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HELMINTH BIODIVERSITY AND HEAVY METAL CONTAMINATION OF *Perca fluviatilis* (Linnaeus, 1758) AND *Eustrongylides excisus* (Jägerskiöld, 1909) LARVAE FROM THE WETLAND MANDRA-PODA

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Abstract

The study presents the first data on the biodiversity of helminths and helminth communities of European perch (Perca fluviatilis Linnaeus, 1758) from the freshwater ecosystem of the anthropogenically affected protected area Mandra-Poda. Thirty specimens of perch were examined by the method of complete helminthological study. The infection characteristics and the dominant structure of the helminth communities were determined. Helminth communities were analyzed at two levels: infracommunities and component communities. Basic biotic indices are presented. The core species in the helminth communities of perch is Eustrongylides excises Jägerskiöld, 1909 larvae (Nematoda; Dioctophymatidae) (P% = 60; MI = 7.12; 1-51 specimens). New data on the content of heavy metals and their circulation in the system water-sediment-perch and its helminth Eustrongylides excisus have been established. New data on the bioindicator significance of helminths and helminth communities of European perch are discussed.

***Key words**: bioindication, Black Sea Water Basin, european perch, ecological indices, Mandra-Poda.*

INTRODUCTION

Mandra-Poda complex is a part of the Burgas Wetlands, Black Sea Basin Region, Ecoregion 12: Pontic Province, Sub-Ecoregion 12-2: Black Sea, Southeast Bulgaria. It is located south of the industrial zone of the city of Burgas. According to the Ramsar Convention, the Mandra-Poda complex has been declared a wetland of international importance. The Mandra-Poda complex is a protected area under the Habitats Directive (Directive 92/43/EEC) and the Birds Directive (79/409/EEC), as well as the Corine Biotope, an Ornithologically Important Site. Izvorska, Fakiyska, Sredetska, and Rusokastrenska rivers flow into the Mandra Lake. The Lake is located in a river valley, across the sea coast. The main economic activities in the Lake are related to the use of water by Lukoil Neftohim for industrial needs. The main threats are related to significant anthropogenic pressure from the construction of areas along the coast of the wetland and the destruction of the natural habitats located there (especially wetland meadows), eutrophication of the wetland, excessive water use, development of the surrounding infrastructure, pollution with chemicals and solid municipal waste from the watershed, overfishing, poaching, significant disturbance, others. On the territory of the complex, three protected areas have been declared, according to the Law for the Protected Areas (‘Poda’; ‘Ustie na reka Izvorska’; ‘Uzungeren’), for conservation of the habitats of endangered and rare bird species. The complex is part of the migration flyway "Via Pontica". Although the freshwater ecosystem is under serious anthropogenic interference, the Mandra-Poda complex is important for biodiversity conservation. Research on parasites and parasite communities of freshwater fish from the Mandra-Poda area was carried out only by Margaritov (1959). There are single scientific studies on chemical indicators of water pollution and the content of pollutants in the muscles of freshwater fish species (Georgieva et al., 2015; Peycheva et al., 2022). The study aims to present research results on helminths and helminth communities of *P. percae* from the Mandra-Poda complex and monitor Se concentrations in waters, sediments, tissues, and organs of European perch and its parasite *E. excisus*.

MATERIALS AND METHODS

In 2023, thirty specimens of *Perca fluviatilis* from the Mandra-Poda Complex were examined for helminths and Selenium (Se) content. The examined fish were caught by gill nets (BSS EN 14757:2015 Water quality. Sampling of fish with multi-mesh gillnets), according to permission from the Ministry of Agriculture of the Republic of Bulgaria. The scientific name is represented according to the FishBase database (Froese and Pauly, 2020). The fish were collected in the Northeastern section of the Complex (42°24'12.31″N, and 27°19'18.05"E; 309 m; Figure 1).

Figure 1. Studied biotopes from the Complex Mandra-Poda

The helminthological study of *P. Fluviatilis* was carried out according to Petrochenko (1956); Zashev & Margaritov (1966); Bauer (1987); Moravec (2013). Collected helminth specimens were fixed in 70% of ethyl alcohol. Species diversity was determined on permanent slides according to the method of staining with iron acetocarmine (Georgiev et al., 1986; Sholz and Hanzelova, 1998) and on temporary slides carried out by the methods of Moravec (2013) and Petrochenko (1956). Helminth community structure was analyzed on two levels: on the level of intracommunity (total number of fish species; total and mean number of fish specimens; Brillouin's diversity index (HB) and on the level of component community (prevalence (P%); mean intensity (MI)). Helminth species in the component community were divided into three groups: core species (P%>20), component species (P%>10), and accidental species (P%<10), according to the criteria of Magurran (1988); Bush et al. (1997), and Kennedy (1997).

Tissue and organ samples of *P. fluviatilis* and *E. excisus* were prepared for determination of Se content according to Nachev et al. (2013). The pre-weighed and thawed samples of liver, muscle and skin of *P. fluviatilis* (to 300 mg wet weight) as well as *E. excisus*, extracted from the cysts (to 100 mg wet weight) were subjected to acid digestion with aqua regia and microwave heating - Method B: "Microwave heating with temperature control at 175±5°C". Samples of 2 g have been used in closed vessels under pressure with 6.0 ml HCl and 2.0 ml HNO_3 and determination of the element using ICP-OES, according to EN ISO 16170:2016. In surface water and sediment samples, selenium concentrations were determined according to EN ISO 11885:2007

Water quality - Determination of selected elements by inductively coupled plasma optical emission spectrometry (ICP-OES) and EN 16170:2016 Sludge, treated biowaste and soil - Determination of elements using ICP-OES.

Bioconcretacion factors were calculated to determine the accumulation capacity of muscle, skin, and liver of European perch and E. excisus from the freshwater environment (Bioconcentration factor, $BCF=C_{SeSkin/SeLiver/SeMuscle/SeE.excisus}/C_{SeWater/SeSediments}$) (Sures et al., 1999), and accumulation capacity of *E. excisus* from organs and tissues of the European perch (Bioaccumulation factor, $BAF=C_{Se\_E.excisus}/C_{Se\_fish\_tissues\_organs}$) (Zaharieva, 2022a). Spearman rank correlation (r_s) was calculated to check the relationship between Se concentrations in samples of environment (water, sediments), in the samples of liver, muscles, and skin from fish hosts, and in samples of its nematode species *E. excisus* (Sokal and Rohlf, 1981). The coefficient of determination (r_s^2) is also presented, which reflects what percentage of the factor variable will cause changes in the outcome variable. A Friedman test ANOVA was applied to determine the significance of differences between the selenium content in the studied fish tissues and organs and *E. excisus* (Sokal & Rohlf, 1981). The results were statistically processed using Statistica 10 (StatSoft Inc., 2011) and MS Exel (Microsoft 2010).

RESULTS AND DISCUSSIONS

Characteristics of the studied fish species

Perca fluviatilis Linnaeus, 1758 (Percidae) is a freshwater, demersal, and brackish fish species. The European perch is a predatory species. It feeds mainly on zooplankton, zoobenthos, and small fish. The species is defined as not endangered in the International Red Book (LC; IUCN) (Kottelat & Freyhof, 2007; Froese & Pauly, 2020; Karapetkova & Zhivkov, 2006). The European perch is not a protected species on the territory of Bulgaria. The species is widespread in different types of water bodies in the country. *P. fluviatilis* could be of more economic importance but is mainly subject to recreational fishing. Maximum length (cm) and weight were determined for each examined specimen (g). The maximal length of studied specimens varies from 15-21 cm (17.78±1.28), and the weight from42-72 g (56.1±8.55).

Helminths and Helminth community structure

In 2023, as a result of the ecologoparasitological examinations of 30 specimens of European perch, *Perca fluviatilis* Linnaeus, 1758 from the Mandra-Poda Complex, three taxa of endohelminths was established: *Proteocephalus percae* (Gmelin, 1790) La Rue, 1911; *Acanthocephalus lucii* (Müller, 1776) *Eustrongylus excisus* (Jägerskiöld, 1909), larvae, belonging to three classes, three orders, three families and three genera (Table 1).

Table 1. Biodiversity and ecological indices of helminths and helminth communities of *Perca fluviatilis* from the Mandra-Poda Complex

Perca fluviatilis (N[1] = 30) Helminth species	n[2]	p[3]	P%[4]	MI[5] (min.-max.)
Class CestodaRudolphi, 1808 **Order** Tetraphyllidea (Beneden, 1849) Carus, 1863 **Family** Proteocephalidae La Rue, 1911 **Genus** *Proteocephalus* Weinland, 1858				
Proteocephalus percae juv. (Gmelin, 1790) La Rue, 1911	2	2	6.67	1 (1)
Class AcanthocephalaRudolphi, 1808 **Order** Echinorhynchida Southwell et Macfie, 1925 **Family** Echinorhynchidae (Cobbold, 1879) Hamann, 1892 **Genus** *Acanthocephalus* Koelreuther, 1711				
Acanthocephalus lucii (Müller, 1776)	2	2	6,67	1 (1)
Class Nematoda Rudolphi, 1808 **Order** Dioctophymida (Skrjabin, 1927) Schulz et Gvosdev, 1970 **Family** Dioctophymidae Railliet, 1915 **Genus** *Eustrongylides* Jägerskiöld, 1909				
*Eustrongylus*excisus (*Jägerskiöld, 1909*), larvae	18	128	60	7.12 (1-51)

Legend: [1]N = total number of examined fish specimens.
[2]n = total number of infected fish specimens.
[3]p = total number of helminth specimens.
[4]P% = prevalence.
[5]MI = mean intensity.

***Proteocephalus percae* (Gmelin, 1790) La Rue, 1911** is an intestinal parasite of *Gymnocephalus cernua* (Linnaeus, 1758); *P. fluviatilis; Esox lucius* Linnaeus, 1758, etc. The developmental cycle is not well understood, but plerocercoids have been reported to develop in representatives of the genus *Cyclops* Müller, 1785 (Bauer, 1987; Kakacheva-Avramova, 1983). *Pr. percae* was reported as parasite species of *P. fluviatilis* from Lake Srebarna (Shukerova, 2010; Shukerova et al., 2010); from the Maritsa River (Kuzmanova et al., 2019; Kuzmanova et al., 2023) and the Danube River, near village of Kudelin (Zaharieva, 2022).

***Acanthocephalus lucii* (Müller, 1776)** develops as an adult in many freshwater fish species from Cyprinidae, Salmonidae, Percidae, Siluridae, etc. The intermediate host is *Asellus aquaticus* (Linnaeus, 1758) (Bauer, 1987; Kakacheva–Avramova, 1983). *A. aquaticus* is a bioindicator for α-mesosaprobity and is in Group D of tolerant forms regarding environmental conditions in habitats (Belkinova et al., 2013). *A. lucii* was reported as parasite species of *P. fluviatilis* from Lake Srebarna (Shukerova, 2010; Shukerova et al., 2010); from the Danube River (Matgaritov, 1966; Kakacheva et al., 1978), and the Maritsa River (Kuzmanova et al., 2019; Kuzmanova et al., 2023).

***Eustrongylus excisus* (Jägerskiöld, 1909)** in the adult state parasitizes the glandular stomach of cormorants [*Phalacrocorax carbo* (Linnaeus, 1758), *Ph. pygmaeus* (Pallas, 1773)].It develops with the participation of two intermediate hosts.The first intermediate host is aquatic oligochaetes [*Lumbricus variegatus* (Müller, 1774), *Tubifex tubifex* (Müller, 1774), *Limnodrilus* sp.]. The localization of the larvae is initially in the intestine and then in the body cavity (larvae I stage), after which the larva of the second stage passes into the abdominal blood vessel, and the larva of the third stage with the bloodstream passes to the head and tail end of the host. A second intermediate host is various species of benthic fish [*Ponticola kessleri* (Günther, 1861), *Neogobius melanostomus* (Pallas, 1814), *Rutilus rutilus* (Linnaeus, 1758)]. Invasion occurs through food.In the organism of the host fish, the larvae of the III stage are localized in the body cavity or curled up in a circle under the serous coating on the surface of the internal organs. After another molt, they migrate into the musculature of the fish (most often the stomach and less frequently the dorsal). The IV-stage larva is coiled, encapsulated, and invasive. Reservoir hosts of *E. excisus*are: *P. fluviatilis*, *Leuciscus aspius* (Linnaeus, 1758), Silurus glanis Linnaeus, 1758, *E. lucius*, *Sander lucioperca* (Linnaeus, 1758), *Sander volgensis* (Gmelin, 1789), *G. cernua*, *Chalcalburnus chalcoides* (Güldenstädt, 1772), *Bentophilus macrocephalus* (Pallas, 1787), *Huso huso* (Linnaeus, 1758), *Acipenser ruthenus* Linnaeus, 1758, *A. gueldenstaedtii* Brandt & Ratzeburg, 1833, *Leuciscus idus* (Linnaeus, 1758), *Luciobarbus brachycephalus* (Kessler, 1872), *Pelophylax ridibundus* (Pallas, 1771), *Natrix tessellata* (Laurenti, 1768) (Bauer, 1987; Kakacheva-Avramova, 1983). *E. excisus* was reported as parasite species of *P. fluviatilis* from the Lake Srebarna (Shukerova, 2010; Shukerova et al., 2010; Hristov, 2013; Kirin et al., 2013a); from the Arda River (Kirin et al., 2013b); from the Danube River (Atanasov, 2012; Kirin et al., 2013a). *E. lucius* has been reported for the Mandra-Poda Complex from *S. lucioperca* as a reservoir host and from *Gobius* sp. as an intermediate host (Margaritov, 1959).

Component community

The presented helminth taxa were found in 18 of the studied 30 specimens of European perch (60%). Prevalence (P %), mean intensity (MI), and rank were determined for each taxon. *E. excisus* (P% = 60) is a core species of the endohelminth communities of *P. fluviatilis* from the Mandra-Poda Complex. The other two species are accidental ($P\%_{Pr.percae}$ = 6.67; $P\%_{Ac.lucii}$ = 6.67). *E. excisus* is was distinguished with the highest mean intensity (MI = 7.12). The mean intensity for the other two species is low ($MI_{Pr.percae} = MI_{Ac.lucii}$ = 1.0). Only two *Pr. percae* and *Ac. lucii specimens* are fixed in the infected specimens of *P. fluviatilis*. *E. excisus*is an allogenic species. *Pr. percae* and *Ac. lucii* are autogenic species. The found taxa are generalists for the helminth communities of *P. fluviatilis* from the Mandra-Poda Complex, Bulgaria (Table 1).

Infracommunity

The twelve examined specimens of *P. fluviatilis* are free of helminths (40%). A mixed invasion of *E. excisus* and *Ac. lucii* was found in two host specimens (6.67%) and in one of the studied specimens of European perch, a mixed invasion of *E. excisus* and *Pr. Percae* (3.34%). The maximum number of helminths found in a single specimen by the host is 51 (*E. excisus*). The average number of all endohelminth specimens is 7.34. The value of Brillouin's diversity index (HB) is low (HB = 0.121) (Table 2).

Table 2. Infracommunity data

Number of helminth species			
Number of infected fish	12	15	3
Number of helminth species	0	1	2
Number of helminth specimens			
Total number	132		
Mean±SD	7.34±14.25		
Range	1-51		
Mean HB±SD	0.121±0.03		

Concentration of Selenium (Se) in the system Water – Sediments - *P. fluviatilis* - *E. excisus*

The content of Se was determined in samples of skin, muscles, and liver of *P. fluviatilis* from the Mandra-Poda Complex, as well as in samples of *E. excisus* as a core species in the helminth communities of the European perch (wet weight and dry weight, $mg.kg^{-1}$). The highest content of Se was found in *E. excisus*, followed by that in liver samples of *P. fluviatilis* as wet weight. The content in water samples is the lowest, followed by that in muscle and skin as wet weight. The Se content of *E. excisus* was 1.46 times that of the perch liver, 4.91 times higher than that in the skin, 7.79 times more than in the perch muscles, and 753.34 times more than in the water samples (Table 3).

When comparing the results in dry weight, the highest content was reported in sediments, followed by that in *E. excisus* and the liver of European perch.

The lowest Se content was again reported in the muscle samples as dry weight. In these cases, the selenium content is the highest in the sediments.The Se content of the parasite was 3.92 times lower than that in the sediments and 1.42 times higher than that in the liver, 9.46 times higher than that in the skin, and 15.28 times higher than that in perch muscle as dry weight (Table 3).

Significant differences were found between Se concentrations in E. excisus and those in the skin, muscle, and liver of P. fluviatilis, waters, and sediments (Friedman's ANOVA, $p < 0.05$).

Table 3. Concentration of Selenium in tissues and organs of fish and its nematode species *E. excisus*

Samples	**Se min.-max. $mg.kg^{-1}$**	**SeMean±SD**
Skin (wet weight) *P.fluviatilis*	0.39-0.56	0.46±0.11
Muscle (wet weight) *P. fluviatilis*	0.15-0.44	0.29±0.20
Liver (wet weight) *P. fluviatilis*	1.28-1.79	1.54±0.36
E. excisus(wet weight) *P. fluviatilis*	2.16-2.36	2.26±0.14
Water	0.003-0.003	0.003
Skin (dry weight) *P. fluviatilis*	0.77-1.07	0.92±0.21
Muscle (dry weight) *P. fluviatilis*	0.29-0.84	0.57±0.39
Liver (dry weight) *P. fluviatilis*	5.11-7.15	6.13±1.45
E. excisus (dry weight)	6.36-11.05	8.71±3.32
Sediments	35.4-40.2	34.1±6.84

BCF comparing samples as wet weight for Se content was highest for the ratio of Se concentrations in *E. excises* to those in water samples, followed by that obtained between Se concentrations in liver and water samples, ofskin and water samples, and lastly BCF, obtained as a ratio between Se concentrations in muscle and water samples (Table 4).

Table 4. Bioconcentration factor (BCF), Bioaccumulation factor (BAF), and Spearman correlation coefficient (r_s) between the content of Se in water, sediments, *P. fluviatilis* and *E. excisus*

Fish – Parasite - Water	**BCF**	**r_s**	**p**
Skin (wet weight) - Water	154.34	-1****	<0.001
Muscle (wet weight) - Water	97.67	-1****	<0.001
Liver (wet weight) - Water	514.67	-1****	<0.001
E. excisus(wet weight) - Water	753.34	-1****	<0.001
Fish – Parasite - Sediments	**BCF**	**r_s**	**P**
Skin (dry weight) - Sediments	0.03	1****	<0.001
Muscle (dry weight) - Sediments	0.02	1****	<0.001
Liver (dry weight) - Sediments	0.18	1****	<0.001
E. excisus (dry weight) - Sediments	0.26	1****	<0.001
***E. excisus* - Fish**	**BAF**	**r_s**	**P**
E. excisus (wet weight) - Skin (wet weight)	4.88	1****	<0.001
E. excisus (wet weight) - Muscle (wet weight)	7.71	1****	<0.001
E. excisus (wet weight) - Liver (wet weight)	1.46	1****	<0.001
E. excisus (dry weight) - Skin (dry weight)	9.43	1****	<0.001
E. excisus (dry weight) - Muscle (dry weight)	15.38	1****	<0.001
E. excisus (dry weight) - Liver (dry weight)	1.42	1****	<0.001

Legend: **** - very significant correlation

BCF comparing samples as a dry weight for Se content was highest for the ratio of Se concentrations in *E. excises* to those in sediment samples, followed by that between samples of liver and sediments, skin and sediments, and in last place, between the concentration of Se in samples of muscle and sediments. Therefore, the wet-weight samples maintain the trend (Table 4).

BAF for tracking the accumulation capacity of *E. excisus* is also presented.BAF was highest for the ratio of Se content in the parasite to that in the muscle samples and lowest for the ratio of Se content in the parasite to that in the liver (Table 4).

Spearman rank correlation coefficient was determined to assess the possible relationships between concentrations of Se in water - sediments - fish tissues and organs - *E. excisus*. Very significant negative correlations were found between the Se content of the water samples and the corresponding samples of the parasite, liver, skin, and muscles.Therefore, as the concentrations of Se in the samples from the biological sites increase, the concentrations of Se in the water samples from the Mandra-Poda Complex decrease.All other relationships are positive, also with very significant correlations. Therefore, with increasing concentrations of Se in sediments, its concentrations in biological samples also increase, and with its increase in the organs and tissues of perch, their increase in *E. excisus* samples, respectively (Table 4).

The coefficient of determination (r_s^2) for all variants is 100%. Therefore, any changes in the content of selenium in the habitat of the perch (water, sediments) will lead to changes in the content of selenium in the tissues and organs of both the European perch and its parasite *E. excisus*.

DISCUSSION

A total of 30 specimens of European perch were examined, and three species of helminths were reported.*E. excisus* is a core species for the helminth communities of *P. fluviatilis*.*The present study used P. fluviatilis* and its parasite *E. excisus* as models to trace Se content and bioaccumulation potential.Selenium is a non-metallic element.It is an essential trace element necessary for the mineral nutrition of animals.Regardless, however, when slightly exceeding the required concentrations, it can cause toxic effects.Sources of water pollution with selenium are industry and agriculture due to the direct or indirect discharge of selenium-laden wastewater and soils with high selenium content through irrigation water or drainage water passing through coal ash repositories.Organisms readily absorb Se, so it can quickly reach toxic concentrations dangerous to fish and wildlife.Increasing Se concentrations lead to young and adult organisms' death or reduced reproduction.Se concentrations of 0.002-0.005 $mg.l^{-1}$ water have been found to cause toxic and reproductive problems in fish.Sediments are temporary reservoirs of Se (Lemly, 1987).In the body of plants and animals, Se is mainly bound to proteins. Therefore, foods with a high protein content also contain high concentrations of Se (e.g., meat, seafood). The recommended daily intake of Se for humans is 0.026-0.035 mg/day (FAO/WHO, 1998); from 2000 years, it has been 0.055 mg/day (NAS, 2000).The upper limit for Se is 0.4 mg/day (FAO/WHO, 1998; NAS, 2000; UK EGVM, 2002).The lowest adverse effect level was 0.003 mg/day.Alkaline, poorly aerated environments favor the formation of selenites and selenates, which are water-soluble and easily absorbed by organisms (WHO, 2011).

According to Compilation of Legal Limits for Hazardous Substances in Fish and Fishery Products (FAO Fish.Circ.,(764)), permissible value for Se in foodstuff is 0.3 $mg.kg^{-1}$. According to Regulation No.18 of 27.05.2009 on the quality of water for irrigation of agricultural crops, in Bulgaria, the maximum permissible value for Se in water used to irrigate agricultural crops is 0.01 $mg.l^{-1}$.

Scientific research on Se content in the system Waters - Sediments - Freshwater fishes and their parasites is extremely limited. Even fewer are these for *P. fluviatilis* and *E. excisus*.Nachev et al.(2013) compared the capacity between *Pomphorhynchus laevis* (Müller, 1776) Porta (1908) and *Eustrongylides* sp., helminths of *Babus barbus* (Linnaeus, 1758) from the Danube River.As in the present study, they found much higher levels of Se in *E. excisus* than in the tissues and organs of the fish (*B. barbus*).According to the authors, the degree of accumulation is closely related to the parasites' taxonomic affiliation and stage of development.According to this study, an important factor is also the species of host and his lifestyle, i.e., in the tissues and gills of the European perch studied by us, Se concentrations are much higher than those in *B. barbus* from the Danube River, as well as in *E. excisus* with host *P. fluviatilis* from the Mandra-Poda Complex. Nachev et al. (2013) also indicate intestinal acanthocephalans mainly accumulate toxic elements, while *E. excisus* mainly accumulates essential elements, including Se. They state that *P. laevis* and *Eustrongylides* sp. can be used as bioindicators of metal pollution. Hursky and Pietrock (2015) investigated the effects of parasitism on Se bioaccumulation in juvenile *Oncorhynchus mykiss* (Walbaum, 1792). They found low Se bioaccumulation in *O. mykiss* infected with the nematode *Raphidascaris acus* (Boch, 1779). Nuutinen & Kukkonen (1998) found that with increasing Se concentrations in lake sediments, the accumulation of methylmercury (MeHg) in the body of oligochaetes (*Lumbriculus variegatus* Müller, 1774) decreased. The authors found that Se concentrations of 15 and 50 $mg.l^{-1}$ in sediments reduced Se accumulation in *L. variegatus* by 75, and 86%, respectively.

CONCLUSIONS

The three species of helminths (*P. percae*, *A. lucii*, and *E. excisus*) are reported for the first time for the helminth fauna of European perch from the Mandra-Poda Complex. *P. percae*, *A. lucii* are reported for the first time for the helminthfauna of the freshwater fishes from the complex. The Mandra-Poda Complex is a new locality for them. The obtained high concentrations of Se and the very significant correlation dependences give grounds for *E. excisus* to be used as a bioindicator of Se pollutionin the biomonitoring systems.

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