibiotic resistance is more common in pathogens compared to commensal* organisms, and is linked to the association between resistance and virulence factors or due to frequent exposure of pathogenic strains to antibiotics (Boerlin *et al*., 2005).

A subset of genes are key players in the ability of a bacterium to cause disease. The products of such genes facilitate the successful colonization and survival of the bacterium in or cause damage to the host (Coulter *et al*., 1998). Bacterial virulence factors may be encoded on the chromosomal DNA, plasmid, transposon or temperate bacteriophage DNA. Other virulence factors are acquired by bacteria following infection by a particular bacteriophage, which integrates its genome into the bacterial chromosome by the process of lysogeny. The virulence factors of bacteria can be divided into a number of functional types, these are 1) The adherence and colonization factors, 2) The invasion factors, 3) Capsules and

other surface components, 4) Endotoxins and 5) Exotoxins (Peterson, 1996). This ability of bacteria establishes the pathogenic success of well-adapted gastrointestinal pathogens such as that differentially coordinate the expression of sets of genes as they pass from one host environment to another in their passage through the gut, including the movement through the gastric barrier and survival within macrophages or intestinal epithelial cells (Chaudhuri *et al*., 2013).

The aim of this study was to determine the presence of pathogenic bacterial isolates in abattoirs during processing. The objectives were to:

i. determine the bacterial count in the sales tables and processing halls.
ii. isolate and characterize the bacterial isolates
iii. determine the antibiotic susceptibility profile of the isolates.
iv. determine the presence of virulence genes in the isolates.

Materials and methods

Study site

A total of four (4)abattoirs in Benin City, Edo State, Nigeria were used for this study. Samples were collected from cow skin, hands of handlers, processing tables and floors. The cows were kept in the lariages before they were slaughtered and afterwards stored in cold rooms.

Sample collection

Samples were collected by swabbing a 100 cm^2 area of the sales tables and floor of processing halls with sterile swab sticks which were pre-moistened with 2 % W/V peptone water. After swabbing, the swab sticks were put into a sterile containers and stored in ice while being transported to the laboratory (ISO 18593, 2004).

Isolation and enumeration of bacteria

All samples were cultured by the pour-plate method on Nutrient agar for total bacterial count. Plates were incubated at 37 ^oC for 24 hours, after which the colonies grown were counted using standard plate count method (ISO 18593, 2004).

Characterization of Isolates

Samples were plated on MacConkey agar, Mannitol salt agar and Xylose Lysin Deoxycholate agar using the spread plate method. This was followed by aerobic incubation at 37 ^oC for 24 hours. Discrete pinkish colonies on the MacConkey agar were isolated and sub-cultured to obtain pure colonies. White to deep yellow colonies that developed on the Mannitol salt agar plates were isolated

and sub-cultured to obtain pure colonies. Red colonies with black centres that developed on the plates were isolated and sub-cultured to obtain pure colonies. Pure colonies from the different media were counted using standard plate count method. Confirmatory tests for *Escherichia coli, Staphylococcus* sp, and *Salmonella* sp. were carried out according to ISO 18593 (2004).

Antibiotic sensitivity test

The antibiotic sensitivity of the 3 bacterial isolates (*Escherichia coli, Staphylococcus* sp. and *Salmonella* sp.) to 14 antibiotics: Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Gentamycin (10 µg), Ampicillin (10 µg), Augmentin (30 µg), Ceftriaxone (30 µg), Streptomycin (10 µg), Ceftazidime (5 µg), Tetracycline (30 µg), Enrofloxacin (5 µg), Amoxicillin (10 µg), Penicillin (10 µg), Septrin (30 µg), Erythromycin (10 µg), was determined by the standard disk-diffusion technique in Mueller-Hinton agar (Clinical and Laboratory Standards Institute, 2013).

Determination of virulence genes

The genomic DNA of the bacterial isolates: *Escherichia coli* (11)*, Staphylococcus* sp. (9), *Salmonella* sp. (15), was extracted using ZYMO (ZR) bacterial genomic DNA extraction kit (Zymo Research, U.S.A.) following the manufacturer's instructions. The presence of three virulence genes *tsh*, *hlg*, and *invA* (which enhance virulence and pathogenicity) in *E. coli*, *Staphylococcus aureus* and *Salmonella* sp. isolates respectively were detected by polymerase chain reaction (PCR). Amplification of the genes was achieved by employing the specific primers corresponding to the virulence genes. PCR was performed in a total reaction volume of 10 µl containing 1.5 µl of template DNA (1 µg), 5.0 µl of 2×PCR master mix (Norgen Biotek Corporation, Canada) which contains Taq DNA polymerase, dNTPs, reaction buffer, $MgCl_2$, KCl and PCR enhancer/stabilizer; 1.0 µl of forward primer (2.5 µM), 1.0 µl of reverse primer (2.5 µM) and 1.5 µl of nuclease-free water. PCR reactions were carried out in a TC-412 Thermocycler (Keison, United Kingdom) employing the following amplification conditions: Initial denaturation step of 95 ^{o}C for 2 minutes, followed by 35 amplification cycles each consisting of denaturation at 94 ^{o}C for 1 min, annealing for 60 seconds and extension or elongation at 72 ^{o}C for 2 minutes. Reactions were terminated at final extension of 72 ^{o}C for 10 minutes. The amplified products were analysed by electrophoresis on a 1 % (w/v) agarose gel, stained with ethidium bromide in the presence of a 1 kb PCR sizer ladder (Norgen Biotek Corporation, Canada). Electrophoresis was performed at 80 V for 60 minutes. The sizes were then read against molecular marker of known size by looking at the banding patterns received after gel electrophoresis results, and to observe the virulence genes of the different bacterial isolates (Oloyede *et al*., 2016).

Statistical analysis

All data were analysed using the IBM Statistical Package for Social Science (SPSS) software. Data were expressed as mean ± Standard Deviation. Analysis of variance (ANOVA) was used to determine if the variation observed between variables is significant. The p-value > 0.05 was considered not statistically significant (Ogbeibu, 2015).

Results and discussion

In this study, the bacteria isolated were *Escherichia coli*, *Salmonella* sp and *Staphylococcus aureus* (Table 1). Results are similar to that reported by Kayode (2014), who observed that *Escherichia coli, Salmonella* sp*., Proteus* sp*., Klebseilla* sp*., Pseudomonas* sp*., Enterobacter* sp., *Streptococcus* sp*., Shigella* sp*., Staphylococcus* sp*., Bacillus* sp. and *Clostridium* sp. where isolated and identified in Kara and Odo-eran abattoirs in Ogun state (Nigeria). Also, Itah *et al.* (2005), isolated *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Staphylococcus epidermidis, Micrococcus roseus, Bacillus subtilis*. Species of *Streptococcus, Klebsiella, Pseudomonas* and *Salmonella* from Uyo abattoir (Nigeria).

Table 1.

Morphological and biochemical characteristics of bacterial isolates.

TEST	MacConkey Agar	Mannitol Salt Agar	XLD agar
Colony	Pinkish	Yellow/Milky	Reddish
Gram stain	-	+	-
Shape	Rod	Cocci	Rod
Arrangement	Single	Irregular	Single
Lactose	+	+	-
Indole	+	-	-
Oxidase	-	Nil	-
Citrate	-	Nil	Nil
Catalase	+	+	Nil
Coagulase	-	+	Nil
Mannitol	Nil	+	Nil
Hydrogen	-	-	+
Urease	-	Nil	-
Motility	Motile	Nil	Motile
Isolates	*Escherichia coli*	*Staphylococcus aureus*	*Salmonella* sp.

Results revealed that the heterotrophic bacterial count from the processing halls ranged from $3.47 \pm 0.19 \times 10^3$ to $9.28 \pm 0.26 \times 10^3$ cfu/cm^2. It was observed that the *E. coli* count, which ranged from $2.50 \pm 0.40 \times 10^3$ to $2.91 \pm 0.22 \times 10^3$ cfu/cm^2, was higher than those of *Staphylococcus aureus* ($0.55 \pm 0.06 \times 10^3$ to $1.97 \pm 0.70 \times 10^3$

cfu/cm^2) and *Salmonella* sp. (0.77 ± 0.08 x 10^{3}to 2.52 ± 0.74 x 10^{3}cfu/cm^2) (Table 2). From the sales tables in the abattoir, similar results were obtained, as the *E. coli* count (0.74 ± 0.37 x 10^3 to 4.07 ± 0.81 x 10^{3}cfu/cm^2) was higher than the *Staphylococcus* (1.28 ± 0.38 x 10^3 to 2.11 ± 0.49 x 10^{3}cfu/cm^2)and *Salmonella* (1.24 ± 0.44 x 10^3 to 1.94 ± 0.63 x 10^{3}cfu/cm^2) count, while the heterotrophic bacterial count ranged from 6.29 ± 1.25 x 10^3 to 7.97 ± 0.03 x 10^{3}cfu/cm^2) (Table 3). This is not surprising as *E. coli* is an enteric organism and would have come from the intestinal tract and faecal matter of the slaughtered animals (Jay, 2005). This also implies that *E. coli* is the fastest in colonizing the environment. Studies have shown that pathogenic microbes especially *E. coli* shed by animals can persist in soil, water, manure, and feed, where it can spread to other uninfected animals (Hancock *et al.,* 1997) and to humans (Dos Santos *et al.,* 2007).

Table 2.

Bacterial load of processing halls at the abattoirs.

Abattoirs	Heterotrophic bacterial count x10^3 cfu/cm^2	*E. coli* count x10^3 cfu/cm^2	*S.aureus* count x10^3 cfu/cm^2	*Salmonella* count x10^3 cfu/cm^2
AB 1 (n = 2)	7.84 ± 2.08	2.90 ± 0.23	1.97 ± 0.70	2.52 ± 0.74
AB 2 (n = 2)	3.47 ± 0.19	2.91 ± 0.22	0.55 ± 0.06	0.77 ± 0.08
AB 3 (n = 2)	7.19± 0.94	2.66 ± 0.35	1.26 ± 0.06	1.51 ± 0.06
AB 4 (n = 2)	9.28 ± 0.26	2.50 ± 0.40	1.86 ± 0.51	1.71 ± 0.47
p-value	0.047	0.811	0.146	0.138

Key: AB= Abattoir, n = number of samples collected

Table 3.

Bacterial load of sales tables in the abattoirs.

Abattoir	Heterotrophic bacterial count (x10^3)cfu/cm^2	*E. coli* count (x10^3)cfu/cm^2	*Staphylococcus aureus* count (x10^3)cfu/cm^2	*Salmonella* count (x10^3) cfu/cm^2
AB 1 (n = 3)	7.43 ± 0.81	3.41 ± 0.46	2.11 ± 0.49	1.70 ± 0.30
AB 2 (n = 3)	6.29 ± 1.25	4.07 ± 0.81	1.28 ± 0.38	1.06 ± 0.15
AB 3 (n = 3)	8.21 ± 0.99	0.74 ± 0.37	1.71 ± 0.19	1.24 ± 0.44
AB 4 (n = 3)	7.97 ± 0.03	2.33± 0.32	1.83 ± 0.41	1.94 ± 0.63
p-value	0.475	0.010	0.522	0.466

Key: AB= Abattoir, n = number of samples collected

Escherichia. coli isolates were observed to be multi-drug resistant (resistant to at least three classes of antibiotics) (Table 4). They were resistant to ampicillin, tetracycline and ceftazidime. This characteristic resistance to ampicillin and tetracycline identified at a high rate, is similar to previous findings in *E. coli* isolates from diarrheic or diseased animals in China (Rehman *et al.,* 2017; Zhang *et*

al., 2017). *Salmonella* isolates in this study were resistant to a number of notable antibiotics. It was observed that they were resistant to ceftazidime (100%), ampicillin (83.3%) and chloramphenicol (79.2%). This implied multi-drug resistance, as the three antibiotics listed above were from different classes of antibiotics, with different mechanisms of action. This is similar to previous study, where Akbar and Anal, (2013) reported that all strains of *Salmonella* isolated from poultry in their study were resistant to three or more antibiotics. The resistance profilewere as follows: ampicillin (87%), chloramphenicol (63%), tetracycline (60%), trimethoprim (42%), sulphonamides (42%) and streptomycin (61%). The *Staphylococcus* isolates in this study were resistant to penicillin (100%), amoxicillin (85%) and augmentin (75%). The aforementioned antibiotics are all beta-lactams showing that these isolates are methicillin resistant. This result is consistent with the findings of Adesiji *et al.* (2011) who reported that *S. aureus* from retail meat products in Oshogbo, Nigeria were all resistant to amoxicillin. Their study also reported that *S. aureus* was susceptible to gentamycin, erythromycin and streptomycin which is in line with the findings of this present study.

In this study, 11 *E. coli* isolates were screened for the *tsh* (temperature sensitive haemagglutinin) virulence gene. The results showed that 7 (63.6%) out of the 11 were positive for the *tsh* gene (Figure 1). The *tsh* gene contributes to the development of lesions and deposition of fibrin in the avian air sacs (Kobayashi *et al.,* 2010). The *tsh* gene is mostly reported in APEC (avian pathogenic *E. coli*) strains (Saidenberg *et al.,* 2013) where it is believed to play a role in mechanisms of adherence to the respiratory tract of poultry (Dozois *et al.*, 2000).

Diseases caused by Staphylococci are the result of a synthesis of several virulence factors including the different hemolysins which are important for virulence of the *S. aureus* and other Staphylococci (da Silva *et al*., 2005). They're four types of hemolysins - alpha, beta, gamma and delta hemolysin produced by coagulase positive Staphylococci. Several studies indicated that hemolysins of *S.aureus* correlated well with infections in human and animals (Tackeuchi *et al.,* 2001; Larsen *et al.,* 2002). In this study, of the nine isolates screened for gamma haemolysin (*hlg*) virulence gene only 2 (22.22%) tested positive for *hlg* (Figure 2). In this study, *invA* (invasion protein) gene of *Salmonella* was investigated using *Salmonella* specific primers.

Of the 15 isolates screened for *invA* gene, 14 were positive and 1 was negative. The *invA*genes amplified by PCR was observed as 248bp amplicons (Figure 3). The *invA*, gene of *Salmonella* contains those sequences that are unique to this genus and has been proven to be a suitable PCR target with potential diagnostic applications (Jamshidi *et al.,* 2009). The *inv*A gene codes for protein in inner membrane of bacteria, which is necessary for invasion to epithelial cells (Shanmugasamy *et al.,* 2011). This gene is involved in the invasion of the cells of

the intestinal epithelium and is present in pathogenic *Salmonella*. Therefore for salmonellosis to occur it is important that a gene responsible for invasion must be present. According to Zahraei *et al.* (2006), this gene is essential for full virulence in *Salmonella* and is thought to trigger the internalization required for invasion of deeper tissue.

Table 4.

Antibiotic sensitivity pattern of bacterial isolates

Antibiotics	*Escherichia coli* isolates		*Salmonella* isolates		*Staphylococcus* isolates	
	No. (%) of sensitive isolates	No (%) of resistant isolates	No (%) of sensitive isolates	No (%) of resistant isolates	No (%) of sensitive isolates	No (%) of resistant isolates
CHL	10(52.6%)	9(47.4%)	5(20.8%)	19(79.2%)	16(80%)	4 (20%)
CPR	12(63.2%)	7(38.8%)	7(29.2%)	17(70.8%)	14 (70%)	6 (30%)
GEN	15(78.9%)	4(21%)	13(54.2%)	11(45.8%)	20(100%)	0
AMP	0	19(100%)	4(16.7%)	20(83.3%)	-	-
AUG	10(52.6%)	9(47.4%)	11(45.8%)	13(54.2%)	5(25%)	15(75%)
CTR	5(26.3%)	14(73.7%)	22(91.7%)	2 (8.3 %)	-	-
STR	15(78.9%)	4(21 %)	14(58.3%)	10(41.7%)	12(60%)	8(40%)
CAZ	1(5.2%)	18(94.7%)	0	24(100 %)	-	-
TET	0	19(100%)	15(62.5%)	9(37.5%)	11(55%)	9(45%)
ENOA	19(100%)	0	13(54.2%)	11(45.8%)	-	-
MC	-	-	-	-	3(15%)	17(85%)
PEN	-	-	-	-	0	20(100%)
SXT	-	-	-	-	11(55%)	9(45%)
ERY	-	-	-	-	19(95%)	1(5%)

Key: CHL= Chloramphenicol, CPR = Ciprofloxacin, GEN = Gentamycin, AMP = Ampicillin, AUG = Augmentin, CTR = Ceftriaxone, STR = Streptomycin, CAZ = Ceftazidime, TET = Tetracycline, ENO = Enrofloxacin, AMC = Amoxicillin, PEN = Penicillin, SXT = Septrin, ERY = Erythromycin.

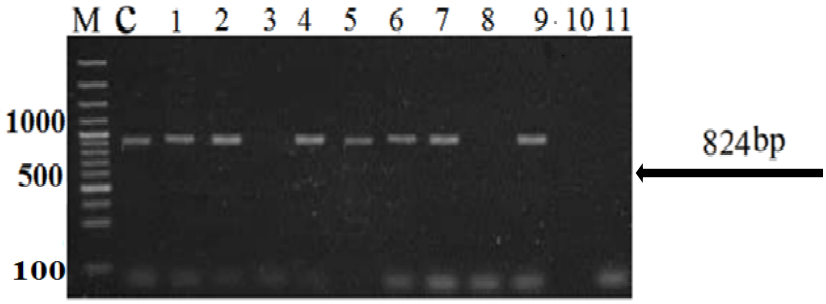

Figure 1. Gel electrophoresis of *E. coli tsh* virulence gene PCR products. Lane M: 100 bp marker, Lane C: positive control, Lanes 1, 2, 4, 5, 6, 7 and 9 indicate positive bands for *E.coli tsh* gene, Lane 3, 8, 10 and 11 are negative bands for *E. coli tsh* gene.

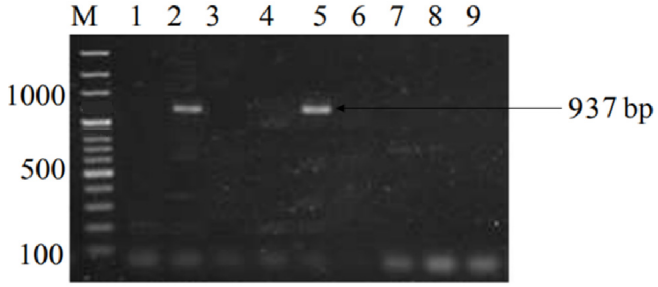

Figure 2. Gel electrophoresis of *Staphylococcus* spp. *hlg* virulence gene PCR products. Lane M: 100 bp marker, Lanes 2 and 5: positive bands for *hlg,* Lane 1, 3, 4, 6, 7, 8, and 9 are negative for *hlg*.

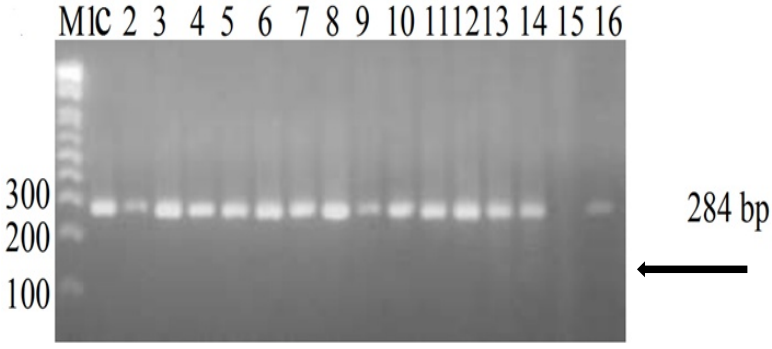

Figure 3. Gel electrophoresis of *Salmonella inv*A virulence gene PCR products. Lane M: 100 bp marker, Lane 1C: positive control, Lanes 2-14 and 16: *invA* gene band. Lane 15 is negative for *invA* virulence gene.

Conclusions

Pathogenic bacteria detected in abattoirs could pose great risk to public health, especially when they posess antibiotic resistance genes and virulence factors. It is recommended, therefore that beef should be properly washed and cooked adequately before consumption. Public enlightenment and proper monitoring of meat and meat products, as well as implementation and surveillance of hygiene measures through the processing and selling by food regulatory bodies is also advised.

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Treatment of rubber effluent from rubber processing plant with fungi

Francis Aibuedefe Igiebor[1,2,✉], Pascal Chukwuebuka Okoye[2] and Oghoreye Nosa Evbuomwan[3]

SUMMARY. Rubber processing industry produces materials used for the manufacturing of rubber industrial products. Large volume of water is consumed and produces a huge amount of effluent which is later discharged into the waterways, thereby causing pollution that affects human health. The effluent was collected from discharge points of a rubber factory. Microbial analyses were carried out before and after pollution. During the incubation, microbial growth in culture tubes was determined using UV-Spectrophotometer by measuring absorbance at wavelength 600nm at 24 hours interval. Four fungi were isolated and identified from rubber effluents which include *Mucor mucedo* and *Aspergillus niger, Aspergillus flavus* and *Penicillium notatum*. Individually, the selected fungi isolates were tested for its efficiency on the bioremediation of rubber processing effluent. The physicochemical properties reduction of the effluent such as BOD, COD, TS, TSS, TDS, phosphate and ammonia were observed after incubating for 7 days. Based on the data obtained in this study, it can be concluded that *Mucor mucedo* and *Aspergillus niger* can be used for bioremediation of rubber processing industry effluent with high efficiency.

Keywords: effluent, industry, indigenous, rubber.

Introduction

Rubber processing industry is one of the essential industries, which produce raw materials used for the manufacture of rubber industrial products such as conveyor belts, rubber rollers, automotive products like fan belts and radiator hoses, latex products which include rubber gloves and toys hygienic products and several kinds of adhesives. The main users of natural rubber are tire and footwear industries (Girish, 2014). The

[1]*Wellspring University, College of Natural and Applied Sciences, Department of Microbiology, Benin City Edo State, Nigeria.*
[2]*University of Benin, Biotechnology and Sustainability Research Group, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, Benin City, Nigeria.*
[3]*University of Benin, Faculty of Life Sciences, Department of Microbiology, Benin City, Nigeria.*
✉ ***Corresponding author: Francis Aibuedefe Igiebor**, Department of Microbiology, College of Natural and Applied Sciences, Wellspring University, Benin City Edo State, Nigeria,*
E-mail: francis.igiebor@lifesci.uniben.edu

processing of these natural rubbers has several environmental impacts as pollution (Tekasakul and Tekasakul, 2006). The production process of rubber products from natural rubber needs enough volume of water for its operation, thus producing large quantities of effluent (Leong *et al*., 2003; Rungruang and Babel, 2008). The disposal of these effluents into surface waters – wells, streams, lakes or even the sea without any treatment can give rise to a severe reduction of dissolved oxygen, thereby affecting the normal environment supporting the aquatic system (Mohammadi *et al*., 2010).

However, environmental damages generated from this industry could become big issues. Natural rubber processing sector consumes a great amount of water, energy and chemicals as well as other utilities. It also discharges enormous quantities of wastes and effluents (Leong *et al*., 2003). Wastewater is an unavoidable by-product of rubber processing: whatever processing procedures are used for preparing products from latex, there will always be an aqueous liquid as a by-product (Rungruang and Babel, 2008). When this wastewater finds their way into surface waters wells, streams, lakes or even the sea without any treatment, will certainly pollute those water bodies. The disposal of these effluents into water bodies can give rise to a serious reduction of dissolved oxygen, hence affecting the environment supporting the aquatic system (Mohammadi *et al*., 2010). The increasing universal concern on the environment demands that wastes should be properly managed in order to lessen and perhaps eradicate their potential harm to public health and the environment. Biodegradation is the process of utilizing indigenous microorganisms for the degradation of complex organic matter into simpler ones.

Rubber and effluents from rubber processing have been reported by researchers to support the growth of the microorganism (Atagana *et al*., 1999a; Bode *et al*., 2001; Cherian and Jayachandran, 2009). Owing to the need of biological treatment of rubber industry wastes and knowing the fact that various fungi and bacteria can grow and degrade the rubber industry wastes, the present study was aimed at isolating and characterizing indigenous fungi that can readily degrade the rubber wastes present in the effluents, with a view to developing an effective biological treatment.

Materials and methods

Sample collection

Rubber effluent samples were collected from discharge points of a rubber factory. For microbiological analysis, samples were collected in 500 ml sterile bottles. Clean plastic containers rinsed several times with the sample were used. The wastewater sample used for DO (dissolved oxygen) and BOD (biological oxygen demand) determinations were collected directly into dark DO bottles and were added some drops of manganous sulphate solution to fix the dissolved oxygen. Samples were collected by lowering the sterile bottle by means of a string into the tank and covered with the screw cap thereafter. The samples were stored at a temperature of 4^0C until required (usually between 24 and 48 hours).

Microbial analyses

Microbial analyses were carried out using the method of Cheesebrough (2000) and Cowan and Steele (1974) before and after pollution. 10 g of Soil sample was collected aseptically, labelled and store in ice packed plastic coolers and transported to the Environmental Biotechnology Sustainability Research laboratory, University of Benin, Nigeria where microbial analysis was carried out within 24 hours of sampling so as to maintain the stability of the sample without significant alteration in the microbial population.

Serial dilution was carried out by weighing 1 g of soil into 9 ml of sterile water contained in a 20 ml test tube and agitated to dislodge the microorganisms from the soil particles. From this initial dilution, a five-fold (10^{-5}) serial dilution was prepared.

Enumeration of heterotrophic fungi

The total heterotrophic fungi count was measured using the method of Taiwo and Oso (2004), by pour plating 1 ml of 10^{-3} dilution into Potato Dextrose Agar (PDA) supplemented with antibacterial agents (50 µg/ml of streptomycin and 30 µg/ml of penicillin) to inhibit the growth of bacterial contaminants. Fungal counts were observed and reported after 72 hours of incubation at room temperature (26^0C). Distinct fungal colonies were subculture repeatedly on freshly prepared Potato Dextrose Agar plates. Pure isolates of the microorganisms were maintained on agar slants as stock, which was preserved in the refrigerator for further use.

Characterization and identification of isolates

Distinct colonies of fungal isolates were characterized and identified based on their cultural and morphological features as described by Barnett and Hunter (1987). The characterizations were achieved through staining techniques-using lactophenol in cotton blue.

Acclimatization of isolates

The fungus was acclimatized by growing it in minimal organic salts medium amended with 10% of rubber processing industry effluent. The minimal medium used in degradation studies contained (mg/mL) KH_2PO_4 – 0.675; Na_2HPO_4 – 5.455; NH_4NO_3 – 0.25; $MgSO_4$ – 0.2; $Ca(NO_3)_2$ – 0.1; and 1 mL mineral solution (Table 1). The fungus was cultured in a 500-mL flask with the medium (100ml/ flask) at 30^0C for 24 hours on a rotary shaker operating at 120 rpm. 1.0 mL of this was transferred aseptically to a second flask containing inorganic salts medium. After this solution became turbid, the culture was transferred to the third flask and incubated. This culture was used for biodegradation studies (Fig. 1).

Table 1.

Composition of minimal organic salt medium
(Source: Gokul and Vijayan, 2015)

Composition	Quantity
KH_2PO_4	0.675 mg/L
Na_2HPO_4	5.455 mg/L
NH_4Cl	0.25 mg/L
$MgSO_4$	0.2 mg/L
$Ca(NO_3)_2$	0.1 mg/L

Biodegradation studies

Different parameters such as biological oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), total dissolved solids (TDS), ammonia ($NH4^+$) and phosphate (PO_4^{3-}) were assayed using standard protocols (APHA, 1995). The effluent was inoculated with 1% inoculum and incubated for 15 days and the estimation was done at the interval of 3 days.

At 24 hours interval during the incubation, microbial growth in culture tubes was determined spectrophotometrically by measuring absorbance at wavelength 600nm with a UV-visible spectrophotometer (Igiebor *et al*., 2017; Osarumwense and Igiebor, 2018).

Results and discussion

Effluents are an inevitable by-product of rubber processing, no matter the processing procedures employed for preparing products from latex (Rungruang and Babel, 2008). Asia and Akporhonor (2007) and Mohammadi *et al*. (2010) reported that effluents from rubber processing industries are very harmful and contain strong colour, a highly fluctuating pH, a large amount of suspended solids, high temperature, BOD and COD. Therefore, the treatment of rubber wastewater is a must before it is being disposed to natural water system (Atagana *et al*., 1999b; Iyagba *et al*., 2008). Most environmentally friendly process for effluent treatment is biodegradation utilizing indigenous microorganisms for the degradation of complex organic matter into simpler ones (Kumar *et al*., 2011). Bode *et al*. (2001), Cherian and Jayachandran (2009) revealed that the effluents from rubber processing have been known to support microbial growth; hence there is a need to isolate from the rubber effluents. The major purpose of the effluent treatment is to remove the suspended and soluble organic constituents measured as chemical oxygen demand (COD) or biochemical oxygen demand (BOD).

In the present study, a successful reduction of both BOD and COD of effluent from rubber processing industry was observed to a level adequate to make the effluent ready to be discharged into the environment, by treating with fungi inoculum. Percentage BOD and COD reduced significantly (within the range of 68 – 76 %) which was higher than the permissible limits. The reduction (64.09 mg/L) in Total Solids (TS) of rubber

effluent after treatment (Table 2) was within the permissible limit of 2100 mg/L (Gokul and Vijayan, 2015). The reduction of TS after treatment might be due to the use of suspended organics by microorganisms for their growth and development.

The reduction in BOD of rubber effluent after treatment (Table 2) showed a significant decrease in BOD values which could be attributed to the consumption of organic material by fungi as a source of food. The reduction in BOD after treatment can result in an instantaneous reduction of the microbial population. Although, high growth of fungi (microbes) had consumed the oxygen present in the treatment container. Furthermore, the continuous and excess aeration may have led to the reduction in BOD. The COD reduction (68.04%) could be due to the presence of high amounts of nutrients in the environment (Table 2), which may have favoured the growth of the isolates. The significant reduction in ammonia (248.97 mg/L – 118.63 mg/L) was observed after treatment, this could suggest that there was degradation of toxic solid components in the effluent by fungi.

Table 2.

Physio-chemical analysis of the effluent treatment

Parameters	Before treatment	After treatment	Percentage reduction
pH	3.36		
TDS (mg/L)	2797	1371	50.98 %
TSS (mg/L)	200	56	72.00 %
TS (mg/L)	2997	1076	64.09 %
Ammonia (mg/L)	248.97	118.63	52.35 %
Phosphate (mg/L)	15.53	8.76	42.31 %
BOD (mg/L)	380	89	76.58 %
COD (mg/L)	6940	2218	68.04 %

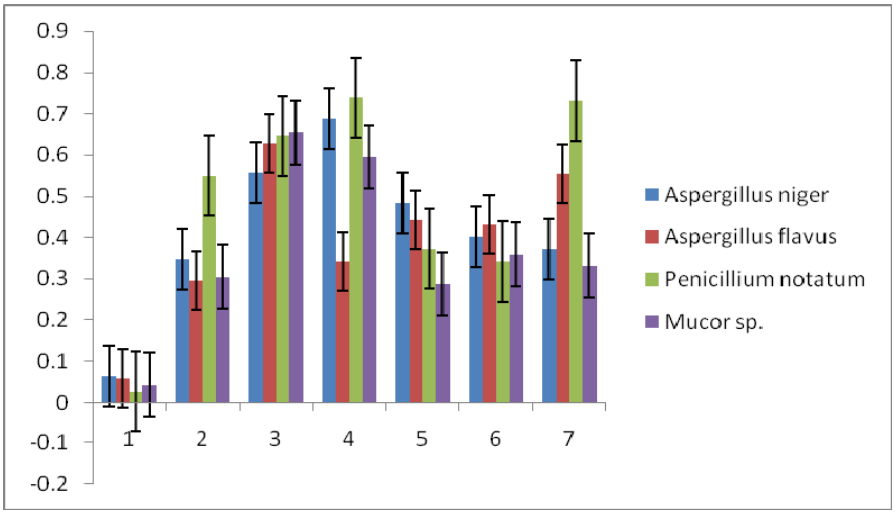

Figure 1. *In vitro* biodegradation studies of isolates

This study showed that the treatment of effluent with fungal isolates resulted in a successful reduction of BOD, COD, TDS, TSS, NH4+ and PO43 to a level of about 50 – 77%. This significant reduction is adequate to make the effluent set to be discharged into the environment. The treatment system that decreases or remove the level of $NH4^{+}$ and $PO4^{3-}$ compounds in the industrial effluent is termed to be highly effective (Ye *et al*., 1988). Microbial treatment (fungal) is also reported to reduce the levels of total suspended solids (TSS) and total dissolve solids (TDS) of industrial effluents (Arun *et al*., 2004; Monica *et al*., 2011), as observed in the present study.

Using Ward's method of cluster analysis, it was observed that *Mucor mucedo* and *Aspergillus niger* were most similar (Fig. 2) in enhancing remediative capacity of the rubber effluent followed by *Aspergillus flavus* than *Penicillium notatum*, which was a stand-alone. This suggests that *Mucor mucedo* and *Aspergillus niger* have the capability to treat rubber effluent.

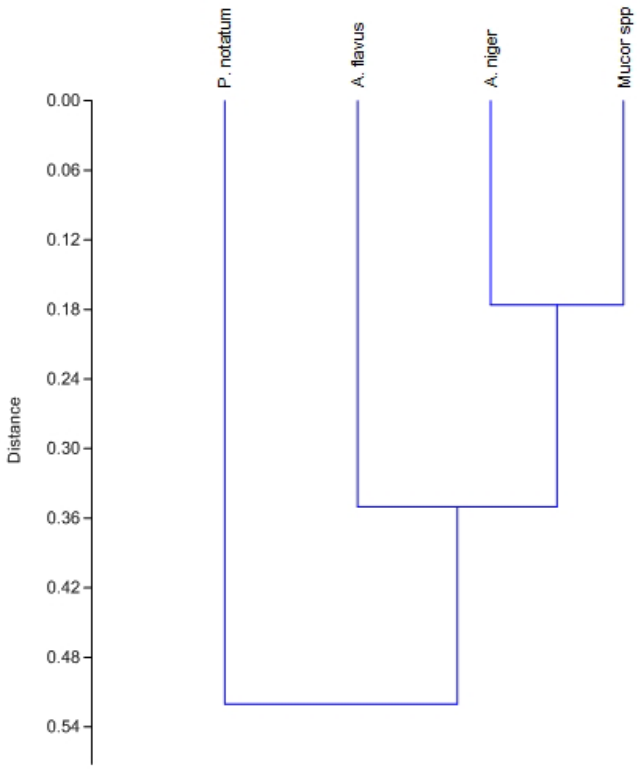

Figure 2. Dendrogram from cluster analyses of fungi isolates accumulated during the study

Conclusions

This study has successfully identified four indigenous fungi that could be used for the treatment of rubber processing effluent. The investigation further suggests that *Mucor mucedo* and *Aspergillus niger* isolated from rubber processing effluent could be employed for the biodegradation of rubber processing industry effluent. There is need for molecular characterization for precise identification.

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Cellulase production and morphology of *Trichoderma reesei* in different experimental conditions

Rahela Carpa[1,✉], Alin Cândea[1], Alexei Remizovschi[1],
Lucian Barbu-Tudoran[1] and Maria Cornelia Maior[1]

SUMMARY. Cellulases production involving alternative substrates has been intensely researched, because it offers perspectives for lowering the costs for enzyme production, costs which are a major obstacle for the development of this field. The concentration, turnout, effectiveness of the enzyme production depends on the content of culture medium. In order to find a way to increase cellulase production, two culture media with different cellulosic substrates in submerged culture experiments were tested. We used a promising off-corn growth media that was efficient and the cellulases output due to substrate action was calculated. The reduction of the sugars released by the enzyme was noticed. On off-corn medium the measurements reached 0.188 mg of released glucose/mL/min/50ºC, while on PSM medium the strains reached the release of only 0.118 mg glucose/mL/min/50ºC. The abundance of the fungi and the pellet morphology were microscopically compared by optic and electronic means. Mycelia with hyphae and spores were also visible in these circumstances, suggesting that when the environment of the mycelium alters, part of the mycelium autolyses and spores are released to propagate. The present study also included the use of newly synthesized cellulases in order to obtain a plant protoplast culture. This study proves that cellulolytic enzymes with further application in laboratory can be provided by less expensive techniques.

Keywords: cellulase activity, off-corn culture media, *Trichoderma reesei*

Introduction

Trichoderma reesei represents an efficient producer of extracellular proteins, therefore it is widely employed in cellulase production. Cellulases play a central role in the biological conversion of lignocellulosic materials to fermentable sugars. Three types of cellulases are known to be produced by *T. reesei* and to interact releasing glucose,

[1] *Babeş-Bolyai University Cluj-Napoca, Faculty of Biology and Geology, Department of Molecular Biology and Biotechnology, 1 M.Kogălniceanu Str., Cluj-Napoca, Romania.*
✉ ***Corresponding author: Rahela Carpa**, Faculty of Biology and Geology, Department of Molecular Biology and Biotechnology, 1 M.Kogălniceanu Str., Cluj-Napoca, Romania, E-mail: k_hella@yahoo.com*

namely: cellobiohydrolases or exo-β-glucanase (EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and β-glucosidases EC 3.2.1.21) (Ma *et al*., 2013; Gupta *et al*., 2016). *T. reesei* also provides xylanase for the digestion of hemicellulase, resulting xylose. The genome of *T. reesei* comprises at least 200 genes involved in encoding glycoside hydrolases (Häkkinen *et al*., 2012), enzymes which free fermentable sugars from lignocellulose. Accessary proteins have a support role, getting involved alongside cellulases, to assist degradation of lignocellulosic biomass, and are also subjected to increasing interest (Gao *et al*., 2011; Zhang *et al*., 2018). These components act synergistically in the conversion of cellulose to glucose (Muthuvelayudham and Viruthagiri, 2006).

Cellulose is the most abundant component of the plant cell wall. If its structure is broken down a wide variety of sugars would become available for other organisms or for life/carbon cycles. Fungi of the genus *Trichoderma* have become very popular due to their capacity to produce a wide range of cellulolytic enzymes (cellobiohydrolases, endo-β-1,4-glucanases and β-glucosidases) (Zhang *et al*., 2012; Somerville *et al*., 2004; Caroll and Somerville, 2009).

Trichoderma reesei has become the most important source of cellulolytic enzymes (Schuster and Schmoll, 2010) and subsequently (has become) a key point in producing second generation biofuels (Sukumaran *et al*., 2005; Bharathiraja *et al*., 2017; Wang *et al.,* 2014). Fuel crisis around the world will be a distant issue to deal with, if an alternative to fossil combustibles is going to be found. New strains of *Trichoderma reesei* have already an improved ability to increase the cellulose production more than 15–20 folds, compared to the wild type strain (Kubicek, 2013; Xia *et al.,* 2018). Even though many authors pursue the goal of producing industrial cellulases (Ahamed and Vermette, 2008; Jourdier *et al*., 2012), the goal of our work was to use a *Trichoderma reesei* strain to perform further laboratory scale experiments in plant/bacterial biotechnology area. Another study highlighted that not only the carbon source is affecting the co-regulation of biomass-degrading enzymes but also the structural characteristics of the substrate (Foreman *et al*., 2003; Peciulyte *et al*., 2014; Zhang *et al*., 2017).

We started our work in order to extend the knowledge on *T. reesei* liquid culture and reduce laboratory costs when cellulases are needed. Two culture media with different cellulosic substrates as carbon and energy source in submerged culture experiments were tested in order to find a way to increase celullase production. We found an off-corn growth media that was efficient, and we calculated the yield of cellulases production. Microscopic observations by optic and electronic means were carried out and a comparison between the fungi frequency and pellet morphology was made. An attempt to use the newly synthesized cellulases to obtain a plant protoplast culture is a part of our work. This study shows that cellulolytic enzymes with further application in laboratory can be obtain with low cost expenses.

Materials and methods

1. The microorganism. The microorganism used in this study was *Trichoderma reesei*, which was maintained on potato dextrose agar culture media (M129 culture media) (Göbel *et al.,* 2004) and grown and recultivated by culture plate method on PSM culture medium with agar (Mandel's agar media) (Mandels, 1975). This fungus was incubated at 30ºC, for 5–7 days, and after that the colonies with evidence of cellulose digestion were selected to be further assayed for cellulose activity.

2. Decomposition of cellulose (filter paper) by Trichoderma reesei. To emphasize cellulose decomposition, *Trichoderma reesei* inoculum is cultivated on a specific medium and incubated at 28ºC for 4 weeks. The characteristics of growth, the color of the paper and the part of the strip indicating disintegration are periodically observed. At the end, the number of test tubes, for each dilution, in which growth took place is determined, and the probable number of cellulosolytic microorganisms is calculated. The degree of paper deterioration is observed, and the phase of paper deterioration is assessed, compared to control (Carpa *et al*., 2014).

3. Cellulase activity at Trichoderma reesei in submerged system. For the submerged system the experiment consisted in two sets. In one set, the mineral culture media (PSM) contained mineral salt solution and the study was carried out according to Mendel's method (Mandels and Reese, 1957; Mandels, 1975). The second set was done on off-corn culture media. The citrate buffer 0,05M, pH=4 was used to dissolve mineral PSM medium and the off-corn medium.

In 250 mL Erlenmeyer flasks, 100 mL of sterilized culture medium were inoculated with 5 mL of fungal suspension in triplicates. The flasks were incubated in orbital shaker incubator (150 rpm) at 30 ºC, for 96 hours. At every 12 hours, the fermented broth was centrifuged at 3,000 rpm for 15 minutes and the supernatant (crude enzyme) was used for further analysis.

The comprehensive activity of cellulase was determined using the filter paper assay by the method recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Li *et al*., 2013). The overall cellulolytic activity was determined by 3,5-dinitrosalicylic acid (DNS), Miller's method (1959). The absorbance was measured at 600 nm using UV/VIS Jasco spectrophotometer. Sugar content was measured using a calibration curve of glucose. Enzymatic activity of cellulase complex was expressed in International Units/mL defined as the amount of enzyme which releases one micro mole of reducing sugar expressed as glucose per minute at 50 °C (Shafique *et al.,* 2009).

4. Microscopic investigations. By optic microscopic investigation of *Trichoderma reesei* species, using simple staining the shape and morphology of *T. reesei* were easy observed. A smear colored with 1% phenicated methylene blue is made and it is examined with immersion lens (100×) (Carpa *et al.,* 2014).

Scanning electron microscopy (SEM) analysis was performed using a Hitachi SU8230 High Resolution Scanning electron Microscope equipped with a cold field

emission gun. For morphological analysis the samples were deposited on aluminum stubs and sputter-coated coated with 10 nm gold on a Q150T ES Quorum. EDX System (X-Max N80TLE Silicon Drift Detector (SDD) from Oxford Instruments.

5. Digestion of plant material. *Arabidopsis thaliana* seedlings were used as plant material to test the enzymes produced by *T. reesei*. The 4 weeks plant material of *Arabidopsis thaliana* was obtained from seeds sterilized and cultured in a growth chamber (temperature of 21 °C and photoperiod of 16 h light and 8 h darkness) on MS1/2 medium (Murashige and Skoog, 1962).

Results and discussion

1. Macroscopic and microscopic investigations. The cellulase synthesizing strain *Trichoderma reesei* was cultured on M129 and PSM solid culture media. The inoculation technique used was to punch the medium in the center. The Petri dishes were incubated 5–7 days at temperature of 30°C. It was noticed that on M129 medium the growth of the hyphae was much stronger compared to the ones on PSM medium (Fig. 1A, B).

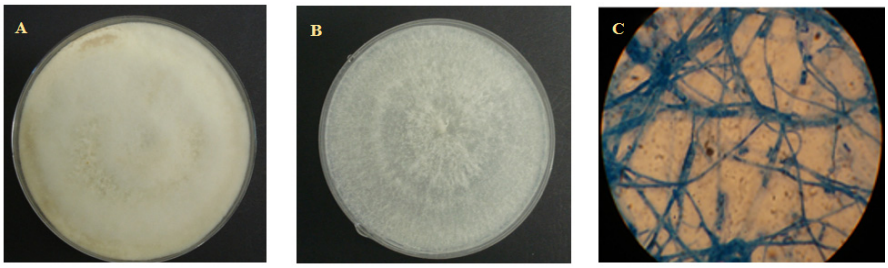

Figure 1. *Trichoderma reesei* cultured on M129 culture media (A) and PSM culture media (B). Dispersed mycelial morphology generated by growing *Trichoderma reesei* in off-corn culture media. The images were captured by optical microscope (C).

From the cultures grown on solid medium microscopic preparations colored with phenicated methylene blue were obtained. By optical immersion microscopy blue colored hyphae specific to *Trichoderma reesei* were highlighted (Fig. 1C). *Trichoderma reesei* is used at a large scale to produce biomass degrading enzymes and is also used in research (Martinez *et al*., 2008; Peterson and Nevalainen, 2012).

The best developed cultures were further used to assess the cellulase activity.

2. Cellulose decomposition (filter paper) by Trichoderma reesei. In order to emphasize the capability of *Trichoderma reesei* to decompose cellulose three experimental versions were tested (with 0.5 mL, with 1.0 mL and with 1.5 mL inoculum). These were observed for 4 weeks. Afterwards the test tubes in which the deterioration of Watmann paper occurred were counted, within each dilution. The most probable number of cellulosolytic microorganisms was next calculated

(Carpa *et al*., 2014). The medium number of cellulosolytic microorganisms was 0.17 x 10^3/mL inoculum and 26 x 10^3/mL inoculum. It was noticed that, at all the experimental versions relative to the control, the strain of *Trichoderma reesei* developed mycelium which extended on the surface of the paper strip. It was also noticed that, the volume of the inoculum influenced its development (Fig. 2A).

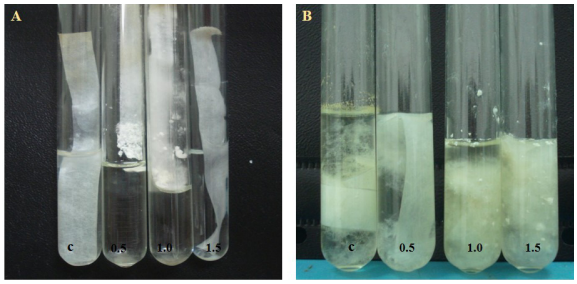

Figure 2. Tubes with filter paper and *Trichoderma reesei* inoculum after 4 weeks of incubation (A), and after shaking (B).

In order to assess the degree of paper deterioration one positive test tube was taken from each dilution and was strongly shake for 1 minute. The same action was repeated in a sample with no inoculum and then, by comparison with the control, the degree of paper deterioration was assessed (Carpa *et al*., 2014). After agitation, in Fig. 2B is visible that at the control the paper remained intact (built up in a ring form in the first test tube). At the experimental alternative with 0.5 mL inoculum, almost 50% of the paper was degraded, while at the versions with 1 mL and 1.5 mL inoculum the paper was 100% deteriorated.

3. Cellulase activity at Trichoderma reesei in a submerged system. For assessing the cellulase activity at *Trichoderma reesei* two types of nutritive medium were used: PSM medium (mineral Mendel), marked with A, and medium with off-corn, marked with B. The cellulase activity was assessed in experimental triplicates. In each Erlenmeyer flask 5 mL of *Trichoderma reesei* suspension were inoculated. Then all the experimental alternatives were incubated for 84 hours in an incubator with shaking of 150 rpm at 30ºC (Fig. 3).

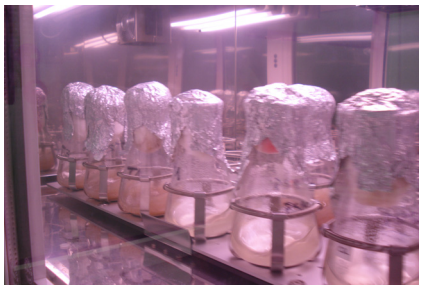

Figure 3. Samples cultivated in the shaking incubator.

At intervals of 12 hours 1 mL of the medium (supernatant) which was centrifuged at 3000 rpm for 15 min is taken for enzyme analysis. After centrifugation the supernatant and the carboxymethylcellulose solution are put into Eppendorf tubes (v/v) and incubated to 50°C for 15 min. The content of the tubes is moved into glass test tubes. 3 mL of 3,5-Dinitrosalicylic acid (DNS) are added on theme and the test tubes were placed into a beaker of hot water. Boiling for 5 min follows. After boiling the content of the test tubes must be yellow (control) and yellow-browny (samples) (Fig. 4). After cooling the content of the test tubes is subjected to spectrophotometer measurements at 640 nm.

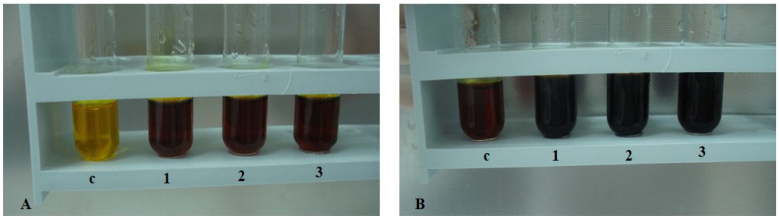

Figure 4. Content of test tubes at PSM medium (A) and off-corn medium (B); c=control, 1, 2, 3=samples.

It was noticed that, both on PSM medium (A) and off-corn medium (B), initially a decrease of optic density to – 0.0092 for PSM medium and – 0.0455 for the alternative on off-corn (B) occurred. Subsequent, on both culture media growth of the species of interest can be observed, noticing that on off-corn medium (B) the growth values are double comparing to the ones on PSM (A) (Fig. 5).

Likewise, there's a difference between the two media at the decrease of the optical density, which was stronger at the culture on off-corn medium (B) after about 60 hours. Regarding the culture on PSM medium (A), the decrease occurred only after 72 hours have passed and was much slighter.

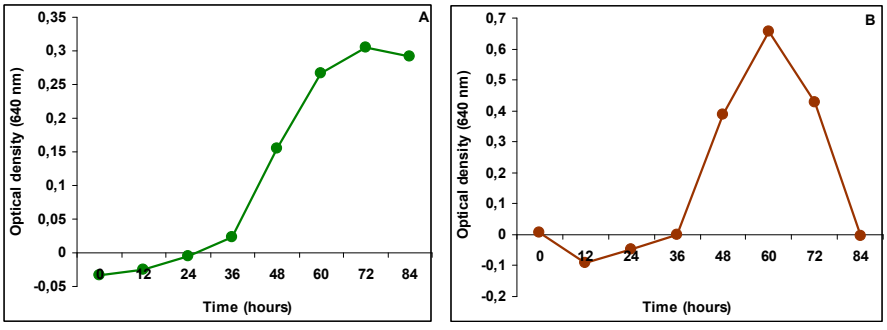

Figure 5. Decreasing sugars at *Trichoderma reesei* in PSM medium (A) and in off-corn medium (B).

The comprehensive activity of cellulase was determined using the filter paper assay by the method recommended by the IUPAC. Following the assessing of cellulase activity it was found that at each experimental alternative, due to the activity of the substrate, the reduction of the sugars released by the enzyme occurred. At the strain grown on off-corn medium the value obtained was 0.188 mg released glucose/ mL/min/50ºC, while at the strain grown on PSM medium the value was 0.118 mg released glucose/mL/min/50 ºC (Fig. 6).

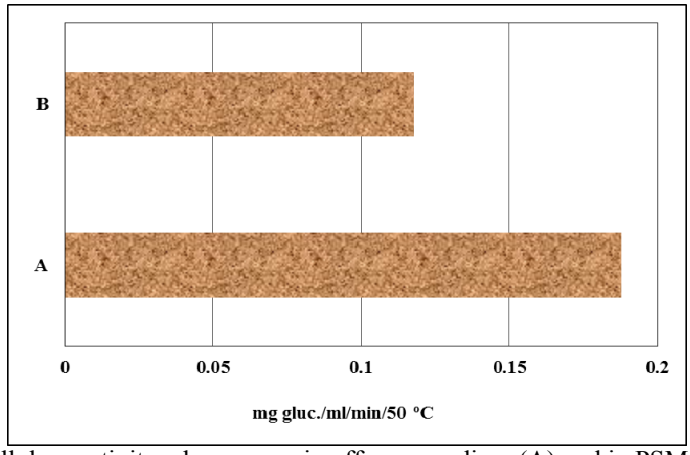

Figure 6. Cellulase activity when grown in off-corn medium (A) and in PSM medium (B).

Also, in this case the stimulating action of off-corn in the culture medium can be seen. Thus, following the experiments it can be concluded that the off-corn medium represents a culture medium richer than the PSM medium and is also preferred by *T. reesei.*

After 84 incubation hours at each experimental version the presence of numerous granules was noticed in the culture medium. At controls these granules were missing. In the samples containing PSM medium, the granules were smaller and fewer (Fig. 7). *T. reesei* granules grown on PSM medium generated difficulties at the supernatant extraction after centrifugation.

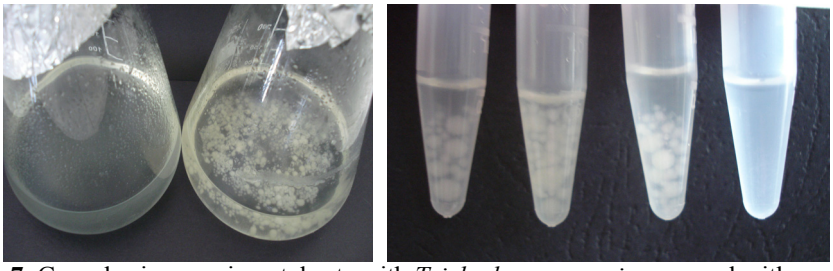

Figure 7. Granules in experimental sets with *Trichoderma reesei* compared with control on PSM medium, after 84 hours incubation.

At the versions on off-corn medium these granules were larger and much numerous than at the versions incubated on PSM medium (Fig. 8). This is correlated with the spectrophotometric values obtained at the kinetics of cellulase production at *Trichoderma reesei.* The control does not contain granules but presents a small quantity of off-corn extracted while pipetting. The presence of numerous granules on off-corn medium would be explained by the fact that this substrate is much better for cellulase production than PSM medium.

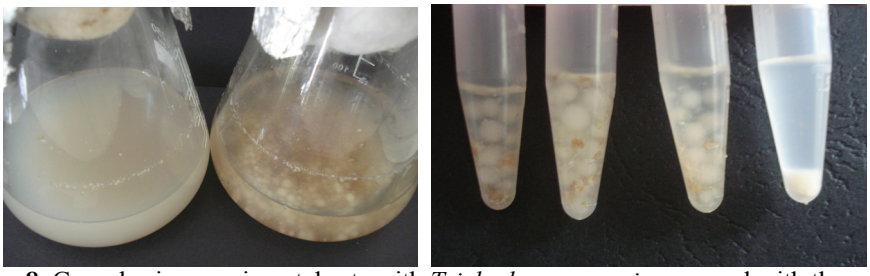

Figure 8. Granules in experimental sets with *Trichoderma reesei* compared with the control left picture, left Erlenmeyer flask; right picture, 1st Eppendorf in the right side) on culture medium with off-corn, at 84 incubation hours.

At the end of the cellulase assessments, microscopic preparations were made out of each experimental version. Thus, granules were taken from each type of tested medium and were pressed on microscopic slides. They were colored with methylene blue. Hyphae specific for *Trichoderma reesei* were observed in all versions investigated with the immersion objective lens (Fig. 9).

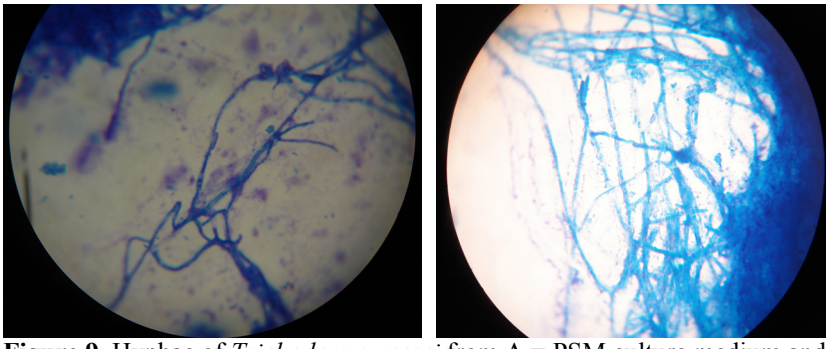

Figure 9. Hyphae of *Trichoderma reesei* from A = PSM culture medium and B = off-corn medium, at 100x optical microscope.

These granules are *T. reesei* fungal hyphae conglomerations that built-up as "bundles", while shaking. These granules were also investigated by Scanning electron microscopy (SEM), whereby both off-cornched mycelia and hyphae, off-cornched conidiophores with sporangia heads, were distinguished (Fig. 10).

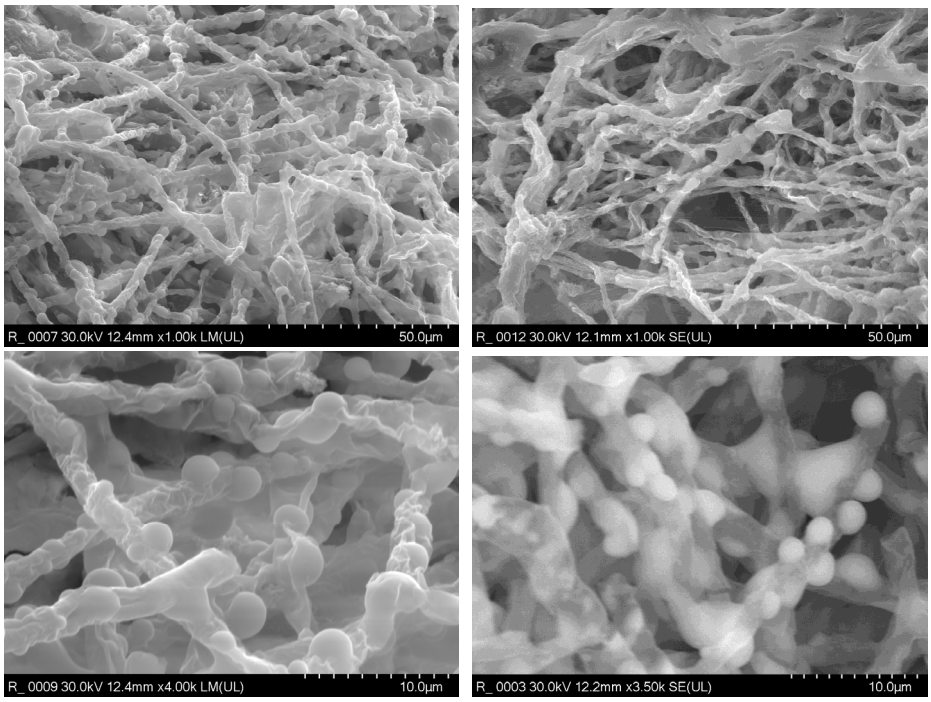

Figure 10. Scanning electron microscopy (SEM) pictures revealing morphological characteristics of *Trichoderma reesei* in off-corn culture medium under submerged fermentation conditions. (A) Highly off-cornched mycelia. (B) Hyphae, off-cornched conidiophores with sporangia heads.

Long and ramified hyphae would increase the surface area of the fungus, possibly enhancing the interaction with the substrate, and thus improving the enzyme productivity. The presence of spores could be also observed under this condition (Fig. 10), which might suggest that the environment of mycelium growth deteriorate, part of mycelium autolyzed and spores were released in order to adapt to the environment.

4. Digestion of the plant material. Cellulose, the major polymer in plant cell walls, is a very stable compound and it is known to be a recalcitrant molecule (Peciulyte *et al*., 2014). The digestion of the plant material occurred after 2.5 h, and this was noticed by macro- and microscopic observations. The main purpose of this experiment was to obtain viable plant protoplasts which could be used in future plant physiology or genetics experiments. The surface of the leaf lamina used as plant material (*Arabidopsis thaliana*) in the experiment was about 1.5 cm (Fig. 11A). The enzyme solution used was extracted by centrifugation out of *T. reesei* culture.

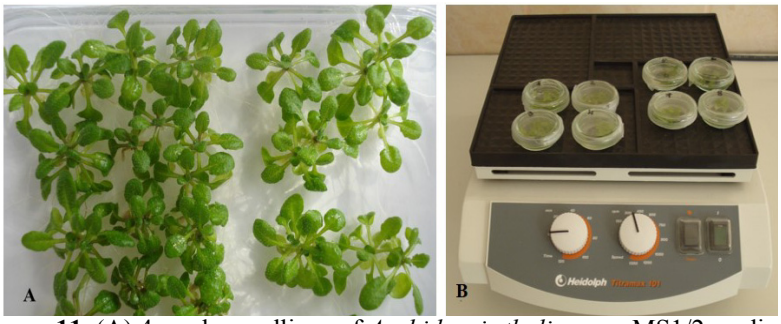

Figure 11. (A) 4 weeks seedlings of *Arabidopsis thaliana* on MS1/2 medium; (B) Leaf fragments in enzyme solution, on shaker.

The plant material was weighed and incubated with the enzyme in Petri dishes of 35 mm. The ration between plant mass and the enzyme solution was about 50 mg at 1 mL solution, with two exceptions (Table 1). The leaf explants were sectioned before being submerged in the enzyme solution and were shaken at 300 rpm, at room temperature, for 2.5 h, using the device Titramax 101 (Fig. 11B).

Table 1.

The enzyme solution types used for the digestion of the plant material

Samples No.	Sample types	Description	Observation
1	Negative control	Distillated water 2 mL	
2	Positive control	Cellulase Onozuka R-10 1% + Macerozyme 0.5% + 0.4 M sucrose	
3	EP + Macerozyme	Enzymatic solution EP 2 mL + Macerozyme 0.5%	
4	ET + Macerozyme	Enzymatic solution ET 2 mL + Macerozyme 0.5%	
5	EP + Macerozyme	Enzymatic solution EP 4 mL + Macerozyme 0.25%	Double EP enzyme quantity compared to sample 3.
6	ET + Macerozyme	Enzymatic solution ET 4 mL + Macerozyme 0.25%	Double EP enzyme quantity compared to sample 4.
7	EP + Cellulase Onozuka R-10 + Macerozyme	Enzymatic solution EP 2 mL + Cellulase Onozuka R-10 0.5% + Macerozyme 0.5%	
8	EP + Cellulase Onozuka R-10 + Macerozyme	Enzymatic solution ET 2 mL + Cellulase Onozuka R-10 0.5% + Macerozyme 0.5%	

EP – Enzyme isolated from *T. reesei* grown on PSM medium; ET – Enzyme isolated from *T. reesei* grown on off-corn medium.

The experimental versions are described in Table 1. At the described samples, Macerozyme R-10 was used as a source of pectinase and Cellulase Onozuka R-10 was used as a supplementary source of cellulase. The enzyme solutions which were separated by centrifugation at the end of the experiment on the growth of *T. reesei* culture in a submerged system were marked with EP (enzyme isolated from *T. reesei* grown on PSM medium) and ET (enzyme isolated from *T. reesei* grown on off-corn medium). The centrifugation was done at 4000 rpm, for 30 min., at 4 °C.

The leaflets were submerged in the enzyme solutions. After 2.5 hours of shaking at room temperature, the digestion of plant material and its dispersion in the enzyme solution could be observed (Fig. 12).

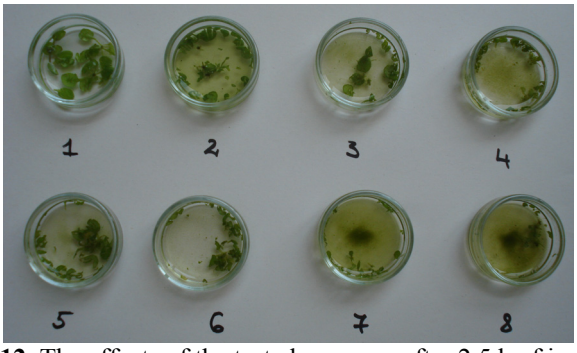

Figure 12. The effects of the tested enzymes after 2.5 h of incubation with shaking, at room temperature.

The following versions can be recorded as being efficient for the digestion of the plant material: 7, 8 and 4. At version 1 the digestion did not occur, because this was the negative control.

Differences were recorded between the enzymes extracted from PSM medium and those on off-corn medium, the last ones being more efficient. The versions 4 and 8 are the ones containing enzymes extracted from the off-corn medium. When the ratio between the enzyme solution and the plant material used was modified, no improvement of the digestion efficiency was noticed, but on the contrary, a decrease of it. This case can be observed in Fig. 12, at versions 5 and 6.

Out of the microscopic observations it was concluded that the regulation of osmolality is an essential factor for obtaining viable protoplasts, but these do not obviously influence the digestion of the plant material (Fig. 13). Similar to the version 3, depicted in the image, protoplasts were also obtained in the other versions but the viability was low.

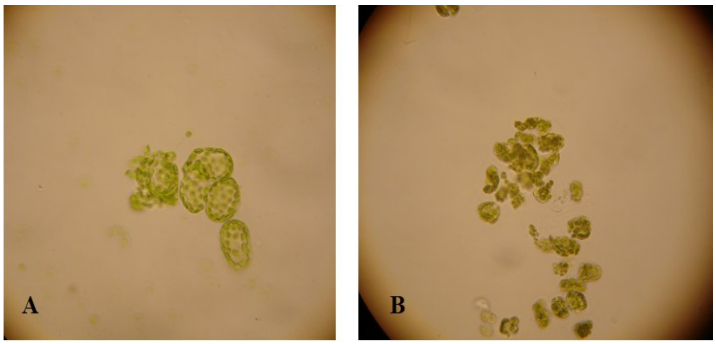

Figure 13. Microscopic images of the protoplasts, version 2 = positive control (A) and version 3 = cellulase obtained from culture grown on PSM medium with 0.5% Macerozyme R-10 (B) (40x).

Thus, obtaining cellulase implies also other determinant factors as the concentration of the enzyme in use, the type of enzyme, pH (Li *et al*., 2013) but also osmolality, whose regulation is very important.

It is essential to enhance the activity of enzymes from *Trichoderma reesei* in the economical production of biofuels from plant materials as a new cost-effective approach. Carbon source plays a vital role in enzyme production because carbohydrates and their derivatives have the ability to induce most of cellulolytic enzymes (Zheng *et al*., 2017).

Conclusions

Initially, *Trichoderma reesei* was recultivated on culture medium with potatoes (M129) plates, and then was transferred on PSM medium with agar, using the culture plate method. These samples grown on plates were further used to obtain the *T. reesei* inoculum for submerged cultures in PSM and off-corn media. Using the microscopic techniques, the morphology of *T. reesei* could be observed, by microscopic preparations with simple phenicated methylene 1% stain.

The composition of the culture medium strongly influenced the development of the studied species, most preferred culture medium for *T. reesei* proving to be the submerged medium based on off-corn.

The action of the cellulosic enzymes on cellulosic materials was observed by testing the enzymes extracted from the submerged *T. reesei* cultures on stripes of filter paper. In time (after 4 weeks), the digestion of cellulose was observed by comparing the samples containing the enzyme with the control, the difference signaling that *T. reesei* produced enough cellulase to cause the degradation of cellulose.

The digestion of the plant material by the enzyme extracted from *T. reesei* cultures was also observed, by obtaining *Arabidopsis thaliana* viable protoplasts.

This method could be improved and further used in plant genetics, plant cytology, physiology and other fields. The cellulase activity was also assessed and a decrease of the sugars freed by the enzyme was noticed.

Trichoderma reesei proved to be a good producer of cellulase, and thus its degradation action is seen as a very valuable property, in the context of its capacity to hydrolyze biomass rich in cellulose.

Trichoderma reesei represents a very important species of fungi due to the bioethanol production, in the context of the environmental problems protruding at the beginning of 21th century, regarding the greenhouse gases emissions. Thus, attention should be focused on developing technologies based on biological techniques.

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Temporal variations in abundance and biomass of fish species inhabiting the K'sob reservoir (Eastern of Algeria)

Fateh Mimeche[1,✉] and Francisco José Oliva-Paterna[2]

SUMMARY. This study presents data on fish community structure in the K'sob reservoirs (semi arid area) in the East of Algeria. The artisanal fishery was conducted from September 2010 to August 2011. The monthly variations in species composition were analyzed by effort and catch per unit of effort (CPUE) and biomass per unit of effort (BPUE). A total of seven species representing three families was recorded in the investigated reservoir. Cyprinids dominated in this reservoir. *Luciobarbus callensis* is the native species captured in this area, the rest of the fish were introduced. The other Cyprinids are: *Cyprinus carpio carpio*, *Hypophthalmichthys molitrix*, *Hypophthalmichthys nobilis*, *Squalius cephalus*. The Poecilidae were represented by *Gambusia holbrooki*. The third family, Cichlidae, was represeted by *Oreochromis niloticus*. The dominant species in terms of abundance was *L. callensis*, however, *C. carpio carpio* and *H. nobilis* showed higher values in biomass. Several species showed significant temporal variation in monthly samples. The fish assemblage is dominated by invasive species, while predatory species were not detected in the study.

Keywords: abundance, Algeria, biomass, fish assemblage, reservoir

Introduction

Land degradation, long an important environmental issue in arid and semi-arid lands, is now acute in Algeria's high plateaus (Hirche *et al*., 2010). The climate of these regions is subject to the influence of the Sahara and is characterised by wet winters, dry and hot summers and high level of evaporation. The northern Africa Freshwater Biodiversity Assessment is a conservation status review of 128 fish taxa (112 species and 16 subspecies) (García *et al*., 2010). In Algeria, about 27 fish species were introduced and at least 303 introduction events, either intentional or accidental, were

[1] *M'Sila University, Department of Agricultural Sciences, M'Sila, 28000, Algeria.*
[2] *Murcia University, Department of Zoology and Anthropology, Murcia, Spain.*
✉***Corresponding author: Fateh Mimeche,*** *M'Sila University, M'Sila, 28000, Algeria,*
E-mail: mimechefateh@gmail.com

recorded in the literature (Kara, 2011). The fish fauna of continental waters of North Africa in general and Algeria in particular is little known (Bacha and Amara, 2007). The Algerian ichthyofaunal region is mostly characterized by a low diversity in freshwater fish. The last time the authority and the research is aimed at the domain freshwater fish, we find the work of Zouakh *et al*. (2004), Blanco *et al*. (2006), Bacha and Amara (2007), Kara (2011), Mimeche *et al*. (2013, 2014), Mimeche and Biche (2015) and Mimeche *et al*. (2015). This study presents data on fish community structure in the K'sob reservoirs in the East of Algeria.

Materials and methods

Study area

The K'sob reservoir is one of the oldest reservoirs made in Algeria during the year 1935. It was constructed on the K'sob stream between 1935 and 1940 for agricultural purposes, with the main objective is to irrigate the plain of M'Sila. It is located 10km north of the M'Sila town (Fig. 1). The surface of the Reservoir is 230ha. Its maximum depth is 47m and a capacity to the origin of 30Mm^3; actually its water storage is less than 06mm^3. The area of the study is characterized by semi-arid bioclimatic on the Saharan Atlas National Park (North Algeria, M'Sila). (Mimeche *et al*., 2013).

Sampling methods and statistical analyses

The study was conducted from September 2010 to August 2011. The fishing gear used by the fishermen consisted of two trammels (each approximately 50 m long). The nets were oriented in a transverse direction relative to the edge of the reservoir. Sampling began between 2 and 3 p.m. and ended the following morning, resulting in a minimum soaking time of 18-20 hours. Species composition and abundance of fish were investigated using selective nets of different size mesh (25, 40, 60 mm). Fish were caught monthly in littoral and pelagic zones. The captured specimens were preserved in neutralised formaldehyde solution (07%) and transported into the laboratory for identification to the lowest taxonomic level according to Kottelat and Freyhof (2007). Total length (TL; ±0.1mm) and weight (W; ±0.1g) were recorded.

The total number of individuals caught by the trammels was counted and expressed as catch per unit effort (CPUEs) and biomass per unit of effort (BPUEs), where 1 unit of effort represented a passive trammel in place for 24 h. The data were logarithmically transformed to allow statistical comparisons.

The total relative abundance was expressed as catch per unit of effort (CPUEs) and total relative biomass per unit of effort (BPUEs):

CPUEs = number of specimens /150m²
BPUEs = fish biomass (g)/150m².

To test for significant differences in the abundance (CPUE and BPUE) of species among months, a ANOVA test was employed, levels of probability $p < 0.05$ were accepted as significant. All statistics were performed using the PAST program (Paleontological Statistics) Version 3.05 (1999-2015).

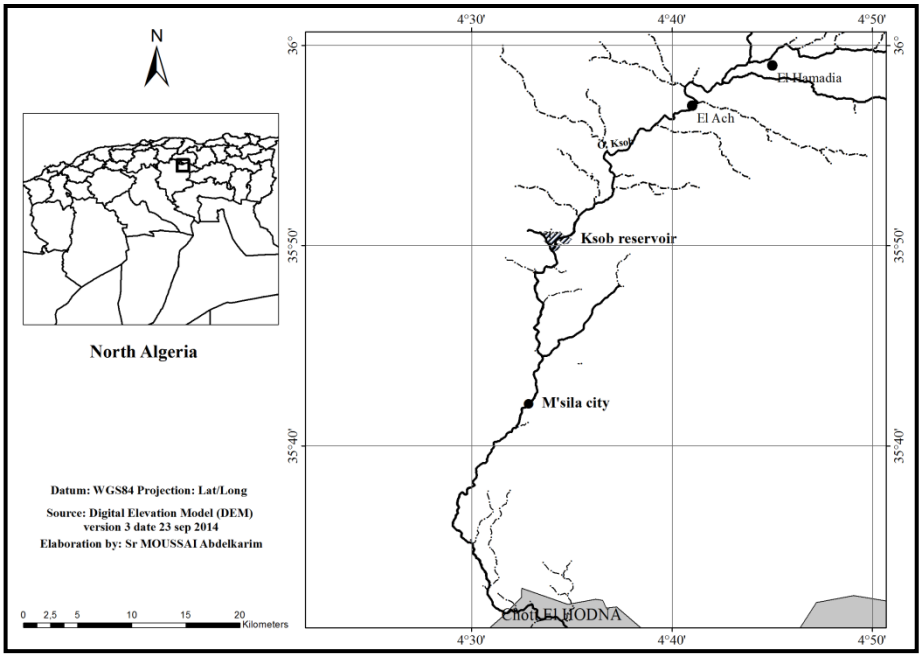

Figure 1. Map of study area

Results

A total of seven species representing three families was recorded (Table 1). Cyprinids represented by *Luciobarbus callensis* (51.1 % of captures during the study period) is the only native species in the reservoir, coexisting with *Cyprinus carpio carpio* (17.3 %), *Hypophthalmichthys nobilis* (9.9%), *Hypophthalmichthys molitrix* (1.5 %) and *Squalius cephalus* (0.2%). The Poecilidae represented by *Gambusia holbrooki* (19.6%) in shallow areas. The third family Cichlidae recorded by *Oreochromis niloticus* (0.4%) .

Overall total CPUEs were 703.33 fishes/m^2 ($F_{(83;6)}$ =28,42, $p<0.0001$) and BPUE were 447291.67kg/ m^2 ($F_{(83;6)}$ = 18.7, $p<0.0001$) during the period of study in Ksob reservoir. The *L. callensis* and *C. carpio carpio* were caught monthly, contributing a total respectively 275.33 fishes/m^2 and 93.33 fishes/m^2 of CPUE (39.15% of a number) with not statistically significant, $p> 0.05$ (Table 2).

Table 1.

List of the taxa collected from 12 sampling events (monthly periodicity) in the M'Sila reservoir. Names are according to FishBase (Froese and Pauly, 2011). (*) Status (n: native; i: invasive) is to the freshwater systems of the ecoregion.

	Taxonomic list	Common name	Status *	Origin	Year of introducion
Cyprinidae	*Luciobarbus callensis* (Valenciennes, 1842)	Algerian barbel	n		
	Cyprinus carpio carpio Linnaeus, 1758	Common carp	i	Hungary	2004
	Hypophthalmichthys molitrix (Valenciennes, 1844)	Silver carp	i	Hungary	2006
	Hypophthalmichthys nobilis (Richardson, 1845)	Bighead carp	i	Hungary	2006
	Squalius cephalus (Linnaeus, 1758)	Chub	i	Europe (France?)	unknown
Cichlidae	*Oreochromis niloticus niloticus* (L., 1758)	Nile tilapia	i	Egypt	2004
Poeciliidae	*Gambusia holbrooki* (Girard, 1859)	Eastern mosquitofish	i	unknown	1986

H. nobilis was captured for 10 months with a CPUE 53.33 fishes/m² (significant difference in catch monthly, $p<0.05$). *G. holbrooki,* caught in littoral area, presents a good place in the fish community composition 105.33 fishes/m^2 (significant difference in catch monthly, $p<0.05$). However, *C. carpio carpio* and *H. nobilis* showed higher values in total BPUE 204420 g/m^2 and 18171.2 g/m^2, respectively (not statistically significant in monthly catch, $p=0.18$ and $p=0.19$) (Table 3). Several species showed significant temporal variation in monthly samples with low caught and value in CPUE and BPUE (Tables 2, 3).

Discussion

Monitoring of CPUE and BPUE provides information on relative changes in the fish populations. The relative abundance of the studied population showed clear monthly variation. The Cyprinid family dominated in the K'sob reservoir; *L. callensis* is known one of the abundant species and to show a certain degree of stability in its seasonal densities (Mimeche *et al*., 2013; Mimeche *et al*., 2014) 2014). It is the only native species captured is this area. This abundance could be an indicator of most variation in the individual size of its population in comparison to other species. The rest of the fish captured were introduced. However the second abundant species in this reservoir is *C. carpio carpio*. Mimeche *et al.* (2015) mentioned the good adaptation and growth in this reservoir.

G. holbrooki introduced in Algeria (Mazafran Oued) since 1926, is considered the most abundant and widespread freshwater fish in the world (Pyke, 2005; 2008).

G. holbrooki presents a clear ability to adapt to lotic systems (Ruiz-Navarro *et al*., 2011), and it is used in the biological control of mosquitoes.

Table 2.

Catch per unit effort (CPUE) (fishes/m²) at monthly sampling of fish community composition in Ksob reservoir, Algeria, between September 2010 to August 2011.

	Lc	*Cc*	*Hm*	*Hn*	*Sp*	*Onn*	*Gh*
Sep	14.00	7.33	0.00	4.00	0.00	0.00	20.00
Oct	12.67	10.00	0.00	5.33	0.00	0.00	16.00
Nov	26.00	15.33	0.00	2.67	0.00	0.00	11.33
Dec	16.67	6.67	0.00	4.67	0.00	0.00	0.00
Jan	16.00	8.67	0.00	0.00	0.00	0.00	0.00
Feb	30.00	4.67	0.00	2.67	0.00	0.00	0.00
Mar	22.00	12.00	1.33	7.33	0.00	0.00	0.00
Apr	23.33	14.67	2.67	10.00	1.33	0.00	12.67
May	16.67	2.67	4.00	12.00	0.00	0.00	15.33
Jun	42.00	6.00	0.00	0.00	0.00	1.33	14.00
Jul	16.67	3.33	0.00	2.00	0.00	0.67	16.00
Agu	39.33	2.00	0.00	2.67	0.00	0.00	0.00
Total	275.34	93.34	8.00	53.34	1.33	2.0	105.33
p	ns	ns	<0.001	ns	<0.001	<0.001	<0.05

Lc: *Luciobarbus callensis, Ccc*: *Cyprinus carpio carpio, Hm* : *Hypophthalmichthys molitrix*, *Hn* : *Hypophthalmichthys nobilis, Sp* : *Squalius cephalus, Onn* : *Oreochromis niloticus, Gh : Gambusia holbrooki,* ns : no significant

The common carp and bighead carp constitute the majority of the fish biomass in these systems (more than 85%). This species presents a wide tolerance range towards the variable environmental conditions in semiarid reservoir, and establishes new viable populations widely dispersed and incorporated in large numbers in the ecosystem (Mimeche and Biche, 2015). The common carp, silver carp and bighead carp represent an important source of protein for the inhabitants of rural communities in this region.

The rarity of the silver carp *H. molitrix* is due perhaps to its unsuccessful reproduction. Bruslé and Quignard (2001) reported that, this fish naturally reproduces in the thermal and hydrological conditions of the average flow of Chinese rivers. It does not seem able to reproduce in Europe, where the spawning is not spontaneous and where the populations must be renewed every year (Pivnicka and Cerny, 1987). The

extinction of *Squalius cephalus* and *Oreochromis niloticus* may be caused by hydrological disturbance, siltation, quality of weirs, mismanagement of the opening and closing of the water distribution channels (Ramdani *et al*., 2001; Kraïem *et al*., 2003), overexploitation, climate change and environmental variability (Brook *et al*., 2008).

Table 3.

Biomass per unit of effort (BPUE) (g/m²) at monthly sampling of fish community composition in Ksob reservoir, Algeria, between September 2010 to August 2011.

	Lc	*Cc*	*Hm*	*Hn*	*Sp*	*Onn*	*Gh*
Sep	1614	17964	0	14582	0	0	30
Oct	1448	25693	0	18020	0	0	22.67
Nov	3613	33744	0	7636	0	0	18.67
Dec	1831	12078	0	14329	0	0	0
Jan	1259	19060	0	0	0	0	0
Feb	3112	8886.7	0	7360	0	0	0
Mar	2495	22447	2103	25287	0	0	0
Apr	2521	29315	4480	35133	200	0	20.67
May	1921	8213.3	6647	40843	0	0	24
Jun	5743	12849	0	0	0	197.3	23.33
Jul	3687	7810.7	0	7373	0	119.3	21.33
Agu	3113	6359.3	0	11149	0	0	0
Total	32357	204420	13230	181712	200	316.6	160.67
p	ns	ns	<0.001	ns	<0.001	<0.001	<0.05

Lc: *Luciobarbus callensis, Ccc*: *Cyprinus carpio carpio, Hm* : *Hypophthalmichthys molitrix*, *Hn* : *Hypophthalmichthys nobilis, Sp* : *Squalius cephalus, Onn* : *Oreochromis niloticus, Gh : Gambusia holbrooki,* ns : no significant

Conclusions

The fish assemblage is dominated by the native species *L. callensis*, which inhabits in the lentic aquatic ecosystem together with invasive species.

Monthly patterns should not necessarily be assumed to be the same for different species or for species in different habitats because differences may exist in (a) recruitment, growth, and mortality within a species, (b) among species and (c) among gears used in sampling. The predatory species were not detected in the study.

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Diversity of entomofauna associated with greenhouse-grown tomatoes in Algiers (North Algeria)

Fatiha Djebara[1,2,✉] Abdelmadjid Benzahra[1], Fateh Mimeche[3] and Lounes Saharaoui[1]

SUMMARY. Tomato is an important economic crop worldwide. Many insect pests are cause both quality and quantity yearly decrease on greenhouse-grown tomatoes. Thus, diversity and relative abundance of both harmful and useful insects were investigated in greenhouse-grown tomatoes in Algiers coastal area. The inventory was realized in 2014 by using yellow sticky platelets traps. The information obtained allowed us to elaborate an inventory with no less than 1449 individuals belonging to 46 different taxons, distributed among eight orders and 26 families. Useful entomofauna is very rich and represented around 32% of the total population with five predators and a dozen of Hymenoptera parasites. Presence ofa significant predatory parasite complex may contribute to the regulation of pests in tomatoes greenhouse and carry out control programs against the main harmful pest of this crop. This assessment of the entomofauna will make it possible to carry out control programs against the main pests of this culture. The results of this study will contribute to finding mechanisms and conditions for reducing the negative impacts of the bio-aggressors of tomato.

Keywords: Algeria, diversity, entomofauna, greenhouse, tomato.

Introduction

Tomato (*Solanum lycopersicon* Mill.) is one of the most important vegetable crops in the world, consumed both fresh or processed form (Blancard *et al.*, 2009). It originated as wild from South America and supposed to be domesticated in Central and Latin America (Kolev, 1976). Tomato was first introduced into Europe by the Spanish conquerors in the sixteenth century and was mostly cultivated in the Sevillian region (Vernouillet, 2007).

[1]*National High School of Agronomy (ENSA, Ex: INA), Department of Agricultural and Forestry Zoology, El-Harrach, 16200 Algiers, Algeria.*
[2]*Algeria's National Institute of Agricultural Research (INRAA), 16200 Algiers, Algeria.*
[3]*University of M'Sila, Department of Agricultural Sciences, BP 166 Echbilia, 28000 M'Sila, Algeria.*
✉ ***Corresponding author: Fatiha Djebara**, Department of Agricultural and Forestry Zoology, E-mail: f.djebara@yahoo.fr*

According to the latest statistics (FAO, 2015) the world production of tomatoes is increasing 161,326,827 tons produced in 2012. The world's leading producers of tomatoes areChina with 22 million tons, followed by the European Union with 15 million tons, and the United States of America producing 10 million tons, Turkey is the 4th world producer, with more than 8 million tons (Desneux *et al.*, 2010). Tomato is an important vegetable crop in Algeria (Nechadi *et al*., 2001), the annual production in 2013 was 975,075 tons (FAO, 2015), however the production does not meet the needs of the population. Algerians are large consumers of tomatoes and prefer the processed tomato with a consumption close to 4 kg / person / year (Baci, 1993).

Tomato is prone to numerous attacks of diseases caused mainly by fungi, bacteria, viruses, viroids and phytoplasmas. It is also threatened by several pests like *Tetranychus mites*, whiteflies, aphids, thrips, leaf miners and greenhouse moths (Vernouillet, 2007). *Tuta absoluta* leaf miner is a microlepidoptera a new pest of tomatoes in the Mediterranean regions (Desneux *et al.*, 2010). It is a microlepidoptera of the family Gelechiidae, originating in Latin America. Its mining caterpillars can cause damages up to 80% and even 100% losses of the crop (Desneux *et al.*, 2010). In Algeria, these biological aggressors were reported for the first time in early spring 2008 in the northwestern region of the country (Mostaganem and Oran), and then spread through all the country reaching south Algeria. Since, several researches on this pest have been carried out in order to evaluate its impact on the tomato crop, such as the studies carried out by Idrenmouche (2011), Mahdi (2011) and Selmane (2011).

The little work about the pest of tomato are published in Algeria, this study is new information about entomofauna of Solanaceae. The aim of this paper is inventoried the entomofauna associated with tomato under greenhouse cultivation system in the western Algerian coastal region, by using conventional survey of both harmful insects and their natural enemies. The approach used in this study is to provide a database of harmful and useful entomofauna diversity and to establish a general state of the main pests and their natural enemies. This assessment of the entomofauna will lead to control programs of the main pests. We will also qualitatively and quantitatively evaluate the main pests of greenhouse tomatoes.

Materials and methods

Study sites

Data collection was conducted ingreenhouses settled in the domain Kheloufi el Djillali in Zeralda (2°49'45" E, 36° 41' 15" N, altitude: 120 MASL), this farm located 40 km west of Algiers (Fig. 1).

The climate of these regions characterised by wet winters, dry and hot summers (Table 1). The site is surrounded by stateforests and is based on a loamclay soil with a neutral pH and low levels of organic matter (0.57%), with a total limestone of 2.25%. The domain specializes in viticulture, citrus and greenhouse vegetable crops, where the main crops are tomatoes and pepper.

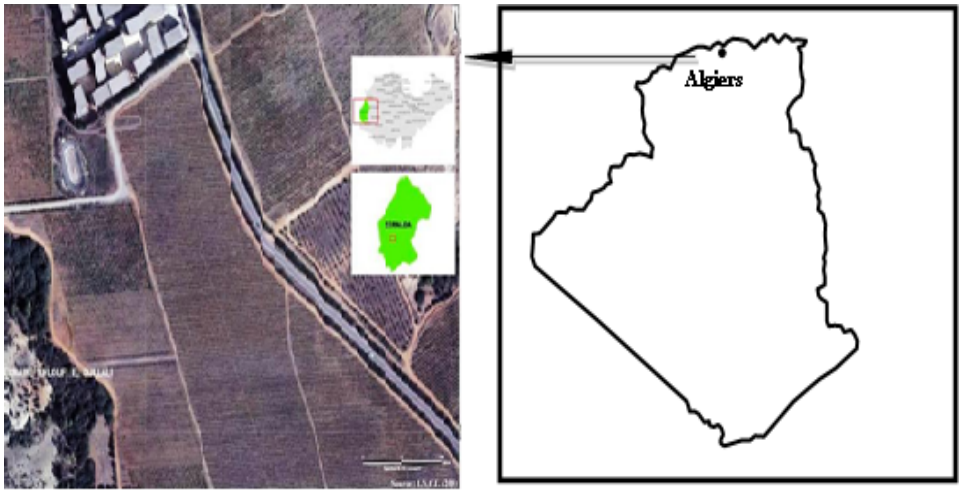

Figure 1. Location of the study area.

Sampling

After the installation of the crop on October 24th, 2014, yellow-glue pads were installed at the entrances and in the middle of each of the two selected greenhouses.The cultivated tomato variety was Agora and no chemical treatment was applied.

Table 1.

Monthly climatic parameters for the study area in north Algeria, between October 2014 to April 2015

Climatic parameters	**Oct.**	**Nov.**	**Dec.**	**Jan.**	**Fev.**	**Mar.**	**Apr.**
Maximum temperature (°C)	26.9	18.0	17.0	17.9	18.2	18.5	23.5
Minimum temperature (°C)	19.9	12.0	10.0	11.4	11.8	09.4	13.0
Maximum air humidity (%)	87.1	87.9	88.7	86.9	-	-	-
Minimum air humidity (%)	52.5	59.3	62.7	57.2	-	-	-
Wind speed [km/h]	14.0	24.0	13.2	24.0	27.2	4.0	4.0
Evaporation (mm/day)	108.6	89.4	69.0	110.5	75.6	64.8	110.1
Precipitation (mm)	21.3	236.6	99.2	54.8	22.7	49.5	3.2

The yellow sticky traps were replaced by new ones twice a month and brought back to the laboratory for analysis. The traps are cut in three parts before their observation under the magnifying glass. The identification of captured individuals is performed by using determination keys or by the help of the Department of Agricultural and Forestry Zoology (National Higher School of Agronomy, El Harrach). Traps installations startedwith tomato transplantation and lasted until the harvest on April 7th, 2015.

Data analysis

To calculate the species abundance, species diversity and the differences in community composition and structure at each month, data were analyzed using PAST software (Paleontological Statistics; Version 2.17). The indexes used to examine insects community were: species richness (S), Frequency of occurrence (F %), Shannon diversity index (H), and equitability (E), where these indices are useful for a comparison between populations at monthly catch. The non-parametric Kruskal-Wallis test (χ^2) was applied to test the variation in species abundances between the months of greenhouse-grown tomatos. The relationships between insect diversity parameters were verified using Pearson correlation tests for each month.

Results and discussion

Taxonomic list and abundances of insect species

The inventory of the entomofauna associated with greenhouse tomato allowed us to capture no less than 1449 individuals belonging to 46 species belonging to 8 orders and 26 families (Table 2). The order of the Homoptera is the most dominant with 13 species, followed by Hymenoptera with 12 species. Diptera are in third position with 8 species, followed by Thysanoptera with 4 species. Coleoptera and Hemiptera contain only 4 species each. Finally, the Neuroptera and Lepidoptera are the least represented with one species each. The Kruskal-Wallis test showed a significant relationship between insect abundances of the six months (χ^2=17.84, P<0.05). Comparing these results with those of other inventories in Algeria and other countries, we can say that they have a relatively important. (Mahdi, 2011) used the same trapping technique and reported the presence of 3.908 individuals distributed among 295 species with 2817 individuals trapped in the field and 1093 caught on greenhouse tomatoes. Species trapped were belonging to the classes of Gastropoda, Arachnida, Crustacea, Chilopoda, Diplopoda, Collembola and Insecta. Clere and Bretagnolle (2001) reported that 4,863 individuals belonging to 35 taxa of arthropods were caught in the Barber pots in the Niort-Brioux (France) cereal plains. Roth (1965) reported 8,222 individuals trapped in yellow insect plates which were distributed among 12 families. Similarly, with the same technique, Chauvin *et al.* (1966) captured 11454 insects in an alfalfa plot during 13 days of collection. Lahmar (2008) trapped no fewer than 62 taxa in a tomato greenhouse in Ouargla (south-east of Algeria).

The constancy classes of the trapped species determined in relation to the occurrence frequencies, according to the Sturge rule, are 11 with an interval equal to 8.75%.The frequency analysis of occurrences shows that among the 45 species trapped, three species are omnipresent (FO% = 100%), namely *A. gossypii*, *M destructor* and *Calliphora* sp. Thirteen taxa are regular with frequencies ranging from 50.0% to 66.7%, among which we can mention aphids: *A. frangulae*, *A. craccivora, B. cardui, B.*

helychrysi and *M. persicae,* thrips: *F. occidentalis* and the predatory *N. tenuis*stink bug *T. absoluta*. Five species are very constant (FO between 75% and 83.3%), they include the species: *Diglyphus sp, Agromyzidae sp*, *M. domestica, Chrysocharis sp* and *T. tabaci*. Twelve taxa are accessory or very accessory with frequencies varying between 33.3% and 41.7%, among which we cite *A. nasturkii aphids, T. triffoli, M. euphorbiae, M. viciae, B. tabaci, M. pallidior, Anthocoridae sp., E. fabae* and *C. carnea*. Unintentional species (FO = 25%) have only two species while there are five others with a frequency of 8.3%. This parasite-predator complex can play a key role in limiting the main pests of the tomato by also ensuring a biological balance in the tomato plot. In order to achieve this goal, it is necessary to use modern tools of comparative analysis.

The different species that can potentially be used in a biological control program must be described and compared taking into account their phylogenetic links (Harvey and Pagel, 1991). The species *N. tenuis* will certainly contribute to the regulation of populations of the species *T. absoluta*, a key pest of tomato in Algeria. Among the predators identified, the ladybug *C. arcuatus* may also limit populations of whitefly *B. tabacia* well as the aphidiphagous predators *H. variegata, C. carnea* and *E. balteatus* in association with the listed Hymenoptera parasites, because no less than 11 species of aphids live on tomato. The idea of looking for an ideal environment (hedges and floral strips) allowing the installation of useful insects: mirids, micro-hymenoptera in order to promote biological control by conservation remains the most promising alternative (Mazollier *et al.*, 2005). The *A. craccivora* and *B. tabaci*, have been recognized to be serious pests of tomatoes worldwide (Lange and Bronson, 1988).

Table 2.

List of greenhouse Tomato Entomofauna at Zeralda station (Ni: number of individuals, Ar %: Relative abundance, FO%: Frequencies of occurrence, P*: Presence) (SR: Slightly regular, OP: Omnipresent, VA:Very accessory, VR: Very regular, A: Accessory, SF: Slightly frequent, R: Rare, AC: Accidental, C:Constant, RE: Regular, Very consistent: VC)

Order	**Families**	**Species**	**Ni**	**Ar %**	**FO %**	**P***
Homoptera	Aphididae	*Aphis craccivora*	20	1.4	50	SR
		Aphis frangulae	43	3.0	50	SR
		Aphis gossypii	163	11.2	100	OP
		Aphis nasturtii	23	1.6	41.67	VA
		Brachycaudus cardui	20	1.4	66.6	VR
		Brachycaudus helychrysi	18	1.2	66.6	VR
		Therioaphis triffoli	20	1.4	33.3	A
		Lypaphis erysimi	4	0.3	16.67	SF
		Macrosiphum euphorbiae	29	2.0	41.7	VA
		Megoura viciae	58	4.0	33.3	A
		Myzus persicae	19	1.3	50	SR
	Aleurodidae	*Bemisia tabaci*	49	3.4	41.7	VA
	Psyllidae	Psyllidae sp.	1	0.1	8.3	R

Order	Families	Species	Ni	Ar %	FO %	P*
Thysanoptera	Thripidae	*Aeolothrips fasciatus*	8	0.6	25	AC
		Melanthrips pallidior	9	0.6	41.7	VA
		Frankliniella occidentalis	53	3.7	66.7	VR
		Thrips tabaci	100	6.9	75	C
Nevroptera	Chrysopidae	*Chrysoperla carnea*	7	0.5	33.3	A
Hemiptera	Cicadellidae	*Empoasca fabae*	16	1.1	33.3	A
	Miridae	*Nesidiocoris tenuis*	47	3.2	66.7	VR
	Anthocoridae	*Anthocoris* sp.	7	0.5	41.7	VA
Lepidoptera	Gelechiidae	*Tutaabsoluta*	3	0.2	16.7	SF
Diptera	Syrphidae	*Episyrphus balteatus*	9	0.6	41.7	VA
	Cecidomyiidae	*Mayetiola destructor*	96	6.6	100	OP
	Agromizidae	*Liriomyza brioniae*	37	2.6	58.3	RE
		Agromyzidae *sp.*	109	7.5	83.3	VC
	Muscidae	*Musca domistica*	35	2.4	83.3	VC
	Calliphoridae	*Lucilia* sp.	20	1.4	66.7	VR
		Calliphora sp.	218	15.0	100	OP
	Tephritidae	*Dacus oleae*	1	0.1	8.3	R
Coleoptera	Coccinellidae	*Clitostethus arcuatus*	3	0.2	25	AC
		Hippodamia variegata	2	0.1	16.7	SF
	Bruchidae	*Bruchus* sp.	2	0.1	16.7	SF
	Curculionidae	*Otiorhynchus* sp.	2	0.1	16.7	SF
Hymenoptera	Apidae	*Apis mellifera*	2	0.1	16.7	SF
	Cephidae	*Cephus spinipes*	35	2.4	58.3	RE
	Braconidae	*Aphidius ervi*	34	2.3	50	SF
		Apanteles glomeratus	2	0.1	8.3	R
	Eulophidae	*Tetrastichus* sp.	3	0.2	8.3	R
		Diglyphus sp.	36	2.5	83.3	VC
		Chrysocharis sp.	39	2.7	75	C
	Aphelinidae	*Aphytis* sp.	10	0.7	41.7	VA
		Aphelinus sp.	15	1.0	50	SF
	Sphecidae	*Pemphredon* sp.	6	0.4	8.3	R
	Ichnomonidae	Ichnomonidae sp.	8	0.6	33.3	A
	Aphidiidae	*Lysiphlebus* sp.	8	0.6	50	SF

Variation of insect diversity parameters

The monthly values of diversity and equitability calculated for the entomofauna trapped with yellow sticky pads are shown in Table 3. Shannon monthly diversity values range from 2.26 bits in October to 3.1 bits in March. Similarly, the equitability (E) values obtained for the captured species are maintained above 0.76. It was in March that E reached its highest level of 0.90. These are values that tend towards 1. As a result, the numbers of species captured tend to be in equilibrium with each other. The differences of species richness, composition and insect diversity parameters between the sampling

months can be explained by the variability of seasons.The climatic factors determine trends of population number of generations of many pest insects of agricultural importance (Chafaa *et al.*, 2013).

Table 3.

Monthly values of species richness (S), Number of individuals (Ni) and diversity indices of insect communities subservient to greenhouse-grown tomatos in Zeralda (Algeria) between October 2014 to April 2015

	Oct.	**Nov.**	**Dec.**	**Jan.**	**Feb.**	**Mar.**	**Apr.**
S	17	27	27	17	24	32	25
Ni	87	307	183	135	203	316	218
Shannon_*H (Bit)*	2.26	2.50	2.85	2.30	2.73	3.10	2.80
Equitability_*E*	0.80	0.76	0.87	0.81	0.86	0.90	0.87

Relationships between diversity parameters

All insect diversity parameters were positively correlated regardless of sampling month in greenhouse-grown tomatos. The species richness were positively correlated with number of individuals and Shannon index respectively ($r=0.89$, $P<0.05$; $r= 0.9$, $P<0.05$). The Equitability positively correlated with Shannon index ($r=0.84$, $P<0.05$) (Table 4). The diversity indices provide more information than simply the number of species present (Drouai *et al.*, 2018).

Table 4.

Correlation matrices displaying correlations between diversity parameters of insects subservient to greenhouse-grown tomatos in Zeralda (Algeria). Pearson correlation tests are given as correlation coefficients (shown by white case) and P-values (grey case).

	S	Ni	Shannon_H	Equitability_E
S	1	**0.007**	**0.006**	0.229
Ni	**0.89**	1	0.105	0.664
Shannon_H	**0.90**	0.66	1	**0.017**
Equitability_E	0.52	0.20	**0.84**	1

Entomofauna composition

Overall population by order

The histograms (Fig. 2) show a high predominance of the order of the Diptera followed by Homoptera, totaling 525 and 467 individuals and representing 36.21% and 32.21% of the total population respectively. The order of the Diptera is more particularly represented by Syrphidae, Ceccidomyiidae, Agromizidae and Calliphoridae. In Homoptera it is mainly the aphids that predominate with no less than eleven species, then come the Psyllidae and Aleurodidae with only one species each. Hymenoptera come in third with 198 individuals (13.66%), mostly

represented by parasites, belonging to the Braconidae, Eulophidae, Aphelinidae, Sphecidae and Ichnomonidae families. Thysanoptera totaling 170 individuals include four species of Thrips from vegetable crops*: A. fasciatus*, *M. pallidior*, *F. occidentalis* and *T. tabaci*. Finally, the orders of the Coleoptera, the Hemiptera, the Neuroptera and the Lepidoptera their presence in greenhouse tomato is insignificant, their number does not exceed 70 individuals.The Kruskal-Wallis test showed a no significant relationship between ordersabundances of the six months (P>0.05).

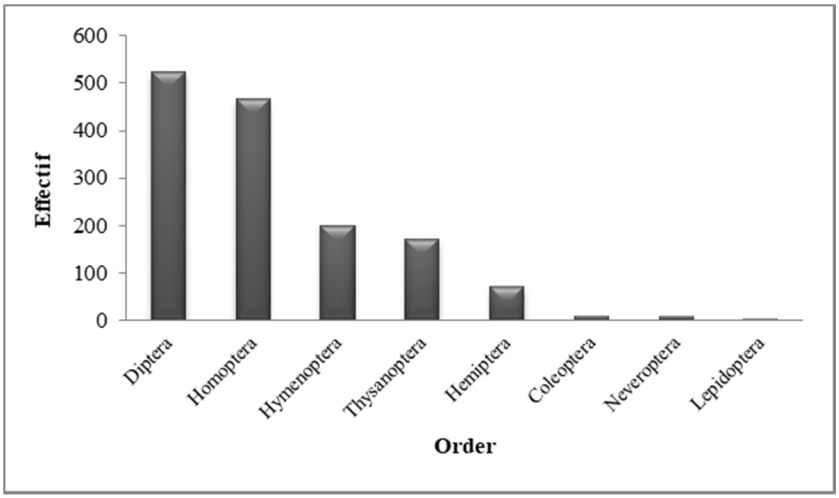

Figure 2. Importance of the entomofauna by orders.

Overall population by family

The entomofauna recorded during our experimentation is distributed in no less than 26 families. The most dominant is the Aphididae (417 individuals), or 28.78% of the total population. They are followed by Calliphoridae (Diptera) with 238 individuals (16.43%). Thripidae and Agromizidae come in third with 170 and 146 individuals respectively, ie 11.73% and 10.08%. Holophidae (Hymenoptera), Ceccidomyiidae (Diptera), Miridae (Hemiptera) and Aleurodidae (Homoptera)with respectively 71, 66, 40 and 39 individuals. Finally, the other families are insignificant, not more than 25 individuals (Fig. 3). The Kruskal-Wallis test showed a no significant relationship between orders abundances of the six months (P>0.05).

The family Aphididae (Fig. 4) occupies the first place in the greenhouse tomato inventoried entomofauna. 11 species have been identified in this study. The species *A. gos*sypii predominates with 163 individuals (39.09%) of the overall aphid population. *Megoura vicia* is the second species with 58 individuals (13.91%), followed by the tomato aphids *A. frangulae* and *M. euphorbiae* with 10.31% and 6.95% respectively. The other species,from the most important to the less important are *A.*

nasturkii (5.52%), *A. craccivora* (4.80%), *T. trifolii* (4.80%), *B. cardui* (4.80%), *M. persicae* (4.56%) and *B. hel*ychrysi (4.32%). Only four isolated individuals of *L.erysimi* were trapped.

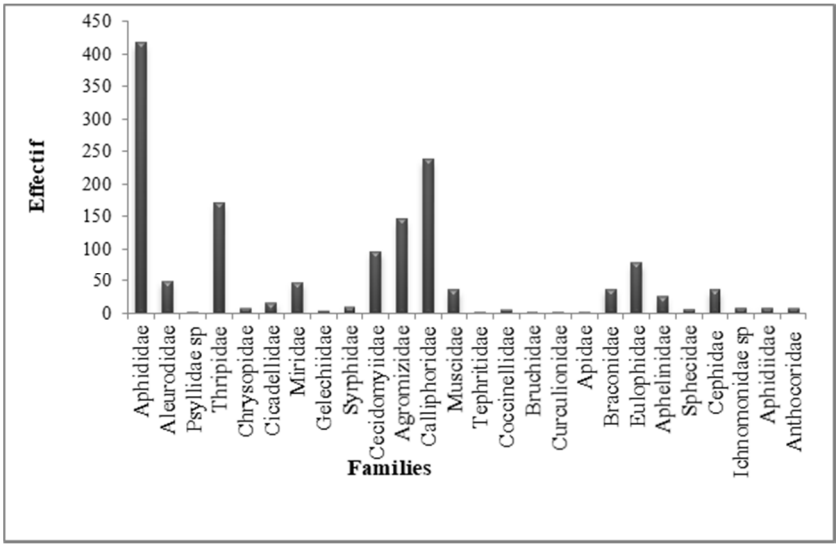

Figure 3. Importance of the entomofauna by families

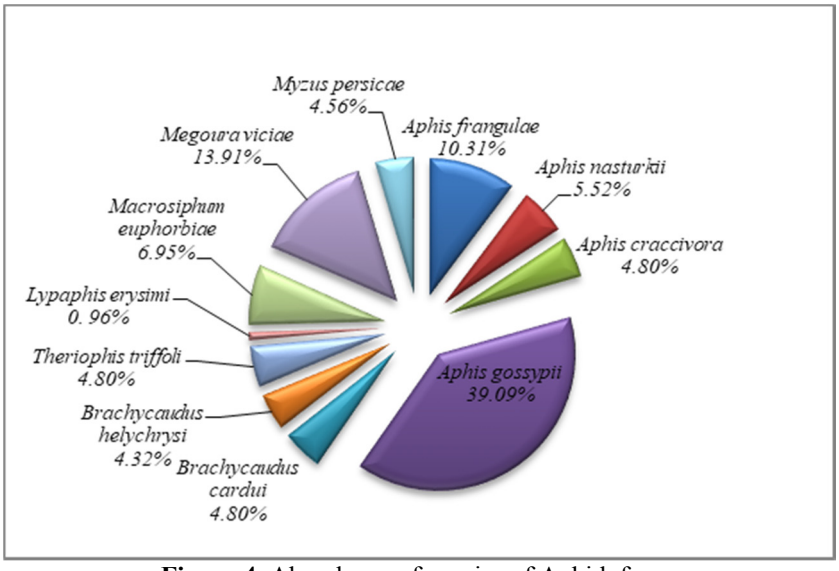

Figure 4. Abundance of species of Aphidofauna

The second family Thrips, represent the order of the Thysanoptera in the inventory. Possessing buccal parts of the picker-sucker type, they cause enormous damage to the crops. On tomato, thrips attack much more young leaves. Among the four taxa listed *T. tabaci* is the most dominant, it accounts for 58.82% with 100 individuals. It is followed by *F. occidentalis* with 53 individuals ie 31.18%. The other two species are *M. pallidor* and *A. fasci*atus their presence is insignificant with only 9 individuals (Fig. 5).

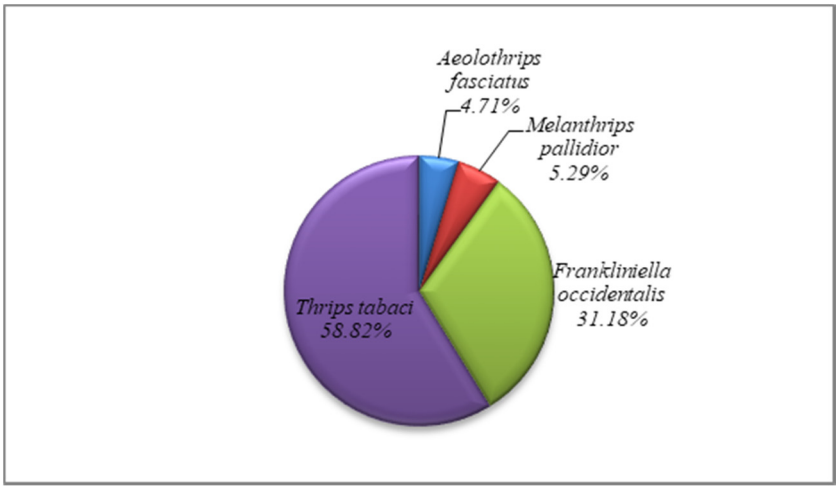

Figure 5. Abundance of species of Thrips

The family of the ordre Diptera are the most important group of trapped entomofauna, with 525 individuals representing 36.21% of the total trapped population. The genus Calliphora predominates with 218 individuals (41.52%) of the total Diptera population. The Agromizidae come in second with 109 individuals ie 20.76%. In the leafminers, the Hessian Fly (*M. destructor*), which is considered to be a key pest of tomato in recent years, totals 96 individuals (18.29%), while the other miner, *L. brioniae*, represents 7.05% of the total population (37 individuals). It is followed by *M. domestica* with 35 individuals. The only inventoried insect predator is the Syrphe *E. balteatus*, its presence is insignificant with only (09) individuals (Fig. 6).

Finally, the Hymenoptera are the richest species group in which 11taxons have been identified. This order comprises ten parasites representing 81.31% of the total trapped Hymenoptera (Fig. 7). These parasites can play an important role in regulating the populations of certain pests such as aphids and leaf miners. The rest consists of a pest*: C. spinipes*and a Hymenoptera poliniser *A. mellifera*. In the parasites group the species *Chrysocharis* sp. predominates with 39 individuals

(19.70%) of the total population. It is followed by: *Diglyphus* sp., *A. ervi* and *Aphelinus* sp. with 36, 34 and 15 individuals, respectively. Finally, parasites as *A. glomeratus, Tetrastichus* sp., *Aphytis* sp., *Pemphredon* sp. and Ichnomonidae species had an insignificant presence, their number does not exceed 8 individuals. As for the pest *C. spinipes* and the bee *A. mellifera*, they total 35 and 2 individuals respectively.

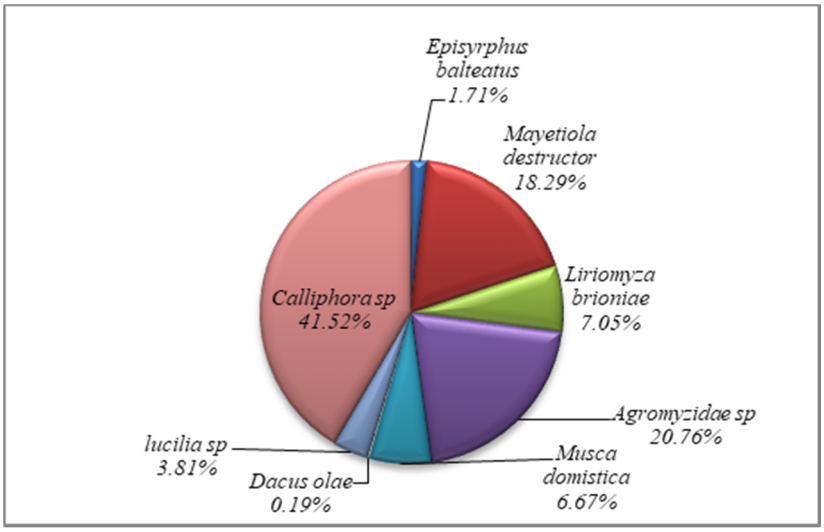

Figure 6. Abundance of species of Diptera

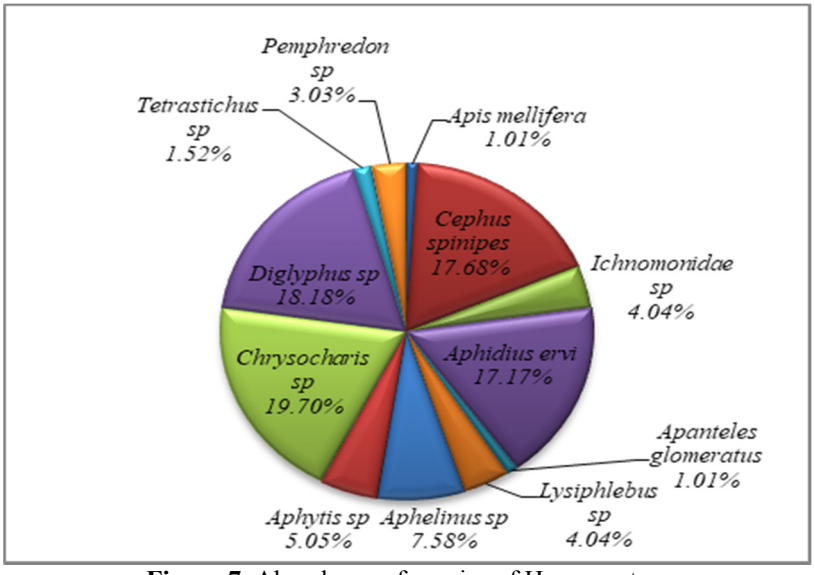

Figure 7. Abundance of species of Hymenoptera

Conclusions

At the end of this work on the diversity of the entomofauna associated with the tomato under shelters, 46 species distributed in eight orders and 26 families have been recorded.

The order of the homoptera is the most dominant with 13 species, followed by that of the hymenoptera with 12 species.

Three taxa are ubiquitous: the *Aphis gossypii* aphid, the *Mayetiola destructor* and *Calliphora* sp.

The useful entomofauna is very rich, accounting for 32.61% of the total population. Five predators: *Chrysoperla carnea*, *Nesidiocoris tenuis*, *Episyrphus balteatus*, *Hippodamia variegata* and *Clitostethus arcuatus*, and about ten parasites. Hymenoptera: *Aphidius ervi*, *Apanteles glomeratus*, *Tetrastichus* sp., *Diglyphus* sp., *Chrysocharis* sp., *Aphytis* sp., *Aphelinus* sp., *Pemphredon* sp., Ichnomonidae species and *Lysiphlebus* sp. were identified. This predatory parasite complex can contribute to the regulation of tomato pests under shelter.

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Plant ascorbate peroxidase: molecular phylogeny and role in oxidative stress

Imola Fábián[1], Edina Török[1], Dorina Podar[2,3] and Gyöngyi Székely[1,3,4,✉]

SUMMARY. Oxidative stress appears as a condition in accumulation and detoxification of reactive oxygen species (ROS). ROS are oxygen-derived free radicals, generated predominantly in mitochondria, peroxisomes and chloroplasts, as natural byproducts of the normal cell aerobic metabolism. In spite of their damaging effect, ROS can act as secondary messengers in different cellular processes, including tolerance to environmental stress factors. To neutralize the harmful effects of ROS, plants have evolved enzymatic and non-enzymatic defense systems. In flowering plants, ascorbate peroxidase (APX) is present in eight isoenzyme forms and constitutes an important enzymatic component in scavenging the harmful hydrogen peroxide to water as part of ascorbate-glutathione cycle. APX proteins, their roles, *in planta* expression location and their phylogenetic relationships are presented in the current paper. The phylogenetic analysis performed with the maximum likelihood method which was established for 118 protein sequences of 45 flowering plants. Our phylogenetic analysis revealed diversification of ascorbate peroxidase in angiosperms, and indicates a close relationship of APX1 with APX2, APX3 with APX4 and APX5, and APX6 with sAPX and tAPX proteins. Evolutionary relationships of plant ascorbate peroxidase isoenzymes indicate the evolution of different plant species genome and their phylogenetic affiliation.

Keywords: antioxidative defense system, evolution, osmoprotectants, phylogenetic

[1]*Babeş-Bolyai University, Faculty of Biology and Geology, Hungarian Department of Biology and Ecology, 5-7 Clinicilor Str., 400006 Cluj-Napoca, Romania.*
[2]*Babeş-Bolyai University, Faculty of Biology and Geology, Department of Molecular Biology and Biotechnology, 1 Kogălniceanu Str., 400084 Cluj-Napoca, Romania.*
[3]*Babeş-Bolyai University, Center of Systems Biology, Biodiversity and Bioresources, 400006 Cluj-Napoca, Clinicilor 5-7, Romania.*
[4]*Babeş-Bolyai University, Institute for Research - Development - Innovation in Applied Natural Sciences, 30 Fântânele Str., 400294 Cluj-Napoca, Romania.*
✉ ***Corresponding author: Gyöngyi Székely,*** *Babeş-Bolyai University, Faculty of Biology and Geology, Hungarian Department of Biology and Ecology, Cluj-Napoca, Romania, E-mail: gyongyi.szekely@ubbcluj.ro*

Introduction

Over the course of their lifetime, plants are exposed to different adverse environmental conditions, like osmotic and oxidative stress. In order to protect themselves against harsh external conditions, plants accumulate a series of protecting compounds, called osmoprotectants, and activate their antioxidative defense system. Appearance of O_2-evolving photosynthetic organisms and aerobic metabolism inevitably generated the occurrence of highly reactive oxygen species (ROS) (Halliwell 2006). During photosynthesis, oxygen is generated in the chloroplasts, and can accept electrons, thus forming $O_2^{\cdot -}$ (superoxide radical). In a multistep reaction, different types of ROS are generated from ground state oxygen i.e. $O_2^{\cdot -}$ (superoxide radical) and leads to the formation of H_2O_2 (hydrogen peroxide), $^1O_2$ (singlet oxygen), $HO_2^{\cdot}$ (perhydroxy radical), $HO^{\cdot}$ (hydroxyl radical), ROOH (alkyl hydroperoxide radical), $ROO^{\cdot}$ (alkylperoxyl radical) and $RO^{\cdot}$ (alkoxyl radical), which are highly reactive molecules causing serious damage to cell components and DNA, conducting to cell death (Gill and Tuteja, 2010). Under steady state conditions, damaging ROS molecules are scavenged by a set of antioxidative defense systems characteristic to chloroplasts, mitochondria and peroxisomes (Foyer and Harbinson, 1994; Alscher *et al.,* 1997; Klotz, 2002; Apel and Hirt, 2004; Navrot *et al.,* 2007; Heyno *et al.,* 2011; Sharma *et al.,* 2012), thus maintaining equilibrium between production and scavenging of ROS. This equilibrium can be disturbed by various biotic and abiotic environmental stress factors, such as pathogen attacks, salinity, drought, extreme temperatures, intense light, heavy metals, air pollution, herbicides and mechanical stress. Due to adverse stress factors, the levels of ROS in cells can suddenly increase and cause serious cell structure damages (Elstner, 1991; Malan *et al.,* 1990; Tsugane *et al.,* 1999).

Plant antioxidative defense mechanisms are of two types: enzymatic and non-enzymatic. Enzymatic system includes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and enzymes of ascorbate-glutathione (AsA-GSH) cycle, such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). Non-enzymatic system comprises of ascorbate (AsA), glutathione (GSH), tocopherols, carotenoids, and flavonoids (Noctor and Foyer, 1998; Sharma *et al.,* 2012) (Fig. 1).

It is important to mention that the osmoprotectant molecule, proline, can act as a non-enzymatic antioxidant needed to counteract the damaging effects of ROS in organisms as microbes, plants and animals (Chen and Dickman, 2005; Székely *et al.,* 2008). Despite their deleterious effect, ROS can act as secondary messengers in different cellular processes, including tolerance to environmental stresses (Desikan *et al.,* 2001; Yan *et al.,* 2007; Sharma *et al.,* 2012). The aim of this article is to present the plant ascorbate peroxidases (APX) (EC 1.11.1.11) phylogeny, as a main participant in neutralizing ROS.

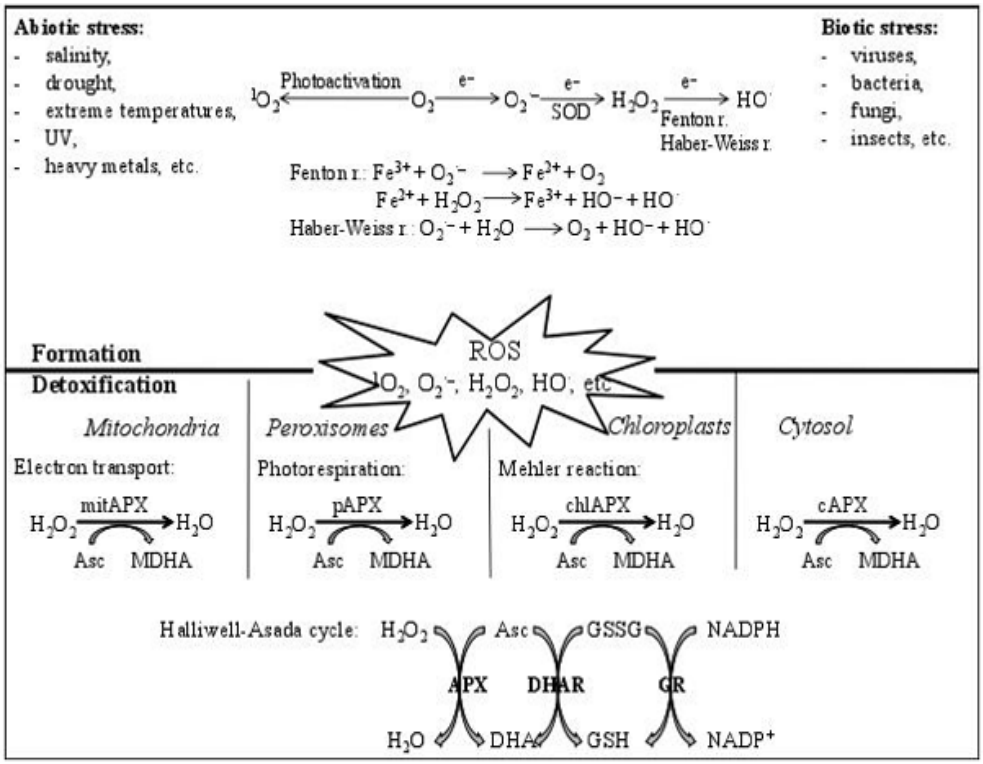

Figure 1. Schematic representation of APX role in ROS formation and detoxification in plants; ROS formation: $^1O_2$: singlet oxygen, O_2: molecular oxygen, $O_2^{\bullet-}$: superoxide radical, SOD: superoxide dismutase, H_2O_2: hydrogen peroxide, $HO^{\bullet}$: hydroxyl radical. ROS detoxification: Asc: ascorbate, MDHA: monodehydroascorbate; Halliwell-Asada cycle: H_2O_2: hydrogen peroxide, APX: ascorbate peroxidase, H_2O: water, DHAR: dehydroascorbate reductase, DHA: dehydroascorbate, GSSG: oxidized glutathione, GR: glutathione reductase, GSH: reduced glutathione, NADPH: reduced nicotinamide adenine dinucleotide phosphate, $NADP^+$: nicotinamide adenine dinucleotide phosphate (based on Noctor and Foyer, 1998; Sharma *et al.,* 2012).

Role of APX enzyme in antioxidative defense system during stress conditions

In plants, APX is the most distributed antioxidant enzyme and is considered to be a key ROS scavenger enzyme and cell protecting molecule (Orvar and Ellis, 1997). APX, together with catalase, controls the level of H_2O_2 in cells, but the main H_2O_2 scavenging role is thought to belong to APX, which converts H_2O_2 to H_2O in water-water and AsA-GSH (ascorbate glutathione or Halliwell Asada) cycles. APX uses two molecules of AsA to reduce H_2O_2 to water with subsequent generation of two molecules of MDHA (monodehydroascorbate) (Fig. 1).

Based on amino acid sequences, several different isoforms of APX family have been found at different subcellular localization in flowering plants, including chloroplast, mitochondria, peroxisomes and cytosol (Jimenez *et al.,* 1997; Madhusudhan *et al.,* 2003; Sharma and Dubey, 2004; Nakano and Asada, 1987). The organelle APX is efficient in scavenging H_2O_2 produced in the organelles, while cytosolic APX neutralizes H_2O_2 from cytosol, apoplast and that diffused from organelles (Sharma *et al.,* 2012). APX isoforms have a much higher affinity for H_2O_2 compared to CAT and are essential in scavenging ROS during stress conditions (Wang *et al.,* 1999). Many publications reported enhanced level of APX enzyme activity during different abiotic stress conditions, such as salinity, drought, extreme temperatures, heavy metal toxicity and presence of high-light intensities (Boo and Jung, 1999; Sharma and Dubey, 2005a; Sharma and Dubey, 2007; Han *et al.,* 2009; Maheshwari and Dubey, 2009; Hefny and Abdel-Kader, 2009). Begara-Morales *et al.,* (2013) reported the increase of APX enzyme activity in pea plants grown under saline (150 mM NaCl) conditions, *Anabaena doliolum* also revealed enhanced APX activity during salt stress (Srivastava *et al.,* 2005), water stress induced APX activity in three cultivars of *Phaseolus vulgaris* (Zlatev *et al.,* 2006) and *P. asperata* (Yang *et al.,* 2008), mild drought stress in rice generated higher chloroplastic-APX activity (Sharma and Dubey, 2005b), Cd stress caused increased APX activity in leaves of *Ceratophyllum demersum* (Arvind and Prasad, 2003), *Brassica juncea* (Mobin and Chan, 2007), *Triticum aestivum* (Khan *et al.,* 2007) and *Vigna mungo* (Singh *et al.,* 2008). Simonovicova *et al.,* (2004) reported enhancement of APX activity in *Hordeum vulgare* roots in the presence of Al stress. However, biochemical methods currently used to assess the enzyme APX activity can evaluate only the total APX activity, without distinguishing between the activities of the different APX isoforms.

Materials and methods

Data collection

The ascorbate peroxidase protein sequences used in this paper were gathered from the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) (McEntyre and Ostell, 2002) database and from arabidopsis.org. The sequences were run through BLAST Sequence Analysis Tool online program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in a non-redundant database (McEntyre and Ostell, 2002).

Sequence similarity

APX sequence similarity between *Arabidopsis thaliana* and the studied species was calculated using matGAT software with first gap penalty 12 and expanding gap 2 settings. (Campanella *et al.,* 2003). We collected separately the monocots and dicots APX sequences, the sequences which did not correspond to

homology were not taken into account. Thus, the similarity score was obtained from comparing 118 protein sequence. The scoring matrix used was BLOSUM50, penalties for first gap 16, and extending gap 4. The query monocots sequences were assessed comparing their similarity with *Arabidopsis thaliana* APXs (reference sequences: P82281, Q05431, Q1PER6, Q42564, Q42592, Q42593, Q7XZP5, Q8GY91). The predicted subcellular localization was obtained from http://cello.life.nctu.edu.tw/ (Yu *et al.,* 2004; Yu *et al.,* 2006) with the specific parameters ("Eukaryotes" and "Protein").

Magnoliophyta APX proteins

APX protein forms of 45 species from Magnoliophyta phylum were analyzed and compared to each other. The protein sequences were classified based on Catalogue of Life database (Table 2). Magnoliopsida class analysis indicated several families: Brassicaceae and Capparaceae family (APX1-3 and APX5); Capparaceae and Chenopodiaceae family (APX1-3 and APX6); Cucurbitaceae family (APX1,2 and APX6); Euphorbiaceae family (APX1, APX3, APX6); Fabaceae family (APX2) Malvaceae (APX1-2); Moraceae (APX1), Nelumbonaceae (APX2); Pedaliaceae and Rosaceae family (APX1-3, APX6), Rutaceae (APX1-3); Salicaceae (APX1-2, APX6); Solanaceae (APX1, APX3, APX6), Sterculiaceae family (APX2); Vitaceae family (APX1-2, APX6). From Liliopsida class Poaceae family (APX1-2, APX4, APX6, sAPX, tAPX) was analyzed.

Classification of APX isoforms

In order to fulfil an accurate analysis, a table containing the eight *Arabidopsis thaliana* AtAPX isoforms was created, as a benchmark for all APX proteins of the examined species (Table 1). The APX protein sequences were grouped according to their similarity index. Table 1 shows the APX isoforms according to NCBI database and the same isoforms based on their sequence alignments. The highest APX similarity values were used in our analyses. Similarity values below 50% were not considered.

Phylogenetic tree

For phylogenetic analyses 118 sequences were used: APX1 (25 sequences), APX2 (26 sequences), APX3 (10 sequences), APX4 (11 sequences), APX5 (11 sequences), APX6 (15 sequences), sAPX (10 sequences), tAPX (10 sequences) (Table 1). The protein sequences were sorted and aligned by multiple sequence alignments with ClustalW in MEGA7 program (Kumar *et al.,* 2016) using default settings. The length of aligned protein sequences were 271 amino acids. The phylogenetic tree was generated in PhyML SMS (Guindon *et al.,* 2010; Lefort *et al.,* 2017) maximum likelihood framework program. The phylogenetic reconstruction

was performed assuming a LG +G+I (Le and Gascuel, 2008) evolution model with gamma distributed variation rate across site (G) and a proportion of invariable site (I). The statistics value was based on Shimodaira–Hasegawa [SH] approximate likelihood ratio test [aLRT]. Phylogenetic tree was created by FigTree (v 1.4.0.) program. Main genetic distance between groups and within groups was estimated using p-distance (bootstrap value 1000) by MEGA7 program.

Results and discussion

Table 1 summarizes the APX isoforms, their cellular localization, role and expression location in *A. thaliana*.

Table 1.

APX isoforms and their role in *Arabidopsis thaliana* according to arabidopsis.org

APX protein	TAIR accession no.	Cellular localization	Role induced by	*In-planta* expression location
APX1	At1g07890	Golgi apparatus, chloroplast stroma, cytoplasm, plasma membrane, plasmodesma, cell wall	Cd ion, cytokinin, heat, oxidative stress, reactive oxygen species, salt stress	root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen, pollen tube cell,) fruit, seed, guard cell
APX2	At3g09640	cytoplasm	hydrogen peroxide catabolic process, oxidation-reduction process, oxidative stress	leaf
APX3	At4g35000	chloroplast envelope, glyoxysomal membrane, mitochondrion, peroxisomal membrane, plasmodesma, plastid, vacuolar membrane	hydrogen peroxide catabolic process, oxidation-reduction process, cytokinin, oxidative stress	root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen, pollen tube cell,) seed, guard cell

APX protein	TAIR accession no.	Cellular localization	Role induced by	*In-planta* expression location
APX4	At4g09010	chloroplast thylakoid lumen, chloroplast thylakoid membrane, cytoplasm, nucleus	oxidation-reduction process, oxidative stress	shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell
APX5	At4g35970	integral component of peroxisomal membrane	oxidative stress, hydrogen peroxide removal, hydrogen peroxide catabolic process, oxidation-reduction process	root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell
APX6	At4g32320	cytosol, extracellular region	hydrogen peroxide catabolic process, oxidative stress, seed germination, seed maturation	root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell
sAPX	At4g08390	chloroplast stroma, mitochondrion membrane	hydrogen peroxide catabolic process, oxidation-reduction process, cytokinin, oxidative stress	root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell
tAPX	At1g77490	integral component of chloroplast thylakoid membrane	chloroplast-nucleus signaling pathway, cold acclimation, hydrogen peroxide catabolic process, hydrogen peroxide mediated signaling pathway, oxidation-reduction process, oxidative stress	root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell

Plant species and their APX isoforms are visible in figures representing the phylogenetic tree of APX proteins. Table 2 shows the cellular localization of the studied 45 plant species.

Table 2.

List of analyzed species, APX isoforms and their subcellular localization

	Species name	**NCBI GenBank ID**	**APX**	**Subcellular localization**
1	*Arabidopsis thaliana*	AEE28201.1	APX1	Cytoplasmic
2	*Arabidopsis thaliana*	Q05431.2	APX1	Cytoplasmic
3	*Beta vulgaris* subsp. *vulgaris*	XP_010691257.1	APX1	Cytoplasmic
4	*Brachypodium distachyon*	XP_003558178.1	APX1	Cytoplasmic
5	*Brassica rapa*	XP_009118405.1	APX1	Cytoplasmic
6	*Citrus maxima*	ACM17464.1	APX1	Cytoplasmic
7	*Cucumis sativus*	AGJ72850.1	APX1	Cytoplasmic
8	*Gossypium hirsutum*	ABR18607.1	APX1	Cytoplasmic
9	*Jatropha curcas*	ACV50426.1	APX1	Cytoplasmic
10	*Malus domestica*	ABP87792.1	APX1	Cytoplasmic
11	*Morus notabilis*	EXC33221.1	APX1	Cytoplasmic
12	*Nicotiana sylvestris*	XP_009784425.1	APX1	Cytoplasmic
13	*Nicotiana tabacum*	AAA86689.1	APX1	Cytoplasmic
14	*Nicotiana tomentosiformis*	XP_009597491.1	APX1	Cytoplasmic
15	*Oryza brachyantha*	XP_006649890.1	APX1	Cytoplasmic
16	*Oryza sativa Japonica*	ABF95353.1	APX1	Cytoplasmic
17	*Oryza sativa Japonica*	Q10N21.1	APX1	Cytoplasmic
18	*Populus euphratica*	XP_011008849.1	APX1	Cytoplasmic
19	*Prunus mume*	XP_008224940.1	APX1	Cytoplasmic
20	*Sesamum indicum*	XP_011089855.1	APX1	Cytoplasmic
21	*Setaria italica*	XP_004984819.1	APX1	Cytoplasmic
22	*Solanum lycopersicum*	AAZ77770.1	APX1	Cytoplasmic
23	*Solanum tuberosum*	NP_001275066.1	APX1	Cytoplasmic
24	*Spinacia oleracea*	BAA12890.1	APX1	Cytoplasmic
25	*Tarenaya hassleriana*	XP_010521780.1	APX1	Cytoplasmic
26	*Vitis vinifera*	NP_001267988.1	APX1	Cytoplasmic
27	*Zea mays*	NP_001152249.1	APX1	Cytoplasmic
28	*Aegilops tauschii*	EMT09178.1	APX2	Cytoplasmic
29	*Arabidopsis thaliana*	AEE74792.1	APX2	Cytoplasmic
30	*Arabidopsis thaliana*	Q1PER6.3	APX2	Cytoplasmic
31	*Beta vulgaris* subsp. *vulgaris*	XP_010696372.1	APX2	Cytoplasmic
32	*Brachypodium distachyon*	XP_003562395.1	APX2	Cytoplasmic
33	*Brassica rapa*	XP_009123280.2	APX2	Cytoplasmic
34	*Brassica rapa* subsp. *oleifera*	CCC55736.1	APX2	Cytoplasmic
35	*Camelina sativa*	XP_010486536.1	APX2	Cytoplasmic
36	*Citrus maxima*	ACM17463.1	APX2	Cytoplasmic
37	*Citrus sinensis*	XP_006480586.1	APX2	Cytoplasmic
38	*Fragaria vesca* subsp. *vesca*	XP_004302839.1	APX2	Cytoplasmic
39	*Glycine max*	AAB01221.1	APX2	Cytoplasmic
40	*Gossypium arboreum*	KHG05754.1	APX2	Cytoplasmic
41	*Malus domestica*	XP_008350397.1	APX2	Cytoplasmic

	Species name	NCBI GenBank ID	APX	Subcellular localization
42	*Momordica charantia*	AIE12238.1	APX2	Cytoplasmic
43	*Nelumbo nucifera*	XP_010253495.1	APX2	Cytoplasmic
44	*Oryza brachyantha*	XP_006658179.1	APX2	Cytoplasmic
45	*Oryza sativa Japonica*	Q9FE01.1	APX2	Cytoplasmic
46	*Populus euphratica*	XP_011048406.1	APX2	Cytoplasmic
47	*Prunus mume*	XP_008239139.1	APX2	Cytoplasmic
48	*Sesamum indicum*	XP_011094725.1	APX2	Cytoplasmic
49	*Setaria italica*	XP_004958804.1	APX2	Cytoplasmic
50	*Tarenaya hassleriana*	XP_010543364.1	APX2	Cytoplasmic
51	*Theobroma cacao*	EOY07733.1	APX2	Cytoplasmic
52	*Theobroma cacao*	XP_007016653.2	APX2	Cytoplasmic
53	*Vitis vinifera*	XP_010651099.1	APX2	Cytoplasmic
54	*Zea mays*	NP_001105500.2	APX2	Cytoplasmic
55	*Arabidopsis lyrata* subsp. *lyrata*	XP_020874660.1	APX3	Cytoplasmic
56	*Arabidopsis thaliana*	Q42564.1	APX3	Cytoplasmic
57	*Beta vulgaris* subsp. *vulgaris*	XP_010679905.1	APX3	Mitochondrial
58	*Brassica rapa*	XP_009138334.1	APX3	Cytoplasmic
59	*Citrus sinensis*	XP_006486751.1	APX3	Cytoplasmic
60	*Nicotiana tomentosiformis*	XP_009618135.1	APX3	Cytoplasmic
61	*Oryza sativa Japonica*	Q0JEQ2.1	APX3	Cytoplasmic
62	*Ricinus communis*	XP_002530823.1	APX3	Cytoplasmic
63	*Sesamum indicum*	XP_011088597.1	APX3	Cytoplasmic
64	*Solanum tuberosum*	XP_006359692.1	APX3	Cytoplasmic
65	*Vitis vinifera*	XP_002278281.1	APX3	Cytoplasmic
66	*Aegilops tauschii*	EMT10887.1	APX4	Cytoplasmic
67	*Aegilops tauschii* subsp. *tauschii*	XP_020163634.1	APX4	Cytoplasmic
68	*Arabidopsis thaliana*	P82281.2	APX4	Chloroplast
69	*Brachypodium distachyon*	XP_003574893.1	APX4	Cytoplasmic
70	*Hordeum vulgare* subsp. *vulgare*	BAB62533.1	APX4	Cytoplasmic
71	*Oryza brachyantha*	XP_006659666.1	APX4	Chloroplast
72	*Oryza sativa Japonica*	Q6ZJJ1.1	APX4	Chloroplast
73	*Oryza sativa Japonica*	XP_015650808.1	APX4	Chloroplast
74	*Setaria italica*	XP_004974146.1	APX4	Cytoplasmic
75	*Sorghum bicolor*	XP_002444620.1	APX4	Cytoplasmic
76	*Stipa purpurea*	AJF34885.1	APX4	Cytoplasmic
77	*Zea mays*	NP_001132505.1	APX4	Cytoplasmic
78	*Arabidopsis lyrata* subsp. *lyrata*	XP_002869048.1	APX5	Cytoplasmic
79	*Arabidopsis thaliana*	Q7XZP5.2	APX5	Cytoplasmic
80	*Arabidopsis thaliana*	NP_195321.1	APX5	Cytoplasmic
81	*Arabidopsis thaliana*	AAP72144.1	APX5	Cytoplasmic
82	*Brassica napus*	XP_013743324.1	APX5	Cytoplasmic
83	*Brassica rapa*	XP_009148286.2	APX5	Cytoplasmic
84	*Camelina sativa*	XP_010432206.1	APX5	Cytoplasmic
85	*Camelina sativa*	XP_010446847.1	APX5	Cytoplasmic

	Species name	NCBI GenBank ID	APX	Subcellular localization
86	*Capsella rubella*	XP_006285911.1	APX5	Nuclear
87	*Eutrema salsugineum*	XP_006412038.1	APX5	Nuclear
88	*Oryza sativa Japonica*	P0C0L0.1	APX5	Chloroplast
89	*Tarenaya hassleriana*	XP_010526807.1	APX5	Cytoplasmic
90	*Arabidopsis thaliana*	Q8GY91.1	APX6	Chloroplast
91	*Beta vulgaris* subsp. *vulgaris*	XP_010674956.1	APX6	Chloroplast
92	*Brachypodium distachyon*	XP_003578843.1	APX6	Mitochondrial
93	*Cucumis sativus*	XP_004149799.1	APX6	Chloroplast
94	*Fragaria vesca* subsp. *vesca*	XP_004290885.1	APX6	Chloroplast
95	*Jatropha curcas*	XP_012078304.1	APX6	Chloroplast
96	*Malus domestica*	XP_008365199.2	APX6	Chloroplast
97	*Nicotiana sylvestris*	XP_009784836.1	APX6	Chloroplast
98	*Nicotiana tomentosiformis*	XP_009629390.1	APX6	Chloroplast
99	*Oryza sativa Japonica*	P0C0L1.1	APX6	Mitochondrial
100	*Oryza sativa Japonica*	ABA96617.1	APX6	Mitochondrial
101	*Populus euphratica*	XP_010999402.1	APX6	Chloroplast
102	*Prunus mume*	XP_008219620.1	APX6	Chloroplast
103	*Sesamum indicum*	XP_011074839.1	APX6	Chloroplast
104	*Setaria italica*	XP_004977222.1	APX6	Mitochondrial
105	*Solanum lycopersicum*	NP_001234631.2	APX6	Chloroplast
106	*Vitis vinifera*	XP_003634424.1	APX6	Chloroplast
107	*Aegilops tauschii* subsp. *tauschii*	XP_020162413.1	sAPX	Chloroplast
108	*Arabidopsis thaliana*	Q42592.2	sAPX	Chloroplast
109	*Brachypodium distachyon*	XP_003579783.1	sAPX	Chloroplast
110	*Hordeum vulgare* subsp. *vulgare*	BAJ97403.1	sAPX	Chloroplast
111	*Oryza brachyantha*	XP_006652305.1	sAPX	Chloroplast
112	*Oryza sativa*	CAH67301.1	sAPX	Chloroplast
113	*Oryza sativa Indica Group*	EEC77311.1	sAPX	Chloroplast
114	*Oryza sativa Japonica*	Q7XJ02.1	sAPX	Chloroplast
115	*Oryza sativa Japonica*	XP_015635863.1	sAPX	Chloroplast
116	*Setaria italica*	XP_004975656.1	sAPX	Mitochondrial
117	*Sorghum bicolor*	XP_021319656.1	sAPX	Chloroplast
118	*Aegilops tauschii* subsp. *tauschii*	XP_020185648.1	tAPX	Chloroplast
119	*Arabidopsis thaliana*	Q42593.2	tAPX	Chloroplast
120	*Brachypodium distachyon*	XP_010235542.1	tAPX	Chloroplast
121	*Oryza brachyantha*	XP_006647364.1	tAPX	Chloroplast
122	*Oryza sativa Japonica*	Q69SV0.2	tAPX	Chloroplast
123	*Saccharum hybrid cultivar*	AGD80597.1	tAPX	Chloroplast
124	*Setaria italica*	XP_004952823.1	tAPX	Chloroplast
125	*Sorghum bicolor*	XP_021316117.1	tAPX	Chloroplast
126	*Triticum aestivum*	AAS80158.1	tAPX	Chloroplast
127	*Triticum urartu*	EMS46926.1	tAPX	Chloroplast

Phylogeny of APX isoforms out of 45 flowering plant species is represented by Figure 2.

The APX isoforms constitute a monophyletic group. The APX phylogenetic tree has split three branches with highly supported values (1 SH). Red band indicates the phylogenesis of APX1 and APX2 proteins, with highest number of species. These groups APX1 and APX2 sequences are mixed. The first lineage (0.677 SH-like support value) consists of species like Vitaceae, Pedaliaceae, Brassicaceae, Capparaceae, Malvaceae, Chenopodiaceae, Rutaceae, Rosaceae, Salicaceae families. Rutaceae family members has sister groups with Chenopodiaceae (APX2), Malvaceae (APX2), Capparaceae (APX2) and Brassicaceae (APX2) family species. The next lineage, Liliopsida class, consists of Poaceae family with APX1-2 groups, and they are sister groups (0.872 SH). Inside the Poaceae family there is a split between APX1 and APX2. The next split is the Malvales class with the highest confidence interval (0.780 SH). In close relation (0.8 SH) appears the Rosaceae with Moraceae and Cucurbitaceae family species. These groups are sister groups (0.826 SH) with Chenopodiaceae, Solanaceae, Brassicaceae, Capparaceae, Pedaliaceae family species. The second big branching are APX3, 4, 5 groups (1SH). The light green band shows the APX3 as a well-supported clade (0.895 SH) with three divergences: Chenopodiaceae, Brassicaceae and the close related Solanaceae, Vitaceae, Euphorbiaceae, Rutaceae, Pedaliaceae (0.895 SH) families. Another well supported clade is the APX4 marked with dark blue band, which comprise Poaceae family species and is separated in two sister groups with the highest divergence (1 SH). A well separated group is the APX5, colored with light green band, and consists the Capparaceae family and a highly separated Brassicaceae family species (0.998 SH). The last big branching is APX6, sAPX and tAPX groups (1 SH). APX6 is marked with light blue and is a well separated clade. The first clade is Liliopsida class (0.97 SH) with a well-supported clade, Poaceae family members (0.966 SH). The next branch is composed of Chenopodiaceae, Euphorbiaceae, Vitaceae, Rosaceae, Salicaceae, Pedaliaceae, Solanaceae, Cucurbitaceae families. The Vitaceae and Cucurbitaceae generates a sibling group and are consisted of one sister group, the Rosaceae family species. sAPX appears as a well-supported clade marked with lilac band. sAPX of *Oryza* genus are weakly supported. The yellow tAPX are well isolated group (1 SH), with two sister clades: *Oryza* genus and the Poaceae family species (0.375 SH). However, the low support of nodes on the phylogenetic tree shows that the relationship between the species is not clearly resolved yet. The high level of differentiation of APX groups is also confirmed by the high p-distance value expressed as a percentage value and appears as a high level of divergence between groups (Table 3).

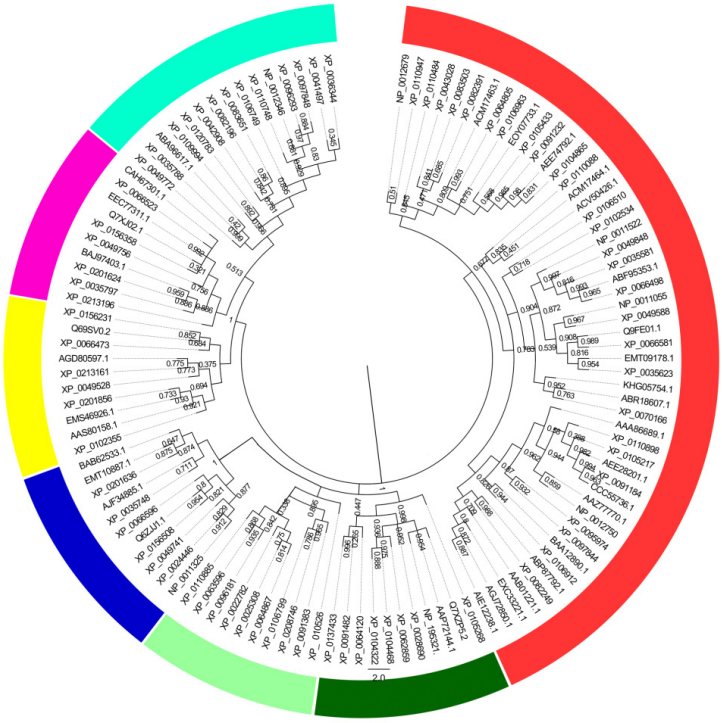

Figure 2. PhyML phylogenetic tree of 45 flowering plant species APX proteins; APX1 and APX2 are marked with red band, APX3 with light green band, APX4 with dark blue band, APX5 with light green band, APX6 with light blue band, sAPX with lilac band and tAPX with yellow band.

Table 3.

Average genetic distance between APX groups (%)

Groups name	APX1	APX2	APX3	APX4	APX5	APX6	sAPX
APX2	16.7						
APX3	33.1	33.2					
APX4	33.6	33.4	19.1				
APX5	38.7	38.3	26.8	31.8			
APX6	50.7	50.6	46.2	44.9	51.7		
sAPX	49.6	49.3	45.5	45.3	50.5	16.4	
tAPX	50.3	50.4	46.3	45.5	52.4	16.0	12.2

The genetic distance analyses within groups were also analyzed. The groups APX1-2-3 and APX6 revealed the higher genetic distance, exceeding the value of 1.1% (Table 4).

Table 4.

Average genetic distance within the APX groups (%)

Groups name	Genetic distance
APX1	1.59
APX2	1.54
APX3	1.19
APX4	0.66
APX5	0.90
APX6	1.26
sAPX	0.58
tAPX	0.55

Our cellular localization analysis indicated that APX1 and APX2 are localized in cytoplasm, APX3, APX4 and APX5 are localized in mitochondria, chloroplasts, nucleus and cytoplasm, APX6, sAPX and tAPX are mitochondrial and chloroplastic. The result of phylogeny reconstruction shows the relationship between the APX isoforms which may be influenced by the cellular localization also.

Conclusions

Ascorbate peroxidase is an essential enzyme in detoxifying the extremely harmful hydrogen peroxide, therefore in plant oxidative stress response. Molecular phylogeny analysis of several plant APX proteins has been presented in this study, in order to investigate the molecular manner of evolution of ascorbate peroxidase isoenzymes family in angiosperms. Evolutionary analysis of ascorbate peroxidase isoenzymes of different plant species, showed that APX is a monophyletic group, probably evolved from a single ancestor, where some isoformes are close related, some are not. Our phylogenetic analysis points close relationships between APX1 and APX2, between APX3, APX4 and APX5, and between APX6, sAPX and tAPX proteins in angiosperms. With an essential role in detoxifying processes of the cell, understanding molecular mechanisms of stress tolerance and phylogeny of APX, can serve to generate an efficient drug for prevention of several diseases in plants, animals and humans caused by severely harmful oxidative stress. Gathering information about molecular function of APX isoforms to scavenge ROS in different cellular compartments, could contribute to stress responsive gene engineering in order to improve crop tolerance against adverse environmental conditions.

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Effects of different cadmium levels on the growth and yield parameters of wild *Vigna*

Beckley Ikhajiagbe[1,✉], Geoffery O. Anoliefo[1], Edokpolor O. Ohanmu[1,2] and Ephraim Aliu[1]

SUMMARY. The assessment of growth and yield parameters of wild *vigna* to to different levels ofcadmium pollution has been investigated in this study. The experimental setup consisted of three (3) treatments namely; 0, 2.5 and 5 ecological screening value (ESV) and parameters recorded were taken 84 days and 20 weeks namely; plant germination factors, plant yield, percentage chlorosis, necrosis and senesced leaves, plant dry matter accumulation as well as plant lifetime morphological changes. Cadmium concentration increased the percentage of foliar chlorosis and necrosis (20.40) in older leaves than younger leaves (4.08) respectively of TVNu-91 sown in 5 ESV cadmium soil and this eventually resulted to an increase in percentage senesced leave with increasing cadmium concentration. Leaf folding and curling symptoms were cadmium-associated in the intermediate partition but same cannot be said for the young plant (YP). However, leaf curling was reported as a prominent morphological feature in this study. With respect to insect foraging, there was total absence of foraging in both control accessions and cadmium polluted accessions. There was also a significant difference ($P>0.05$) in the number of pods per plant as evidenced in TVNu-95 (5ESV) 5.67 when compared to 14.07 in the control of TVNu-95. Thus, there was a gradual decrease with increasing ecological screening value. Generally, there was significant difference in the seed number per pods ($P<0.01$), seed weight per pods ($P<0.01$) plant yield ($P<0.01$), and flower bud size ($P<0.05$). 20 weeks after sowing, plant dry matter accumulation was reduced with increase in metal concentration. There was variability in plant yield response to metal toxicity with a general decrease reported with increased cadmium concentration. However, TVNu-93 had a better yield as compared to the other studied accessions.

Keywords: cadmium, heavy metal, legumes, phytoassessment,Wild *Vigna*.

[1] *University of Benin, Environmental Biotechnology and Sustainability Research Group, Department of Plant Biology and Biotechnology, Nigeria.*
[2] *Edo University, Department of Plant Biology and Biotechnology, Iyhamo, Nigeria.*
✉ ***Corresponding author:*** *Ephraim Aliu, Environmental Biotechnology and Sustainability Research Group, University of Benin, Nigeria,*
E-mail: Ephraimogiealiu@gmail.com

Introduction

Metal pollution has developed into one of the utmost severe ecological problems today as a result of increasing ecological pollution from human actions such as mining of metals, gas exhaust, fuel production, electroplating, fertilizer, sewage and use of pesticides (Kabata-Pendias and Pendias, 2001). Heavy metals are branded to have detrimental effects on plant development and soil microflora thus bringing about losses in plant yield. Heavy metals have been shown, particularly in non-tolerant plant species, to affect an extensive range of plant cellular activities such as photosynthesis, mineral nutrition, respiration, membrane structure function, and gene expression (Maksymiec and Baszynski, 1996; Prasad, 1999; Hall, 2002). Cd, Cu, Hg, Pb and Ni denote the most common heavy metal pollutants. Of concern though, is cadmium (Cd).

Cadmium is from a Latin word "cadmia" and Greek word "Kadmeia" which are ancient names for calamine (zinc carbonate). It was discovered in Germany in 1817 by Fredrick Strohmeyer, a German chemist, as an impurity in zinc carbonate (calamine). A typical source of cadmium pollution in the surrounding is the geologic parent material (generally, high concentrations of chromium, manganese, zinc, copper, cadmium, mercury, tin, and lead are found in the geologic plant materials). The organic and inorganic fertilizers are also important sources of heavy metals to agricultural soils. Cadmium however, is of particular concern in plants as it accumulates in leaves at very high levels, and may be consumed by animals or human beings. Measurable amount of cadmium occur in many soils, animals and plant materials, and an increasing attention is been paid to its concentration in these materials for biological, medical, geochemical, and agricultural prospecting (Stewart *et al*., 1974).

Studies have shown that cadmium tend to accumulate in plant tissues at concentrations exceeding that of the soil solution (Onweremadu *et al*., 2008). Plants tissues like roots, shoots, trunks and leaves have been studied and cadmium is seen as a cumulative toxicant by most scientists. Previous studies on the effect of cadmium on seeds have been restricted to seedlings that have already germinated. Increase in cadmium also occurs due to the use of sewage sludge, manure and limes (Yanqun *et al.,* 2005). Although the levels of heavy metals in agricultural soils are very small, however constant use of phosphate fertilizer may result in the dangerously high accumulation of some metals (Verkleji 1993). Liming also increases the level of heavy metals in the soil more than the compost refuse, and the nitrate fertilizers. Among heavy metals, cadmium is one of the toxic elements that have no function in living organisms. It has long biological persistence as it causes leaf rolls, chlorosis, growth inhibition, water imbalance, phosphorus and nitrogen deficiency, reduced manganese transport and reduction of root and stem growth (Mishra *et al.,* 2006). It can be found in soils because it is present in insecticides, fungicides, sludge and commercial fertilizers (Ravichandran *et al.,* 2011).

Cadmium treatment with 1μM in 24 hours reduces the root-growth up to 30% and this inhibition has positive correlation with the reduction of root cells viability (Siroka *et al.,* 2004). It is widely recognized that cadmium taken up by plants is the main source of cadmium accumulation in food (Lopez-Millan *et al.,* 2009). Cadmium can be easily absorbed by plant roots and transported to shoots results and this results in the disorder of biochemical and physiological processes, and then affects plant growth and morphology (Sgherri *et al.,* 2002). It has also been suggested that growth inhibition by cadmium is due to a direct effect of cadmium on the nucleus or its interaction with hormones in the aerial parts of the plants.

In Nigeria, legume provides a wide variety of high protein diet. Thus, the choice of wild vigna was informed due to its large cultivation and consumption in most industrial cities of Nigeria, such as Kano and Kaduna, which are also located in regions where significant metallurgic activities are ongoing. Farmers in these regions are also known to depend heavily on fertilizers for improved crop yield. The present study was carried out with the aim to observe impact of different cadmium levels on growth as well as yield parameters of different accessions of wild vigna and to provide a theoretical basis for the risk assessment of cadmium pollution and the maintenance of sustainable agricultural production.

Materials and methods

Experimental site

The experiment was carried out in the Botanica garden of Plant Biology and Biotechnology, University of Benin, Benin city. The experiment lasted between the periods of November, 2016 to March, 2017.

Experimental material

Soils used were collected from 10 random points at a depth of 0 – 15cm using an auger from the botanical garden, University of Benin. The soils were bulked and crushed together to obtain homogeneity. The homogenized soils were placed into polythene bags weighing 15kg and sun dried for 48 hours. Soil samples were sent to the laboratory for physiochemical analysis and generally characterized as sandy loam.

Procurement of seeds

Seeds of five accessions of wild vigna namely TVNu-91, TVNu-93, TVNu-94, TVNu-95 and TVNu-96 were procured from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria.

Treatment preparation

The preparation of treatments is listed in (Table 1) below.

Experimental procedure

The experimental setup consisted of three blocks replicated thrice in a randomized block design making a total of 45 treatments. The seeds obtained from the various accessions were planted directed into each polythene bags. The plants were watered everyday till the end of the experiment. Data was collected weekly and biweekly. Parameters measured are listed below;

Morphological stress responses

Morphological observations of the physical appearances of the plant in response to the experimental conditions were recorded on periodic basis. These observations include the colour, form or the appearance of the leaves and the stem of the plant as well as positioning of flowers and nodes. Care was also taken to ensure that the progression of chlorosis was recorded. In this case, whenever chlorosis was noted, the leaf was immediately tagged so that chlorotic progress would be followed up till when the leaf became entirely chlorotic; in this regard, the progress of chlorosis measured in hours was therefore provided. This same procedure was followed to describe the progression of necrosis.

The rate at which plant lost their leaves as well as which portion of the plant lost a particular leaf was also taken into consideration. Thereupon, every plant was therefore divided into three major partitions according to (Ikhajiagbe and Guobadia 2016), and herein referred to as old partitions which consisted of any plant part comprising its stem and leaves, and other appendages from the soil level measuring up till 45cm above soil level; young partition which also consist of all leaves, stem, flowers and other appendages taking up the portion from the meristem of the plant measuring 45 cm downwards. The intermediate partition therefore was taken in this study as the portion in between the demarcated old partitions and new partitions.

Haven followed this demarcation based on partitioning, changes with regards to necrosis, or any other physical observation was made and reported on time basis. Care was taken to ensure that the total number of leaves that folded, curled or showed signs of foraging were taken into notes and as such were counted and presented as percentage of the total number of leaves that appeared in the plant at any given time. These were therefore presented in the results sections as percentage of folded leaves.

Plant yield

The plant yield (kg/m^2) was calculated using the formula;

$$\frac{S \times P \times 120}{1000 \times 22}$$

where S = seed weight per pod; P = number of pod per plant

Data analysis

Data was subjected to analysis of variance (ANOVA) using SPSS 16.0 version. Least significant difference (LSD) was used to separate the means at 5% level of significance.

Results and discussion

The study was undertaken to investigate the impact of different cadmium levels on the growth and yield parameters of different accessions of wild vigna and some morphological as well as yield parameters were on hand to provide necessary interpretations of the research outcome. (Table 1) shows the treatment designations required for the study.

Table 1.

Treatment designations for metal concentrations

Designations	Description
0 ESV	Control (unpolluted soil)
2.5 ESV	0.15 g of cadmium chloride diluted in 2L of water and mixed in 15 kg soil
5 ESV	0.30 g of cadmium chloride diluted in 2L of water and mixed in 15 kg soil

ESV –Ecological screening value (4mg/kg) (Efroymson, 1997)

Germination factors

The effects of cadmium treatment on emergent time of different accessions of wild vigna at 7days after sowing have been presented on Table 2. The lowest (fastest) average emergence value was 2.30 observed in TVNu-95 as opposed to the highest (slowest) average emergence value of 3.70 obtained in TVNu-93. First day of emergence in TVNu-91 was 3.00 which increased to 4.60 with increasing ecological screening value of 2.5 ESV (4.20) and 5 ESV (4.60) respectively. In TVNu-93 it took an average of 3.70 days which eventually increased to 4.00 with increasing ESV. Similar trend was observed for TVNu-94, TVNu-95 and TVNu-96. Over all, the number of days it took to emergence of the various accessions of wild vigna was not significantly different ($P > 0.05$) hence heavy metal incidence did not significantly affect first day of emergence of the plant. Similarly, final emergent percent in the metal treated accessions was 66.67% except in 2.5 and 5 ESV of TVNu-96 ESV which gave a final emergence percent of 33.33%; again this was not significantly different ($P=0.00$). Table 2 also shows an average mean daily emergent of 4.76 to 9.53 days in the various accessions of wild vigna ($P<0.01$).

Percentage foliar chlorosis, necrosis and senesced leaves

The percentage of total foliar chlorosis and necrosis of wild vigna have been shown in (Table 3a and 3b). Cadmium pollution on the incident of foliar chlorosis was more predominant in the older leaves than in the fresh leaves of the plants. TVNu accessions sown in control soils had no chlorosis in the fresh leaves as

compared with those sown in cadmium polluted soils (Table 3a). However, there was no significant difference (P>0.05) in cadmium pollution of the various accessions studied. With respect to foliar necrosis (Table 3b), necrosis was more prevalent in the older leaves than the fresh leaves of the plants in both control and cadmium polluted soils of the various accessions. It was suggested by (Ohanmu *et al.,* 2018) that cadmium may have been transported to older leaves as a survival strategy in order to overcome the stress exerted by the metal. Although there was no significant difference (P>0.05) in both control and cadmium pollution of the various accessions. It has been reported by Ghoshroy and Nadakavuharen (1990) that within few hours of its supply, cadmium is readily absorbed by roots and then transported to other parts of the plant. Cadmium pollution also significantly affected the percentage of senesced leaves 84 days after sowing (DAS). TVNu-91 sown in control soils had a percentage senesced leaf of 18.6 % as compared to TVNu-91 (47.8 %) sown in the 5 ESV (Fig. 1). Plant responds to environmental stresses by a variety of means. It is noted frequently that many crop species tolerance to prevailing stress conditions increases with the advancing age of the plant (Ohanmu *et al.,* 2018).

Table 2.

Effects of Treatment on emergent time of wild *Vigna* at 7 days after sowing

Plant Accessions	**Cd. Conc. (ESV)**	**First day of emergent (days)**	**Final Emergent percentage(%)**	**Mean daily emergent (%/day)**	**Mean emergent time**
TVNu-91	0	3.00	66.67	9.53	5.33
	2.5	4.20	66.67	9.53	6.00
	5	4.60	33.33	4.76	6.00
TVNu-93	0	3.70	66.67	9.53	5.33
	2.5	3.90	66.67	9.53	6.00
	5	4.00	66.67	9.53	6.00
TVNu-94	0	3.30	33.33	4.76	5.33
	2.5	3.60	66.67	9.53	5.33
	5	3.90	33.33	4.76	5.33
TVNu-95	0	2.30	66.67	9.53	5.00
	2.5	2.80	33.33	4.76	5.00
	5	2.87	66.67	9.53	5.00
TVNu-96	0	2.34	33.33	4.76	4.67
	2.5	2.56	33.33	4.76	4.67
	5	2.77	33.33	4.76	5.00
P-value		0.147	0.000	0.012	0.034
Sig*		P>0.05	P=0.000	P<0.01	P<0.05

Table 3a.

Percentage of total foliar chlorosis of wild *Vigna* at 84 days after sowing

Plant Accessions	Cd. Conc. (ESV)	No. of affected leaves per Plant partition			Total
		Younger	Intermediate	Older	
TVNu-91	0	0	0	4.30	4.30
	2.5	0	4.22	16.90	21.12
	5	4.08	4.08	20.40	28.56
TVNu-93	0	0	2.05	2.05	4.10
	2.5	4.43	4.43	13.29	22.14
	5	5.39	5.39	21.57	32.36
TVNu-94	0	0	2.24	4.48	6.72
	2.5	0	7.96	11.94	19.90
	5	4.53	9.07	18.13	31.73
TVNu-95	0	1.65	1.65	3.30	6.60
	2.5	5.43	2.72	13.58	21.72
	5	6.55	6.55	19.65	32.74
TVNu-96	0	0	1.73	3.46	5.19
	2.5	2.58	5.17	10.33	18.08
	5	6.55	6.55	16.37	29.47
P-value		0.614	0.553	0.984	0.999
Sig*		P>0.05	P>0.05	P>0.05	P>0.05

Table 3b.

Percentage of total foliar necrosis of wild *Vigna* at 84 days after sowing

Plant Accessions	Cd. Conc. (ESV)	No. of affected leaves per Plant partition			Total
		Younger	Intermediate	Older	
TVNu-91	0	2.15	2.15	6.44	10.74
	2.5	4.22	12.67	16.90	33.80
	5	4.08	16.32	20.40	40.80
TVNu-93	0	0	4.10	4.10	8.21
	2.5	8.86	8.86	17.71	35.43
	5	10.79	16.18	21.57	48.54
TVNu-94	0	2.24	2.24	4.48	8.96
	2.5	7.96	7.96	19.90	35.81
	5	9.07	13.60	27.20	49.86
TVNu-95	0	1.65	3.30	4.95	9.89
	2.5	2.72	8.15	16.29	27.15
	5	3.27	9.82	22.92	36.02
TVNu-96	0	1.73	1.73	6.93	10.39
	2.5	5.17	5.17	15.50	25.83
	5	6.55	6.55	22.92	36.02
P-value		0.520	0.685	0.995	0.969
Sig*		P>0.05	P>0.05	P>0.05	P>0.05

Other observable morphological parameters

Other observeable morphological changes of wild vigna during the plant lifetime such as leaf folding, leaf curling and insect foraging have been showed on (Table 4). Leaf folding was totally absent in all control plants of the various accessions. With respect to leaf curling, only the older portions of TVNu-91, TVNu-94, and TVNu-95 in the control accessions experienced leaf curling. Although, the cadmium polluted accessions showed presence of leave folding and curling which progressed from the younger portion to the older portion. However, there was total absence of insect foraging in both control and cadmium polluted accessions. Leaf curling was reported as a prominent morphological feature in the study. From (Table 4), leaf folding and curling symptoms were cadmium-associated in the intermediate partition but same cannot be said for the young plant (YP). The mechanism behind this could not be explained. It is therefore suggested that this may just be an indicative characteristic that Plant Biologists might use to suggest cadmium toxicity. With respect to insect foraging, there was total absence of foraging in both control accessions and cadmium polluted accessions.

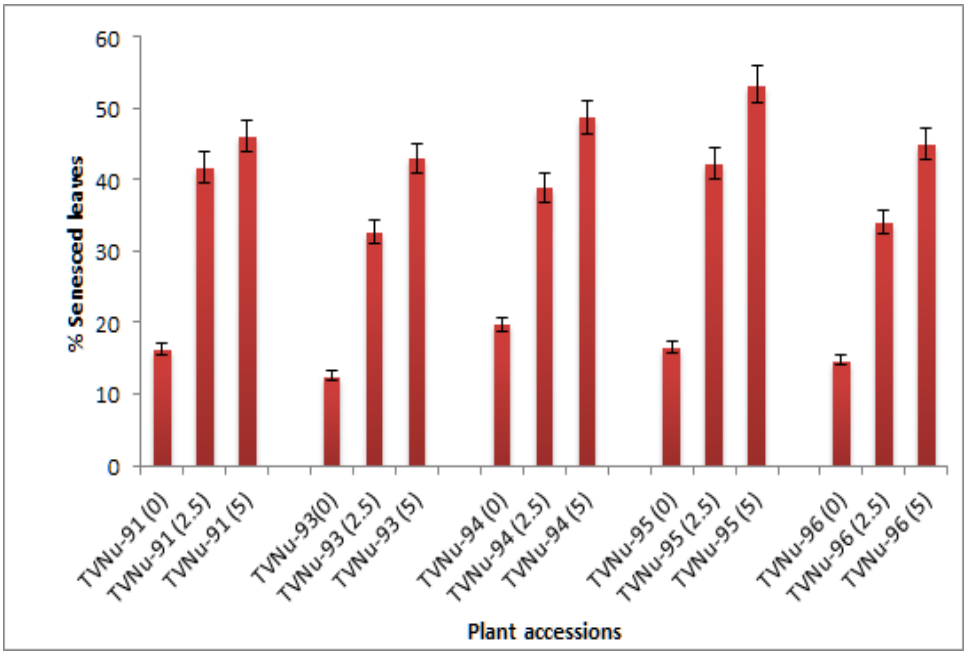

Figure 1. Percentage senesced leaves of wild vigna accessions 84 days after sowing

Table 4.

Other observable morphological presentations of wild *Vigna*.
Results represent total observation during the lifetime of the test plant

Plant Accessions	Cd. Conc. (ESV)	Folded leaves			Curled leaves			Leaves with sign of foraging by insects		
	Control	YP	IP	OP	YP	IP	OP	YP	IP	OP
TVNu-91	0	0	0	0	0	0	2.15	0	0	0
	2.5	0	4.22	12.67	8.45	8.45	0	0	0	0
	5	0	4.08	16.32	12.24	12.24	0	0	0	0
TVNu-93	0	0	0	0	0	0	0	0	0	0
	2.5	0	0	0	8.856	8.86	4.43	0	0	0
	5	0	0	0	21.57	21.57	5.39	0	0	0
TVNu-94	0	0	0	0	0	0	2.24	0	0	0
	2.5	0	15.9	11.94	7.96	7.96	11.94	0	0	0
	5	0	22.7	27.2	13.6	13.6	22.67	0	0	0
TVNu-95	0	0	0	0	0	0	3.3	0	0	0
	2.5	5.43	8.15	5.43	2.72	2.72	10.86	0	0	0
	5	6.55	26.2	16.37	6.55	6.55	22.92	0	0	0
TVNu-96	0	0	0	3.46	0	0	0	0	0	0
	2.5	0	10.3	5.17	0	0	5.17	0	0	0
	5	3.27	19.7	16.37	0	0	6.55	0	0	0

YP= Young plant, IP= Intermediate Plant, OP= Older Plant

Plant Dry Matter Accumulation

Cadmium pollution significantly reduced ($P<0.01$) the overall foliar yield of TVNu-91 (5ESV) to 29.15 as compared to TVNu-93 (44.20) sown in the control soil (Table 5). There was also significant reduction ($P>0.01$) in the plant dry weight of the different accessions studied with increasing cadmium concentration. TVNu-93 (5ESV) had a reduced dry plant weight of 23.79 when compared to 37.34 obtained in the control soil of TVNu-93 and this is supported by earlier reports showing that legumes were sensitive to increased cadmium levels (Van Assche *et al.,* 1998). The effect of cadmium pollution on the root dry weight as well as the shoot to root ratio was also reported. There was significance difference ($P=0.00$) in the root dry weight as well as shoot to root ratio of both control and cadmium polluted accessions. This could be attributed to the fact that increased cadmium concentration in the root environment resulted in reduction of absorption of water and nutrients, reduction of water transpiration and disturbance in water balance, inhibition of enzymes activities and of cell metabolism (Ohanmu *et al.,* 2018).

Table 5.

Effects of cadmium pollution on Plant Dry Matter Accumulation of wild *Vigna* at 20 weeks after sowing

Plant Accessions	Cd Conc.	Overall foliar yield (g)	Plant dry weight(g)	Root dry weight (g)	Shoot: Root Ratio
TVNu-91	0	44.2	19.45	1.45	12.41
	2.5	34.11	12.62	1.02	11.37
	5	29.15	7.44	0.97	6.67
TVNu-93	0	114.17	37.34	13.82	1.7
	2.5	85.38	29.09	11.77	1.47
	5	59.34	23.79	10.49	1.27
TVNu-94	0	52.07	17.89	0.87	19.56
	2.5	33.93	12.87	0.78	15.5
	5	28.79	8.5	0.58	13.66
TVNu-95	0	28.66	3.67	0.49	6.49
	2.5	17.01	1.92	0.16	11
	5	13.45	1.54	0.15	9.27
TVNu-96	0	86.93	37.5	3.88	8.66
	2.5	45.84	19.07	3.74	4.1
	5	42.75	17.17	3.52	3.88
P-value		0.01	0.004	<0.001	<0.001
LSD(0.05)		21.7	13.2	12.35	16.03

Plant yield

The effects of cadmium on the reproductive capacity of wild vigna have been presented (Table 6a and 6b). There was a significant difference ($P>0.05$) in the number of peduncle per plant as evidenced in TVNu-95 (5ESV) 5.67 when compared to 14.07 in the control of TVNu-95. Generally, there was significant difference in the length of peduncle ($P<0.01$), days to flower bud initiation ($P<0.01$), days to 50% flower bud initiation ($P<0.01$), flower bud size ($P<0.05$), number of flowers per plant ($P<0.05$), flowering duration ($P<0.05$) as well as days to maturity ($P<0.01$). The number of peduncle per plant, length of peduncle, days to flower bud initiation, flower bud size, number of flowers per plant, flowering duration as well as days to maturity were absent for TVNu-93, TVNu-94 and TVNu-96. However, 2.5 ESV and 5 ESV of TVNu-91 also showed absence of the above named parameters. Days to flowering, duration of flowering and the time

required for physiological maturity each increased with the degree of climbing ability of the plants (Hernandez *et al*., 1979). It has been suggested that one of the first traits observed by the ancient seed gatherers of wild legume species could have been earliness in flowering and formation of fruit and seeds (pod), which, along with uniformity of these periods, continue to be used as criteria for cultivar selection (Hernandez *et al*., 1979). Thus, control soil of TVNu-95 (8.05) had a faster maturity rate when compared to TVNu-91 (8.13).

Table 6a.

Effects of cadmium pollution on reproductive capacity of wild *Vigna*

Plant Accessions	Cd Conc.	No. of peduncle/ plant	Length of peduncle (cm)	Days to flower bud initiation (DAS)	Days to 50% flower bud initiation (DAS)	Flower bud size (mm)	No. of Flowers/ plant	Flowering duration (days)	Days to maturity (days)
TVNu-91	0	12.1	1.78	99.04	109.21	1.69	14.05	12.03	8.13
	2.5	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0
TVNu-93	0	0	0	0	0	0	0	0	0
	2.5	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0
TVNu-94	0	0	0	0	0	0	0	0	0
	2.5	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0
TVNu-95	0	14.07	1.92	98.07	107.21	1.57	13.07	12.92	8.05
	2.5	8.31	1.74	101.02	111.07	1.33	9.37	9.08	10.1
	5	5.67	1.31	107.5	112.65	1.17	8.09	7.14	11.72
TVNu-96	0	0	0	0	0	0	0	0	0
	2.5	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0
P-value		0.04	0.007	0.002	0.003	0.014	0.03	0.02	0.001
LSD(0.05)		11.01	1.14	57.95	67.21	0.98	7.63	6.38	6.09

There was a significant difference (P>0.05) in the number of pods per plant (Table 6b) as evidenced in TVNu-95 (5ESV) 5.67 when compared to 14.07 in the control of TVNu-95. Thus, there was a gradual decrease with increasing ecological screening value. Generally, there was significant difference in the seed number per pods (P<0.01), seed weight per pods (P<0.01), the plant yield (P<0.01), and flower bud size (P<0.05). However, the above parameters were absent for TVNu-93, TVNu-94, TVNu-96 as well as 2.5 ESV and 5 ESV of TVNu-91.

Table 6b.

Effects of cadmium pollution on reproductive capacity of wild *Vigna*

Plant Accessions	Cd Conc.	No. of pods/plant	Seed No./ pods	Seed wt./ pods (g)	Plant yield (g/m^2)
TVNu-91	0	1.76	4	0.19	2.68
	2.5	0	0	0	0
	5	0	0	0	0
TVNu-93	0	0	0	0	0
	2.5	0	0	0	0
	5	0	0	0	0
TVNu-94	0	0	0	0	0
	2.5	0	0	0	0
	5	0	0	0	0
TVNu-95	0	2.48	2	0.19	3.55
	2.5	1.23	2.67	0.15	1.93
	5	1.09	2.94	0.13	1.48
TVNu-96	0	0	0	0	0
	2.5	0	0	0	0
	5	0	0	0	0
P-value		0.024	0.032	0.014	0.0072
LSD(0.05)		1.01	1.68	0.11	0.67

Conclusion

Due to the incidence of cadmium pollution, there was reduced plant growth with increase in chlorosis, necrosis, curled as well as folded leaves. Thus, the plant had to senesced older leaves in order to remove most of the cadmium-toxicant. With respect to the reproductive capacity as well as foliar chlorosis and necrosis, TVNu-95 showed a high tolerance to cadmium stress than other accessions, which makes TVNu-95 suitable to cadmium contaminated soil. Although cadmium significantly reduced the yields of wild vigna accessions, wild vigna accessions can serve as a good indicator to quantify the presence of cadmium toxicity in soils. However, further work is recommended in area of mechanisms of action in the inhibition of enzymes as well as interaction of the root nodule with rhizospheric bacterial and if this also had a determining effect on the plant yield.

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A computational insight into cardiac beat-to-beat intervals variability: from normal to pathological

Ștefan-Claudiu Mirescu[1,2,✉], Firuța-Liliana Ardelean[1]
and Octavian Popescu[1]

SUMMARY. Over the past 100 years, electrocardiography (ECG) has provided essential information for diagnosing patients with cardiovascular diseases. Despites the large clinical usefulness of classic paper printed 12-lead ECG, along with the accelerated development of computerized data acquisition, beat-to-beat methods of ECG analysis were developed, providing new information in the field of heart rate variability (HRV). The objectives of the study were to establish HRV parameters behavior in healthy subjects and in diabetes mellitus patients, as well as to evaluate HRV during acute cigarette smoking. The results concluded that HRV parameters describe statistically significant lower values in diabetic patients compared to healthy subjects, signifying a decreased response of the heart conduction system to autonomic stimuli. Concerning smoking, the study concluded that during smoking and ten minutes after, HRV parameters presented lower values than before smoking, as revealed by visual (2D and 3D) and analytical HRV methods. Our study is one of the few in literature that focuses on the acute effects of cigarette smoking, rather than the well-known long term effects of this wide-spread habit.

Keywords: computerized instrumentation, electrocardiography, fast Fourier transforms, MATLAB, nonlinear dynamical system.

Introduction

The electrocardiogram (ECG) is a powerful clinical tool in diagnosing heart conditions, providing information on heart rhythm and perfusion of myocardial territories. Since its discovery in 1901 by the Dutch physiologist Willem Einthoven, ECG has been continuously improved and nowadays it is a fundamental tool for screening and diagnosing patients of all ages, in all medical fields, especially cardiology, internal medicine, anesthesiology and intensive care units (Noble *et al.,* 1990; Aubert *et al.,* 2002; Aubert *et al.,* 2003; Qu and Weiss, 2006).

[1]*Babeş-Bolyai University, Department of Molecular Biology and Biotechnology, Cluj-Napoca.*
[2]*Municipal Hospital Cluj-Napoca, Department of Pathology, Cluj-Napoca.*
✉***Corresponding author: Ștefan-Claudiu Mirescu**, Babeș-Bolyai University, Department of Molecular Biology and Biotechnology,*
E-mail: claudiu.mirescu@gmail.com

Traditionally, an ECG examination is characterized by a real time paper recording of a 12-lead signal, which is further evaluated by the physician (Aubert *et al.,* 2002). Recently, computerized ECG measurements gained ground as a consequence of various benefits:

- Lower cost of the ECG device (although a computer is necessary);
- The possibility of unlimited data storage (including the ECG history of the patient, thus eliminating the patient burden of carrying medical documents);
- The possibility of transmitting ECG data by internet to another healthcare specialist (telemedicine);
- The possibility of further analysis of the ECG signal by software methods (Carel, 1982; Hongo and Goldschlager, 2006).

The last category includes heart rate variability (HRV), which is a temporal variation between sequences of consecutive heart beats, as a consequence of the influences of the autonomic nervous system (ANS) on the heart rate (Lokhandwala and Rodriguez, 1999; Acharya *et al.,* 2006). Thus, the fluctuations of heart rate (as analyzed by HRV) are a noninvasive and accurate method of evaluating the balance between the ANS components: the sympathetic component, which prepares the body for the fight-or-flight reaction and translates in an increase of the heart rate, and the parasympathetic component, which prevails when the organism is in a relaxed state, translating in a decrease of the heart rate (Task Force of the European Society of Cardiology, 1996; Acharya *et al*., 2006; Tsai *et al.,* 2014). Certain studies state that there are certain diseases that alter the function of the peripheral nerves, like diabetes mellitus or some neuropathies, resulting in decreased values of HRV parameters (Task Force of the European Society of Cardiology, 1996; Acharya *et al.,* 2006; Orlov *et al.,* 2012; Gardim *et al*., 2014).

HRV is evaluated by three categories of parameters, summarized in Table 1 (Task Force of the European Society of Cardiology, 1996; Aubert *et al.,* 2002; Carvajal *et al*., 2005; Acharya *et al.,* 2006; Billman, 2013).

In relation to interpretation of HRV parameters, most studies admit that decreased HRV values are associated with a loss of heart conduction system sensitivity to autonomic stimuli or with decreased autonomic activity (Task Force of the European Society of Cardiology, 1996; Taelman *et al.,* 2009; Chevalier and Sinatra, 2011; Orlov *et al.,* 2012; Jaiswal *et al.,* 2013; Gardim *et al*., 2014). Also, many authors consider the LF/HF ratio as a measure of sympathetic to parasympathetic balance (von Borell *et al.,* 2007; Taelman *et al.,* 2009), an increase in this ratio being associated with a shift towards sympathetic dominance; however, other authors demonstrate that this hypothesis is false, and state that LF/HF ratio cannot accurately quantify the balance between autonomic nervous system components (Billman, 2013).

The literature is controversial regarding the normal values of HRV parameters. By that reason, most of HRV studies conduct measurements on healthy subjects, in order to establish their own normal values (Nunan *et al.,* 2010).

In addition to the above-mentioned parameters, HRV can be promptly analyzed by visual methods, such as the tachogram, the histogram and the Poincaré plot of beat-to-beat intervals (Task Force of the European Society of Cardiology, 1996; Mirescu and Harden, 2012a; Makivic *et al.,* 2013).

Table 1.

HRV parameters

Parameter	**Unit**	**Significance**
Time-domain parameters		
Mean RR	ms	Mean of consecutive beats intervals
STD RR	ms	Standard deviation of consecutive beats intervals
Mean HR	beats/min	Heart rate mean
SD HR	beats/min	Standard deviation of heart rate mean
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
Triangular index	-	The integral of the density distribution divided by the maximum of the density distribution
TINN	ms	The baseline with of the intervals histogram
Frequency-domain parameters (obtained by fast Fourier transforms)		
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	-	

Materials and methods

Essentially, any method that records a heartbeat-derived signal is appropriate for HRV parameters evaluation. There are two fundamental methods used for HRV: electrocardiography and photopletismography (PPG). Both portray cardiac activity indirectly: the ECG representing the cyclic electrical signal evoked by the cardiac cells, and the PPG expressing peripheral blood volume variation, which is dependent of the cardiac output (Figure 1).

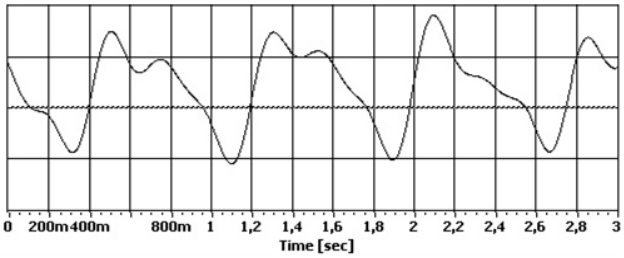

Figure 1. Example of a PPG signal

Each of these signals is characterized by cyclic waves that can be identified by software methods, and further used to calculate beat-to-beat intervals that are used for HRV studies. The cyclic waves correspond with systole (the period where the heart contracts and expels the arterial blood to the arteries) and diastole (the period where the heart is relaxed and receives venous blood from the veins).

A PPG-based recording device is an optical system composed of an infrared light emitting diode (LED) and a photodiode, which are placed on the sides of a finger (or earlobe). Due to the fact that oxygenated blood absorbs infrared light and blood flow is pulsatile as a result of the intermittent cardiac pump function, the result is a pulsatile wave (Figure 2) (Mirescu, 2015).

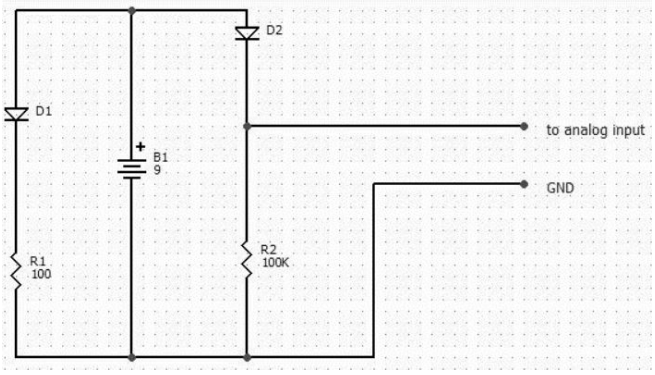

Figure 2. Schematics of a simple PPG device designed for HRV purposes (Mirescu, 2015 – reproduced with permission)

Albeit PPG is a simple and convenient method for HRV purposes, and its results are equivalent to the ones obtained from ECG traces, there are a series of disadvantages in using this procedure, the most important being an increased sensibility to movement artifacts (Mirescu *et al*., 2016).

Although there is a functional equivalence between the two methods (Mirescu and Harden, 2012b), ECG proved to be the much more reliable in recording and tracing heart beat signal for HRV. Figure 3 shows the block diagram of a classical digital ECG device.

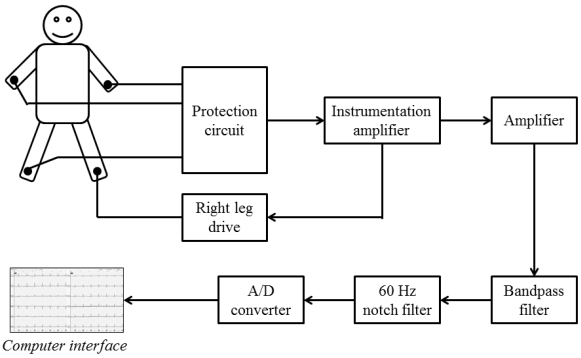

Figure 3. Block diagram of a classical digital ECG device

At the base of ECG recordings is the so called „triangle of Einthoven" (Figure 4), describing in a graphical manner the main ECG leads, which basically represent the potential difference between three key points located on the body surface: the right arm, the left arm and the left leg.

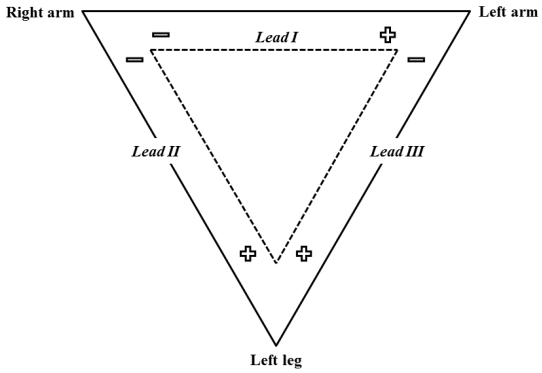

Figure 4. The triangle of Einthoven

Experimental design

The aims of the study were (1) to establish normal values of HRV variability parameters on a Romanian sample population of healthy individuals; (2) to examine HRV parameters values and visual descriptors of diabetic patients; (3) to evaluate the influence of acute cigarette smoking on HRV parameters.

In order to establish normal values for HRV parameters, 50 healthy subjects were taken into consideration (age 22-57 years old; 23 females). For each subject, we recorded a 10 minutes ECG trace using a Neurosoft Poly-Spectrum® device, in a 6-lead configuration (standard ECG leads DI, DII, DIII, aVR, aVL, aVF). The device software automatically identified the R waves of the ECG trace and calculated the intervals between consecutive beats, in milliseconds (RR intervals), as shown in Figure 5.

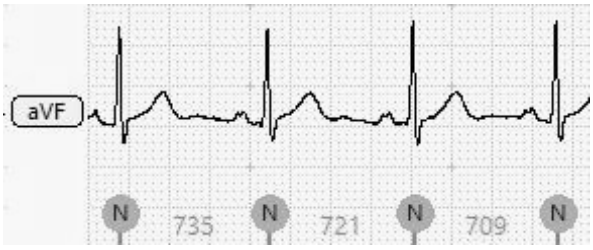

Figure 5. Three consecutive RR intervals as measured by Poly-Spectrum® software, in aVF lead (N symbolizes that the corresponding beat is normal, not ectopic)

The subjects were asked not to smoke or drink caffeinated beverages or other psychostimulants for four hours prior to the recordings. They were also asked not to speak or move during the recording, in order to avoid artifacts.

For evaluating the effects of diabetes mellitus on HRV parameters, we selected 25 diabetic patients admitted to the Diabetes, Nutrition and Metabolic Diseases Center, Emergency County Hospital Cluj, (22-70 years old, 12 females). Each patient was subjected to a 10 minutes ECG recording, using the same device and experimental setting as described before. Medical data was anonymously collected from the patients' medical records. In collecting data from the patients, ethical standard was considered, and patients were not required to discontinue any prescribed treatment and care was taken for the patients not to suffer any discomfort during the recordings.

For evaluating the acute effects of cigarette smoking in healthy subjects, a number of 25 volunteers were recruited (age 20-22 years, 15 females). The recordings were made according to the following protocol:

- The subjects were asked not to smoke or consume psychostimulants at least 4 hours prior to the experiment;
- 6-lead ECG was recorded in a resting position for 5 minutes (baseline recording);
- 5 minutes – ECG recording while smoking a cigarette;
- 10 minutes recording after smoking. This interval was divided into two 5 minutes intervals, and for each HRV parameters were calculated.

The ECG measurements were performed using the same device as described before.

In all three experimental settings, HRV parameters were calculated using *Kubios HRV 2.2* software. Statistical analysis was performed using *Microsoft Office Excel 2010*, and 3D plotting was accomplished using the *scatter3* function of the MATLAB environment.

Data collected from diabetic patients were compared to data obtained from the healthy subjects.

Parameters obtained from the ECG recordings during (and after) cigarette smoking were compared to the ones obtained from the baseline recordings of the same subjects.

Results and discussion

A. Normal values of HRV parameters

HRV parameters values obtained from healthy subjects and considered normal in our studies are illustrated in Table 2.

Table 2.

Normal values of HRV parameters

Parameter	**Unit**	**Value**		
Time-domain parameters				
Mean RR	ms	745.024	±	100.426
STD RR	ms	51.69673	±	18.80544
Mean HR	beats/min	82.32475	±	9.924824
SD HR	beats/min	5.66064	±	34.49615
NN50	#	70.58333	±	86.61208
pNN50	%	14.04793	±	16.26272
Triangular index	-	12.72905	±	4.72086
TINN	ms	246.875	±	89.3929
Frequency-domain parameters				
LF/HF	-	3.191386	±	2.383248
Nonlinear dynamics parameters				
SD1	ms	27.77991	±	15.11479
SD2	ms	68.34148	±	24.21562
ApEn	-	1.16733	±	0.146363
SampEn	-	1.392332	±	0.264679
Correlation dimension	-	2.63317	±	1.3481
DFA α1	-	1.213387	±	0.279406
DFA α2	-	0.859523	±	0.160867

The data obtained in our study are consistent with the literature data gained from other populations of matching sex and age (Aubert *et al.,* 2003; Nunan *et al.,* 2010). Standard deviation of some parameters (NN50, pNN50) was higher than the average of the parameter value, signifying a broad range of values, hence an important variability among healthy subjects.

B. HRV parameters in diabetic patients

We compared all three categories of HRV parameters (time-domain, frequency-domain and nonlinear dynamics parameters) for healthy subjects and diabetic patients. T test was used to compare the parameters between the two groups (p values lower than 0.05 were considered statistically significant). The results are presented in Table 3.

Table 3.

HRV parameters in diabetic patients and the p-value of the t test compared to healthy subjects

Parameter	Unit	Value	p-value
Time-domain parameters			
Mean RR	ms	762.80 ± 100.77	0.57
STD RR	ms	25.96 ± 14.58	< 0.001
Mean HR	beats/min	79.79 ± 13.3	0.45
SD HR	beats/min	2.56 ± 1.12	< 0.001
NN50	#	19.76 ± 37.69	0.04
pNN50	%	2.47 ± 4.85	0.01
Triangular index	-	7.01 ± 3.73	< 0.001
TINN	ms	145 ± 88.6	< 0.001
Frequency-domain parameters			
LF/HF	-	3.03 ± 1.96	0.8
Nonlinear dynamics parameters			
SD1	ms	11.56 ± 9.56	0.004
SD2	ms	34.3 ± 19.25	< 0.001
ApEn	-	1.23 ± 0.16	0.16
SampEn	-	1.13 ± 0.66	0.72
Correlation dimension	-	0.69 ± 0.6	< 0.001
DFA α1	-	1.19 ± 0.26	0.8
DFA α2	-	1.06 ± 0.23	< 0.001

According to our results, all HRV parameters of diabetic patients expressed decreased values, compared to healthy subjects. These results are consistent with other studies in the literature performed on diabetic subjects (Task Force of the European Society of Cardiology, 1996; Orlov *et al.,* 2012; Gardim *et al*., 2014). SDNN, a marker of overall HRV, was 25.96 ms in diabetic patients, compared to 51.69 in healthy subjects ($p < 0.001$), which is in accordance with other studies (Jaiswal *et al.,* 2013).

Another finding of our study was decreased values of NN50 and pNN50 in diabetic subjects, compared to the control group ($p = 0.04$ and $p = 0.01$, respectively). Many of the diabetic patients had the value 0 at these parameters, a situation not described so far in the literature, implying that a decreased value of these parameters could be an independent descriptor of HRV in DM patients. However, further research needs to be conducted in order to strengthen this conclusion.

A powerful visual descriptor of HRV is the tachogram of the RR intervals, which represents the plotting of the RR intervals against time. In a typical 10 minutes recording of a healthy person, numerous inflections and deflections can be seen, due to the antagonistic and continuous influence of the two components of the autonomous nervous system: the sympathetic nervous system creates a downwards inflection (signifying an increase in heart rate) and the parasympathetic nervous system creates an upwards inflection (signifying a decrease in heart rate). Another cause of these irregular changes in the heart rate is the so-called respiratory sinus arrhythmia, a physiological phenomenon which consists of heart rate increasing during inhaling and decreasing during exhaling (von Borell *et al*., 2007; Tininenko *et al.,* 2011; Mirescu and Harden, 2012a,b; Mirescu, 2015). As seen in Figure 6, the tachogram of diabetic patients describes a visible decrease in variability compared to a healthy subject, the tachogram being almost flat. As it is also shown in other studies (Orlov *et al.,* 2012; Jaiswal *et al.,* 2013; Gardim *et al*., 2014), the almost flat appearance of the RR intervals tachogram is an independent marker of overall decreased HRV in diabetic patients and is correlated with the severity of the disease.

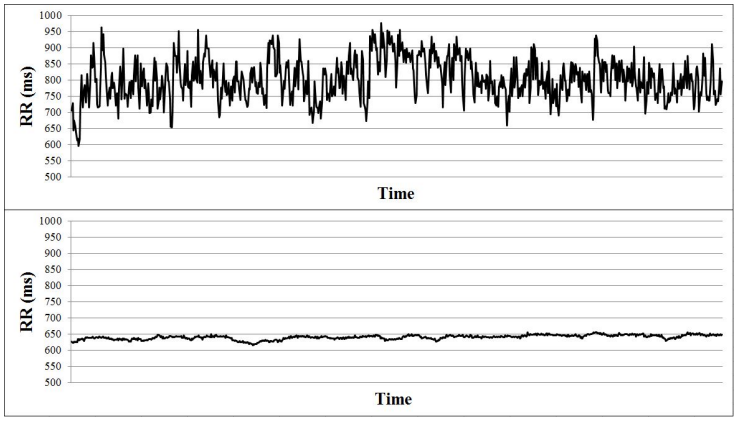

Figure 6. Up – Tachogram of RR intervals in a healthy subject; down – tachogram of a diabetic patient, which displays a decrease in the variability of the intervals; both recordings were made on the standard 10 minutes interval.

Another valuable visual descriptor of HRV is the Poincaré diagram (scattergram), which is a scatter plot of every RR interval against the previous one. As many authors state, the Poincaré diagram is a graphical projection of RR_{i+1} as a function of RR_i (Task Force of the European Society of Cardiology, 1996; Medeiros, 2010; Makivic, 2013). It reflects the graphical correlation between consecutive RR intervals and has become increasingly more popular due to its simple visual interpretation and its proven clinical applicability (Brennan et al., 2001). The Poincaré plot from a healthy subject expresses a large surface of the scattered points, without many isolated points from

the main cloud (which may represent ectopic beats or artifacts). As seen in Figure 7, the points of the Poincaré plot of a diabetic patient are significantly aggregated into a much smaller cloud than the ones of a healthy control subject. These visual findings are in conformity with the mathematical descriptors of the Poincaré plot (SD1 and SD2) for diabetic patients.

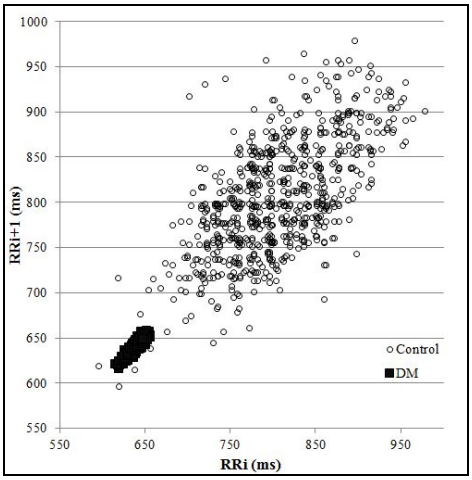

Figure 7. A Poincaré plot of a healthy subject (Control) compared to a diabetic patient (DM)

In an attempt to enhance the information provided by this method, we tested the value of 3D Poincaré plots (RRi+2 and RRi+1 as a function of RRi). The 3D plotting was performed in MATLAB environment, using the *scatter3* function, for all diabetic subjects. As shown in Figure 8, apart from a better visual representation, the 3D scattergram does not provide further information for HRV interpretation purposes.

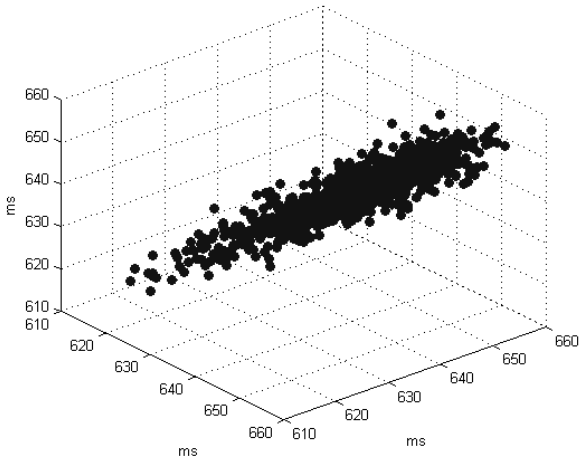

Figure 8. Up – A 3D Poincaré plot of a diabetic patient

As previous studies state, the decrease of HRV in diabetic patients is the result of nerve fiber alteration.

Our study concludes that HRV analysis (by visual and computational methods) is a valuable instrument in autonomic (both sympathetic and parasympathetic) response in diabetic patients, especially in patients which present other autonomic dysfunctions (e.g. altered bowel movement, decreased sweat response or skin ulcers). Most of the cited studies evaluate HRV response in diabetic children and young patients (with type I diabetes mellitus), but the results are consistent with the ones obtained in our study.

C. Influence of acute cigarette smoking on HRV parameters

Although the effects of chronic consumption of tobacco are well known, few studies explored the immediate consequences of cigarette smoking on the functioning of the cardiovascular system. We characterized these effects by the visual and computational methods of HRV.

Time-domain (Mean RR, STD RR, Mean HR, STD HR, pNN50), frequency-domain (LF/HF) and nonlinear dynamics (SD1, SD2) parameters were calculated for each measure interval described in the protocol (5 minutes before smoking, five minutes during smoking and two consecutive 5 minutes interval after smoking). T test was used to compare amongst recordings (p values lower than 0.05 were considered statistically significant).

As expected, the average of RR intervals increased during smoking ($p < 0.001$), decreasing in the next 10 minutes ($p = 0.03$, $p = 0.05$ respectively) – Figure 9A.

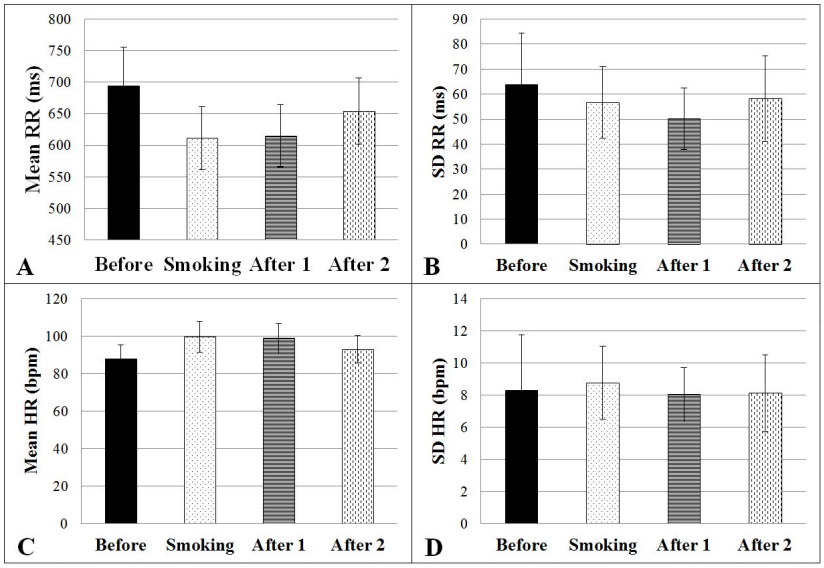

Figure 9. Time-domain parameters (A – Mean of RR intervals; B – Standard deviation of RR intervals; C – Mean heart rate; D – Standard deviation of momentarily heart rates).

The standard deviation of RR intervals (a global measure of HRV) decreased during smoking and continued to decrease 5 minutes after smoking, following an upward trend in the last 5 minutes of the recording, but none of these differences were statistically significant compared to the control recording (Figure 9B). Average heart rate and the standard deviation of the momentarily heart rates followed the same pattern (Figure 9C, D). pNN50, another global descriptor of HRV, had the same evolution as the standard deviation of RR intervals, but, as stated in other studies study, the values were dispersed, and the standard deviation was very high, making this parameter inaccurate for interpretation (Figure 10A).

Figure 10B shows the variation of LF/HF (ratio of the frequency-domain parameters) during the four recording periods. Although there were no significant differences, LF/HF increased during smoking and in the 5 minutes period after smoking, being in accordance with the alteration of other sympathetic prevalence parameters (the decrease of STD RR and pNN50).

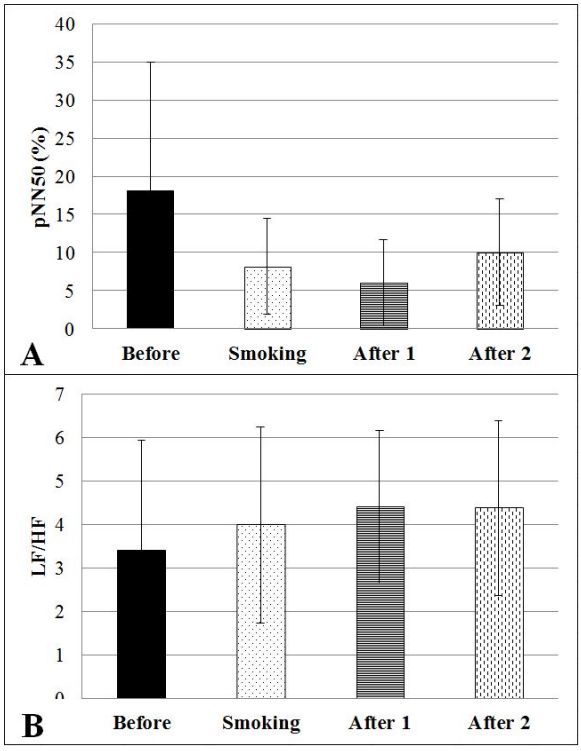

Figure 10. A– The independent descriptor of HRV, **pNN50** decrease during smoking. The decrease continued in the first interval after smoking, but the parameter showed a tendency to return to baseline in the second interval; B – LF/HF ratio increased during smoking, similar to the sympathetic activity increase.

The Poincaré plot derived from the four recording periods is the most valuable visual instrument in evaluating HRV alterations. During smoking, the increasing in sympathetic tonus is represented by the clustering of the points in smaller cloud than in the control cloud (Figure 11), which is also sustained by the mathematical parameters of the Poincaré plot (SD1 and SD2 are lower during the smoking period, $p > 0.05$). During the following two periods, the dispersion of the points (thus, the area of the scattergram) began to increase, but, even after 10 minutes from the cessation of smoking, it had not reached the initial appearance.

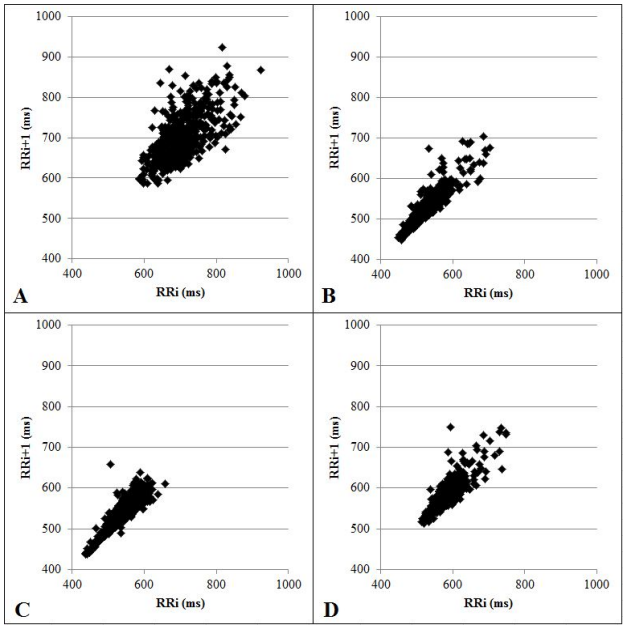

Figure 11. Poincaré plot of a subject derived from HRV recordings during the four periods (A – Before smoking, the Poincaré plot has a large dispersion of points, which shows a heart rate variability increase; B – During smoking the dispersion of points decreased and they move to the left-down, suggestive for a increase sympathetic activity ; C – 5 minutes after smoking the effect is similarly to B period; D – 10 minutes after smoking the points begin to deviate from the origin, which means an increase in variability and a decrease in the sympathetic activity).

Our study regarding the acute effects of smoking on heart rate and HRV is one of the few in literature and one of the most complex, considering that it considered many HRV parameters and visual indicators of HRV.

Other authors obtained similar results in a smaller scale study, concluding that smoking has a global decreasing effect on HRV parameters and that after some time they return to the values before smoking (Gondim *et al.*, 2015). On the

contrary, a larger scale study concluded that smoking does not have any influence on HRV parameters in male adolescents, nor does passive smoking, thus the habit does not have any effects on autonomic modulation changes (Karakaya, 2007). Also, the above-mentioned study concluded that there were no differences in HRV parameters between smokers and non-smokers. These differences could come from not taking into consideration all smoking habits of the subjects (number of cigarettes smoked, concentration of nicotine, and personal history of smoking in years).

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=== REVIEW ===

Endemic species of terrestrial isopods (Isopoda, Crustacea) in the Romanian fauna

Nicolae Tomescu[1,✉]

SUMMARY. In the Romanian fauna 32 endemic species of terrestrial isopods were described; most of them are epigenous, few are troglobiont, troglophilous and edaphic species. **Troglobiont species:** *Biharoniscus racovitzai*, *Biharoniscus fericeus*, *Haplophthalmus tismaniscus*, *Haplophtalmus caecus*, *Trachelipus troglobius*. **Troglophilous species:** *Trachelipus trilobatus*, *Hyloniscus flamuloides*. **Edaphic species:** *Trichoniscoides danubianus*, *Haplophtalmus napocensis*, *Haplophtalmus banaticus*, *Haplophtalmus medius*, *Haplophtalmus ionescui*, *Thaumatoniscellus orghidani*. **Epigenous species:** *Ligidium intermedium*, *Hyloniscus dacicus*, *Hyloniscus siculus*, *Hyloniscus motasi*, *Trichoniscus carpaticus*, *Haplophthalmus orientalis*, *Buddelundiella serbani*, *Trichorhina dobrogica*, *Platyarthrus dobrogicus*, *Cylisticus transsilvanicus*, *Cylisticus brachiurus*, *Porcellium transsylvanicum*, *Trachelipus ater*, *Trachelipus vareae*, *Trachelipus bujori*, *Trachelipus pleonglandulatus*, *Trachelipus spinulatus*, *Orthometopon romanicus*, *Armadillidium banaticum*.

Keywords: endemic species, terrestrial isopods.

Introduction

In Romanian fauna 32 endemic species of terrestrial isopods were described. The majority of them has been described by Radu (1949, 1950, 1951, 1955, 1956, 1959, 1973, 1976, 1977, 1983) and Tăbăcaru (1962, 1970, 1971, 1972, 1973, 1974, 1989, 1996). Radu had generally studied the epigenous isopod species, while Tăbăcaru the troglobiont (cave-dwelling) species.

All the endemic isopod species are described separately by Radu (1983, 1985) in the two sections of the Romanian Fauna: volume IV section 13/1983 and volume IV section 14/1985. Schmalfuss (2003) mentions the endemic species in the World Catalogue of terrestrial isopods (Isopoda Oniscoidea), except three

[1]*Babeş-Bolyai University, Faculty of Biology and Geology, Department of Taxonomy and Ecology, 5-7 Clinicilor Str., 400006, Cluj-Napoca, Romania.*
✉***Corresponding author: Nicolae Tomescu**, Babeş-Bolyai University, Cluj-Napoca, Romania, E-mail: nicolaetomescu36@gmail.com*

species, described by Radu: *Trachelipus vareae* (Radu 1949), *Trachelipus bujori* (Radu 1950) and *Trachelipus pleonglandulatus* (Radu 1950), which Schmidt (1997) synonymised with other species from the genus *Trachelipus*.

In our publication (Tomescu *et al*., 2015) we presented a comparative description of the three species described by Radu and the species they were synonymised with. The results of our research are based on the study of a vast biological sample (tens of males and females from each species), collected in the period 1990-2013, from habitats of several geographic regions, including those mentioned by Radu. We prepared microscopic slides with male organs of taxonomic value, and used them to re-describe the species and to draw some well-documented conclusions. We found that the three species described by Radu (1949, 1950) are valid species and the synonymising by Schmidt (1997) does not correspond to reality. Probably Schmidt did not have biological material from Romania. We include in this paper comparative figures of the three species that were synonymised (Tomescu *et al*., 2015).

The diversity of geographical units and habitats in Romania favoured the isolation of several terrestrial isopod populations and the evolutionary process of forming new species. The biological traits of the terrestrial isopods also contributed to the isolation of their populations. The majority of the species are stenobiont and have a low mobility, that is particularly low in troglobiont and edaphic species, but also in epigenous species.

The majority of endemic species lives in montane areas and their populations have a limited distribution in their area. There are many caves in the montane areas, inhabited also by isopods among other organisms. Five troglobiont and two troglophilous isopod species have been described.

The species of endemic isopods:
Diplocheta: family Ligiidae
Ligidium intermedium Radu 1950
Ecology: epigenous species, lives in the litter layer of forests, in humid microhabitats.
Distribution: Northern Romania, prevalent in montane areas.

Synocheta: family Trichoniscidae
Hyloniscus dacicus Tăbăcaru 1972
Ecology: epigenous species, lives in the litter layer of forests and under rocks, in humid microhabitats.
Distribution: Făgăraş Mountains, Prahova Valley

Hyloniscus flamuloides Tăbăcaru 1972
Ecology: troglophilous species, lives in caves and in the litter layer of forests around caves, in humid microhabitats.
Distribution: Retezat Mountains, Şureanului Mountains, Cozia Mountains, Căpăţânii Mountains

Hyloniscus siculus Mehely 1929
Ecology: epigenous species, lives in the litter layer of forests in montane areas, in humid microhabitats.
Distribution: Eastern and Southern Carpathians, Apuseni Mountains

Hyloniscus (=Ropaloniscus) motasi Radu 1976
Ecology: epigenous species, lives in the litter layer of forests in montane areas, in humid microhabitats.
Distribution: Gutâi Mountains

Trichoniscus carpaticus Tăbăcaru 1974
Ecology: epigenous species, lives in the litter layer of forests in montane areas, in humid microhabitats.
Distribution: Eastern and Southern Carpathians.

Biharoniscus racovitzai Tăbăcaru 1962
Ecology: troglobiont species, lives in caves.
Distribution: caves of the Apuseni Mountains.

Biharoniscus fericeus Tăbăcaru 1973
Ecology: troglobiont species, lives in caves.
Distribution: the cave in Ferice, Bihor Mountains.

Trichoniscoides danubianus Radu 1973
Ecology: edaphic species, lives in the hummus layer in humid forests.
Distribution: forests located on the left side of the Danube, Plavişeviţa and Berzasca, Caraş-Severin County.

Haplophtalmus orientalis Radu G., Radu V., Cădariu 1955
Ecology: edaphic species, lives in the humid litter layer of deciduous forests.
Distribution: Dobrogea.

Haplophtalmus napocensis Radu 1977
Ecology: edaphic species, lives in the hummus layer of deciduous forests.
Distribution: the forests around Cluj-Napoca city.

Haplophtalmus banaticus Radu 1977
Ecology: edaphic species, lives in the hummus layer of deciduous forests.
Distribution: Porţile de Fier area, Orşova, Moldova Nouă.
Haplophtalmus medius Radu G., Radu V., Cădariu 1956
Ecology: edaphic species, lives in the hummus layer of deciduous forests.
Distribution: Perşani Mountains.

Haplophtalmus tismanicus Tăbăcaru 1970
Ecology: troglobiont species, lives in caves.
Distribution: the cave at Tismana Monastery, Gorj County.

Haplophtalmus caecus Radu G., Radu V., Cădariu 1955
Ecology: troglobiont species, lives in caves.
Distribution: in the cave on Biborţ Valley, village Presaca Ampoiului, Alba County.

Haplophtalmus ionescui Radu 1983
Ecology: edaphic species, lives in the hummus layer of deciduous forests.
Distribution: forests in the Porţile de Fier area.

Thaumatoniscellus orghidani Tăbăcaru 1973
Ecology: edaphic species, lives in the hummus layer of deciduous forests.
Distribution: forests around the cave from Topolniţa, Mehedinţi County.

Buddelundiella serbani Tăbăcaru 1971
Ecology: edaphic species, lives in the very humid litter layer of deciduous forests.
Distribution: forests in Tismana, Gorj County.

Crinocheta: family Platyarthridae
Trichorina dobrogica Radu 1959
Ecology: epigenous species, lives under rocks, fallen trees, on arid soils.
Distribution: Dobrogea, Black Sea littoral.

Platyarthrus dobrogicus Radu 1951
Ecology: epigenous species, lives in the litter layer of deciduous forests.
Distribution: deciduous forests in Dobrogea.

Crinocheta: family Cylisticidae
Cylisticus transsilvanicus Verhoeff 1908
Ecology: epigenous species, lives in forests on areas with plant detritus at the basis of rocks.
Distribution: Muntele Rece, Apuseni Mountains.

Cylisticus brachiurus Radu 1951
Ecology: epigenous species, lives in the litter layer of forests.
Distribution: Ciucului Mountains, Eastern Carpathians.
Crinocheta: family Trachelipoidae
Porcellium transsylvanicum Tomescu, Teodor, Ferenţi 2012
Ecology: epigenous species, lives in the litter layer of forests and on herbaceous forest plants.
Distribution: Bârgăului Mountains.

Trachelipus ater Budde-Lund 1896
Ecology: epigenous species, lives in the litter layer of beech and spruce forests, under fallen trees.
Distribution: Southern Făgăraş Mountains, Lotrului Mountains, Căpăţânii Mountains.

Trachelipus trilobatus Stein 1859
Ecology: troglophilous species, lives in humid and old oak forests, at lower temperatures, in the litter layer, under rocks, around cave entrances and on rock walls.
Distribution: forests around Băile Herculane, Ponorul Pecinişcăi, Eastern Mehedinţi Mountains.

Trachelipus vareae Radu 1949
Ecology: epigenous species, lives in the litter layer of deciduous forests.
Distribution: Lunca Cernii, Cioclovina, Turnu Roşu, Călimăneşti, Trascău Mountains, Muntele Mare, Metaliferi Mountains (Tomescu *et al*., 2015).
Note: Schmidt (1997) synonimized *Trachelipus vareae* Radu 1949 with *Trachelipus ater* Budde-Lund 1896. Tomescu *et al*. (2015) studied tens of male and female individuals from both species, collected in the period 1990-2013 from different localities in Romania, and concluded that there is a clear morphological difference between the species. The scientifically argued conclusion states that *Trachelipus vareae* Radu 1949 is a valid species and cannot be synonimized with *Trachelipus ater* Budde-Lund 1896. We present several figures representing morphological characters of the two species, published by Tomescu *et al*. (2015) (Figs. 1- 4). We mention that their spatial distribution is also different.

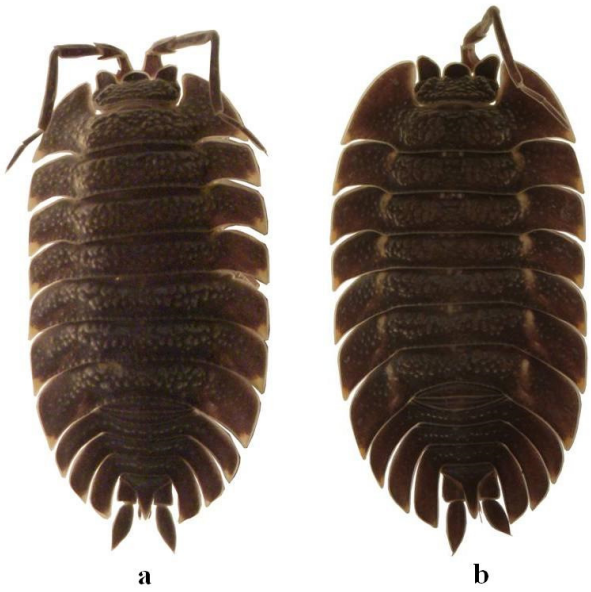

Figure 1. Yellow spots on the coxal plates base of males: **a.** *Trachelipus ater*, ♂ 14 x 7 mm – Vâlsan river Gorges (Argeş county), **b.** *Trachelipus vareae*, ♂ 17 x 9 mm – Feneş Valley (Alba county).

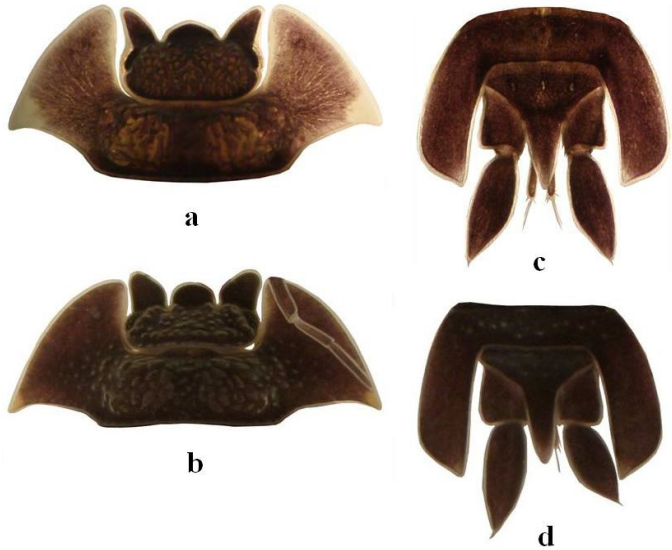

Figure 2. Comparative cephalic lobes: **a.** *Trachelipus ater*, ♂ 12 x 6 mm – Bistriţa river Gorges (Vâlcea county), **b.** *Trachelipus vareae*, ♂ 17 x 9 mm – Feneş Valley (Alba county); **pleotelson**: **c.** *T. ater*, ♂ 14 x 7 mm – Vâlsan river Gorges (Argeş county); **d.** *T. vareae*, ♂ 17 x 9 mm – Feneş Valley.

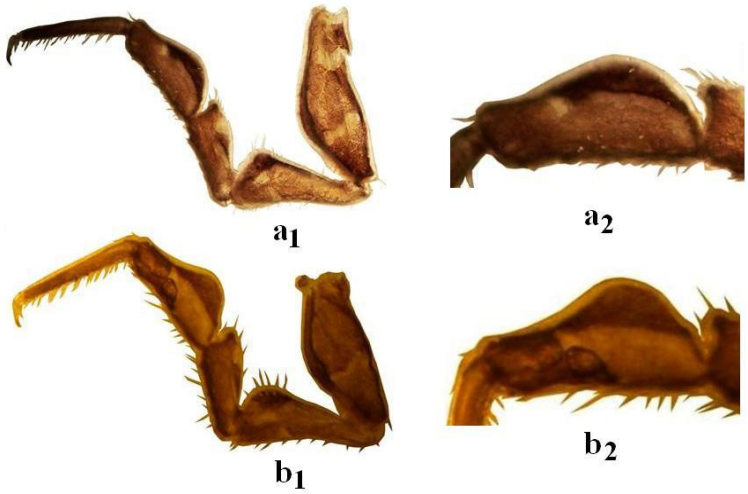

Figure 3. Comparative crest of carpus pereiopod 7 of males: **a_1-a_2.** *Trachelipus ater*, ♂ 14 x 7 mm – Vâlsan river Gorges (Argeş county), **b_1-b_2.** *Trachelipus vareae*, ♂ 17 x 9 mm – Feneş Valley (Alba county).

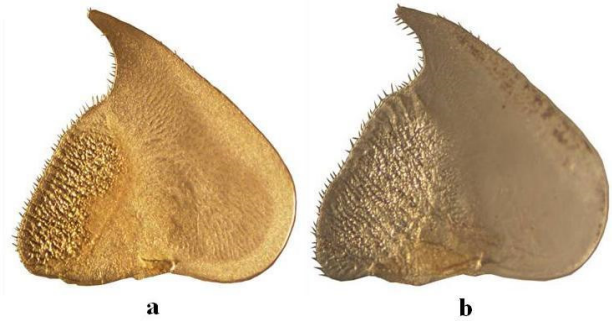

Figure 4. Comparative of pleopods 1 of males, the exopod of pleopods 1 of males: **a.** *Trachelipus ater*, ♂ 14.5 x 8 mm – Vâlsan river Gorges; **b.** *Trachelipus vareae*, ♂ 17 x 9 mm – Feneş Valley.

Trachelipus bujori Radu 1950

Ecology: epigenous species, lives under fallen trees, under bark and in the litter layer of beech and oak forests, at altitudes of 120-360m.

Distribution: Poiana Ruscă Mountains, Parâng Mountains, Almăjului Mountains, Aninei Mountains, Lipovei hills.

Note: Schmidt (1997) synonimized the species *Trachelipus bujori* Radu 1950 with *Trachelipus ratzeburgi* Brandt 1833. Like in the case of *Trachelipus vareae* Radu 1949, Tomescu *et al*. (2015) found morphological differences between *Trachelipus bujori* Radu 1950 and *Trachelipus ratzeburgi* Brandt 1833, proving that *T. bujori* is a valid species. Further, we present several figures with the morphology of the two species (Figs. 5-7).

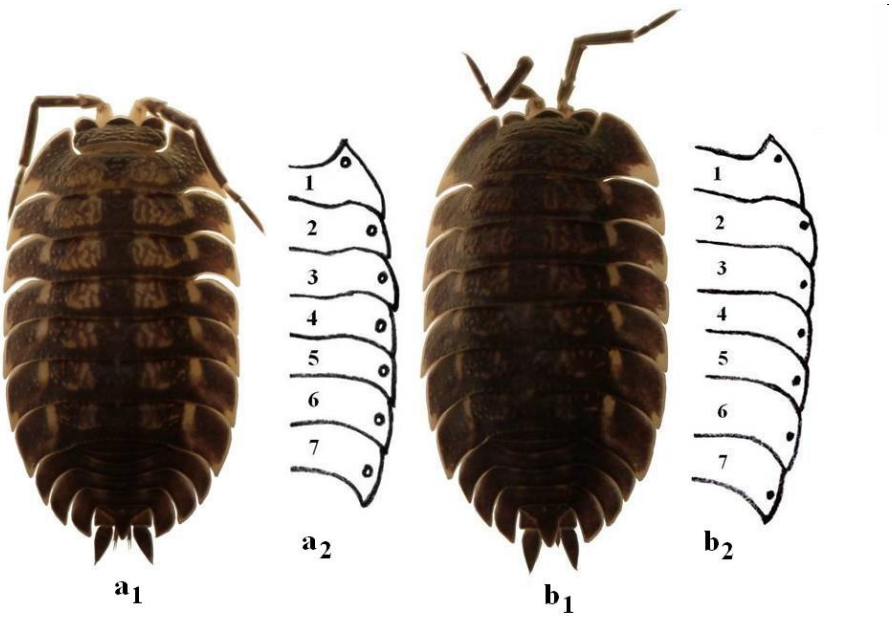

Figure 5. Comparative dorsal view and glandular pore fields: **a1-a2.** *Trachelipus ratzeburgi*, ♂ 10 x 5 mm, – Ersig (Caraş-Severin county), **b1-b2.** *Trachelipus bujori*, ♂ 10.8 x 5.5 mm – Herneacova (Timiş county).

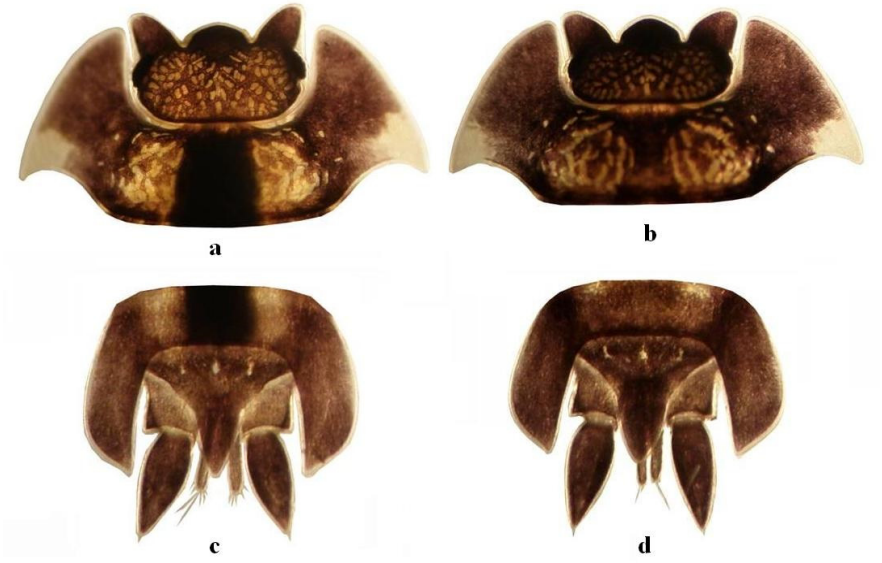

Figure 6. Comparative cephalic lobes: a. *Trachelipus ratzeburgi*, ♂ 10 x 5 mm, – Ersig, **b.** *Trachelipus bujori*, ♂ 10.8 x 5.5 mm – Herneacova; **telson: c.** *Trachelipus ratzeburgi*, ♂ 10 x 5 mm, **d.** *Trachelipus bujori*, ♂ 10.8 x 5.5 mm, those males.

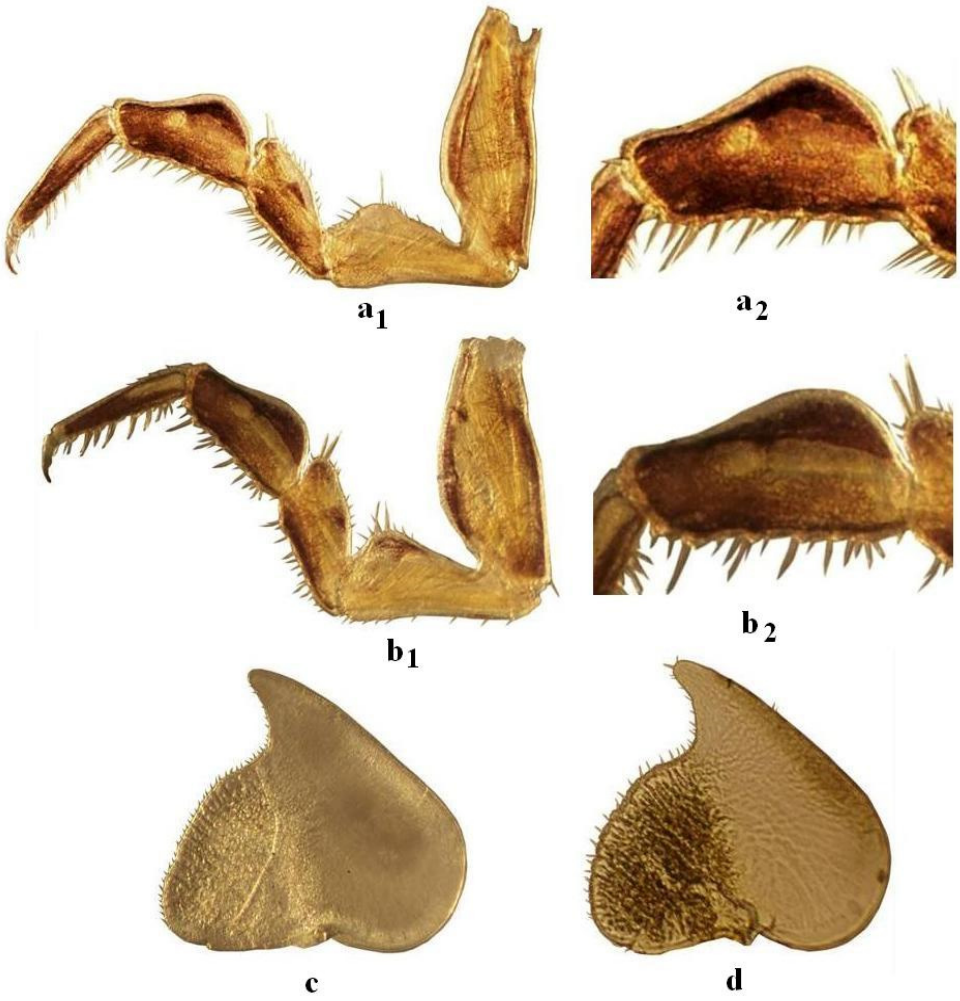

Figure 7. Comparative pereiopods 7 of the males: a1-a2. *Trachelipus ratzeburgi*, ♂ 10 x 5 mm, – Ersig, **b1-b2.** *Trachelipus bujori*, ♂ 10.5 x 5 mm – Meri; **Comparative exopod plopods 1 of males: c.** *Trachelipus ratzeburgi*, ♂ 10.5 x 5 mm, –Gârliştei Gorges, **d.** *Trachelipus bujori*, ♂ 10.5 x 5 mm – Meri.

Trachelipus pleonglandulatus Radu 1950
Ecology: epigenous species, lives in the litter layer of deciduous forests, in carstic regions, on rocky surfaces, but also on open areas near forests.

Distribution: Poiana Ruscă Mountains, Danube Gorges, Baia de Aramă, Parâng Mountains, Mehedinţi Mountains.
Note: Schmidt (1997) synonimized the species *Trachelipus pleonglandulatus* Radu 1950 with *Trachelipus ratkü* Brandt 1833. Tomescu *et al*. (2015) found specific morphological differences, which we present further, published by the authors (Figs. 8-9).

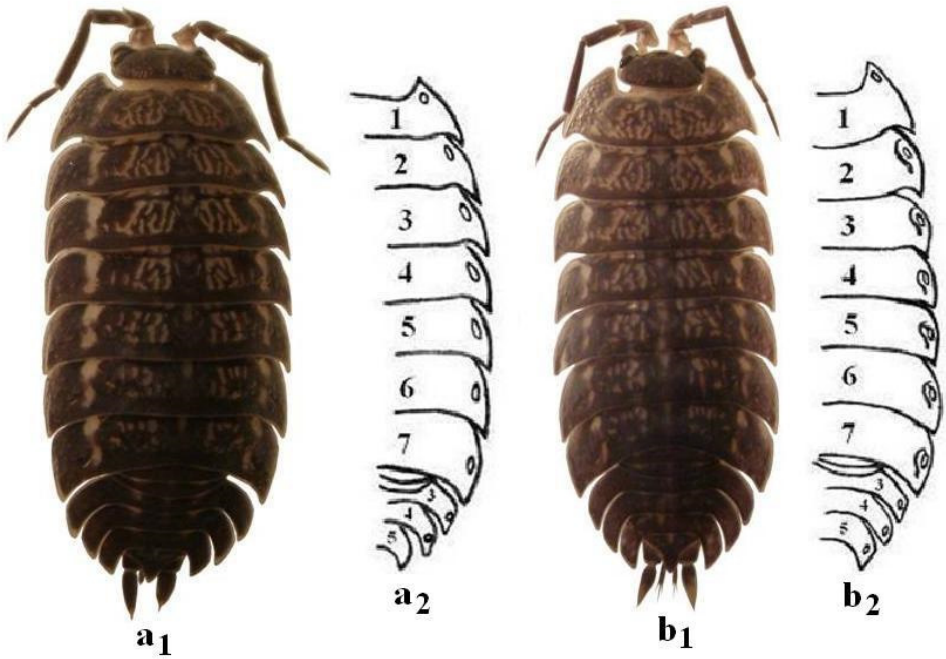

Figure 8. Comparative dorsal view and glandular pore fields: **a1, a2.** *Trachelipus rathkii* (Brandt, 1833), ♂ 11.5 x 5.5 mm – Milova (Arad county), **b1, b2.** *Trachelipus pleonglandulatus* Radu, 1950, ♂ 15 x 6 mm – Pecinişcăi Gorges, Cernei Mounteins.

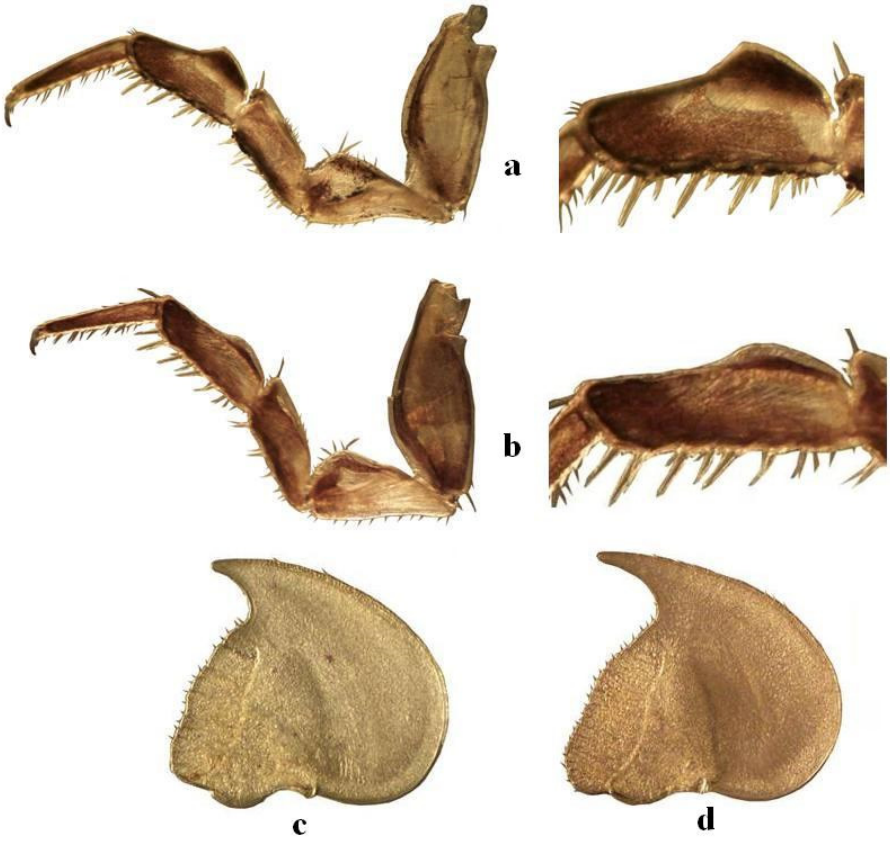

Figure 9. Comparative appendages of males by *Trachelipus rathkii* (Brandt, 1833) and *Trachelipus pleonglandulatus* Radu, 1950: **pereiopod 7**, **a.** *T. rathkii*, ♂ 11.5 x 5.5 mm, **b.** *T. pleonglandulatus*, ♂ 15 x 6 mm; **pleopod-exopodites 1**, **c.** *T. rathkii*, ♂ 11.5 x 5.5 mm, **d.** *T. pleonglandulatus*, ♂ 15 x 6 mm.

Trachelipus spinulatus Radu 1959
Ecology: epigenous species, lives in the litter layer of deciduous forests.
Distribution: forests in the vicinity of the Câmpeni locality, Arieşului Valley.

Trachelipus troglobius Tăbăcaru 1989
Ecology: troglobiont species, lives in caves.
Distribution: Movila Cave, in Dobrogea.

Crinocheta: family Agnaridae
Orthometopon romanicus Tomescu, Teodor 2016
Ecology: epigenous species, lives on sandy areas with reeds and sedges, in layers of thick detritus and high soil humidity.
Distribution: Sacalin Island, Portiţa, Grindul Lupilor in the Danube Delta.

Crinocheta: family Armadillidiae
Armadillidium banaticum Verhoeff 1907
Ecology: epigenous species, lives in the litter layer, under rocks and at the base of rocky walls.
Distribution: Banat, Mehadia, Beului Valley, Şuşarei.

Conclusions

In the Romanian fauna there are 32 endemic isopod species: 5 troglobiont species, 2 troglophilous species, 6 edaphic species and 19 epigean species.

Twenty endemic isopod species are classified in lower taxonomic categories: the families Trichoniscidae and Platyarthridae, small-sized species, of several mm in length and low mobility.

All endemic species have a limited distribution; the majority lives in habitats located in montane regions. With a single exception, the epigenous species live in the litter layer of forests.

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