Oceanological and Hydrobiological Studies

International Journal of Oceanography and Hydrobiology

Volume 51, No. 1, March 2022
pages (74-89)

sciendo

ISSN 1730-413X
eISSN 1897-3191

Molecular identification and distribution of insect larvae in the Lower Danube River

by

Selma Menabit[1], Lavinia Iancu[2], Ana B. Pavel[1], Adrian Popa[1], Naliana Lupascu[1], Cristina Purcarea*[,2]

DOI: **https://doi.org/10.26881/oahs-2022.1.07**
Category: **Original research paper**
Received: **July 15, 2021**
Accepted: **November 18, 2021**

[1]*National Institute of Marine Geology and Geo-Ecology (GeoEcoMar), Bucharest, Romania*

[2]*Institute of Biology Bucharest of Romanian Academy, Department of Microbiology, 296 Splaiul Independentei, 060031 Bucharest, Romania*

* Corresponding author: cristinapurcarea5@gmail.com

Abstract

As a major component of freshwater ecosystems, insect species play an important role in nutrient cycling and are often used as bioindicators of water pollution. Although extensive studies have characterized insects from freshwater habitats, little is known about the distribution of these species along the Lower Sector of the Danube River. Therefore, this survey conducted in the Danube section within the Romanian territory aimed to identify insect larvae belonging to seven different species of Odonata, Trichoptera, Ephemeroptera, Lepidoptera and Megaloptera by DNA barcoding and to investigate their distribution, density and frequency. A total of 41 quantitative macrozoobenthic samples were collected during two consecutive years (2019 and 2020). Species showed large differences in the distribution and density along different sections, and an overall tendency to populate downstream areas, except for *Sialis morio*. On the other hand, only *Hydropsyche bulgaromanorum*, *Triaenodes bicolor* and *S. morio* larvae were identified in the upstream section (Sulina branch). These data provide baseline information on the larger range of some of the most common aquatic insects in the Romanian Danube section in relation to several environmental parameters based on the first molecular identification of these species using COI gene sequencing.

Key words: aquatic insect larvae distribution, COI gene sequencing, Danube River, DNA barcoding, Sulina branch

online at www.oandhs.ug.edu.pl

1. Introduction

Aquatic insects represent a large and diverse group derived from various terrestrial ancestors that re-inhabited aquatic environments, comprising about 76,000 species adapted to all types of freshwater habitats (Samways & Deacon 2021). Although most insects are characterized by a subaquatic life cycle, few species spend their entire life submerged in waters. Most aquatic insects undergo an aquatic immature stage followed by a terrestrial adult (e.g. Odonata, Ephemeroptera, Trichoptera, Megaloptera), being considered semiaquatic, associated only with aquatic and semiaquatic vegetation (Bouchard 2004). These organisms are an important component of aquatic (and sometimes terrestrial) food webs, playing an essential role in nutrient cycling processes (Balian et al. 2008). Most of the larval stages are active and passive filter or deposit feeders, representing an intermediate step between microorganisms and fish in the food chain and constituting an important food source for the latter (Rosenberg & Resh 1993). Due to their large biomass, high reproduction rates with short-lived generations and rapid colonization, insect larvae are successfully used in assessing the structure and function of aquatic ecosystems as important tools in ecology (Hershey & Lamberti 2001), genetics (Sivaramakrishnan et al. 2014) and evolutionary studies (Kristensen 1981; Mazzuccoa et al. 2015). Aquatic ecosystems are exposed to multiple anthropogenic pressures that significantly affect their health condition (Buczyńska and & Buczyński 2019; Gómez-Baggethun et al. 2019; Mureşan et al. 2019; Teacă et al. 2019; Begun et al. 2020) due to habitat change, pollution (Davidson et al. 2013) and soil erosion (Broadman 2013). Being highly sensitive to pollution, aquatic insects are frequently used as bioindicators to assess the impact of environmental stressors on lentic and lotic water systems and the water quality (Nasirian & Irvine 2017), as any change in their composition or density indicates a change in the water quality (Varma & Pratap 2006). Compared to fish and plankton, they have a higher ability to tolerate pollution-induced environmental stress (da Rocha et al. 2010; Andem et al. 2012).

Taxonomic assignment of macroinvertebrates at the species level has proven challenging when using a morphology-based identification technique (Barrett & Hebert 2005). Moreover, identification of early life stages of aquatic insects (larvae and nymphs) based on morphological characters is particularly difficult since most of the identification keys are available only for adults. During the last decades, complementary molecular techniques were used based on DNA analysis for accurate identification and uncertainties linked to the morphological approach (Navajas & Fenton 2000). To date, DNA barcoding based on amplification of the mitochondrial cytochrome C oxidase subunit I gene region has been the most frequently used technique for discriminating invertebrate species (Hebert et al. 2003). In addition to morphological identification, which in most cases could only be performed at the family or genus level, DNA barcoding has allowed identification of specimens up to the species level (Baird et al. 2012).

The distribution and abundance of most aquatic insect communities are considered to be affected by several factors such as current velocity, temperature, altitude, season, total suspended solids, availability of food, and the like (Crisci-Bispo et al. 2007). In general, these organisms require sufficient amounts of dissolved oxygen to survive, and this parameter has the potential to limit aquatic insect diversity (Kamsia et al. 2007). Studies have reported that their dispersal is strongly related to water depth (Hasmi et al. 2021), and the occurrence of certain species is correlated with the presence of vegetation (Crisci-Bispo et al. 2007). Furthermore, the nature of the substrate plays an important role in determining the species distribution and the structure of their communities (Ciutti et al. 2004).

Over the last decades, the distribution, ecology and diversity of aquatic insect species populating the Danube River have been the subject of several studies (Graf et al. 2006; Tubić et al. 2013; Farkas et al. 2014; Krno et al. 2018; Navara et al. 2020), including the Danube sector that crosses the Romanian territory (Chiriac 2004; Pavel et al. 2018). Meanwhile, a very limited number of studies have used molecular identification by DNA barcoding based on COI gene fragments of insects collected from terrestrial environments in Romania, not counting a survey of 180 butterflies (Dincă et. al. 2011), and no identification of aquatic insects using genetic methods has been carried out so far.

In this context, the current study aimed to identify insect larvae belonging to seven different species of the following orders: Odonata – *Erythromma viridulum* (Charpentier 1840) and *Gomphus flavipes* (Charpentier 1825), Trichoptera – *Triaenodes bicolor* (Curtis 1834) and *Hydropsyche bulgaromanorum* (Malicky 1977), Ephemeroptera – *Heptagenia flava* (Rostock, 1878), Lepidoptera – *Acentria ephemerella* (Denis & Schiffermüller, 1775) and Megaloptera – *Sialis morio* (Klingstedt, 1932), using DNA COI barcoding, and to investigate their distribution and quantitative occurrence in the Lower Sector of the Danube River in relation to different environmental parameters

such as substrate type, water depth and oxygen concentration. To our knowledge, this study presents the first molecular identification and characterization of the targeted species from the aquatic environment in Romania.

2. Materials and methods

2.1. Study area, sampling and insect larvae preparation

The Lower Sector of the Danube River, between Baziaş (44°48'57"N; 21°23'28"E) and the estuary where the river meets the Black Sea, has a length of 1075 km, including the Danube Delta (Sulina 45°15'74"N; 29°65'92"E). In this section, 10 main tributaries from the territory of Bulgaria and Romania flow into the Danube. The section of the river traversing a plane area (altitude 38–102 m) becomes shallower and broader, with several major islands. In this region, the currents slow down considerably and the water quality is significantly affected by anthropogenic pollution consisting of excessive loads of nutrients, organic material, and hazardous substances (ICPDR 2009). The Sulina channel, a distributary of the Danube with a total length of 71.7 km, begins at a bifurcation of the Tulcea distributary and carries about 20% of the water and suspended sediment discharge of the river (Bondar & Panin 2000). The water along this branch is particularly affected by human activities (agriculture, fish farming, tourism), with frequent discharges of detergents, domestic waste, and oil products, leading to enrichment with dissolved nutrients (Cretescu et al. 2021).

Sampling was carried out in late spring (May 2019, 2020) when the average temperature ranged from 17.2°C to 19.1°C.

Sediment samples were collected along the lower section of the Danube River from km 626 to km 811, including the Sulina branch, during 2019 and 2020 trips (Fig. 1; Table 1). Of the total 41 samples, 27 were collected in the lower Danube section (Fig. 1A), including seven samples from the Corabia area (between km 626 and km 631), eight samples from the Bechet area (from km 674 and km 676.5.5), nine samples from the Pişculeţ area (km 760 – km 765), and three samples from the Cetate area (from km 806 and km 811), and 14 samples were obtained from the Sulina branch (Fig. 1B). Samples were collected at different depths, ranging from 2.5 to 17.5 m (Table 2).

The sediments were sampled using two different Van Veen seabed sediment grabs with a surface of 0.039 m^2 (small) and 0.135 m^2 (large), respectively. The number of individuals collected per unit surface (1 m^2) was calculated based on their number contained in each sample, using a multiplication factor of 25.2 for the small Van Veen grab, and 7.4 for the large Van Veen grab (SR EN ISO 10870:2012).

Insect larvae samples were washed immediately after collection using 250 and 125 µm mesh sieves to remove excess sediment particles and preserve macrofauna. Each specimen was washed with sterile water and preserved in 200 µl Tris-EDTA pH 8 buffer at –20°C for genetic identification (Ross et al. 1990). For morphological identification, a mixed solution of Rose Bengal and 4% buffered formaldehyde was used for sample preservation (SR EN ISO 5661-1:2008).

Statistical analysis of insect larvae populations was performed by calculating the univariate index (density as ind. m^{-2}).

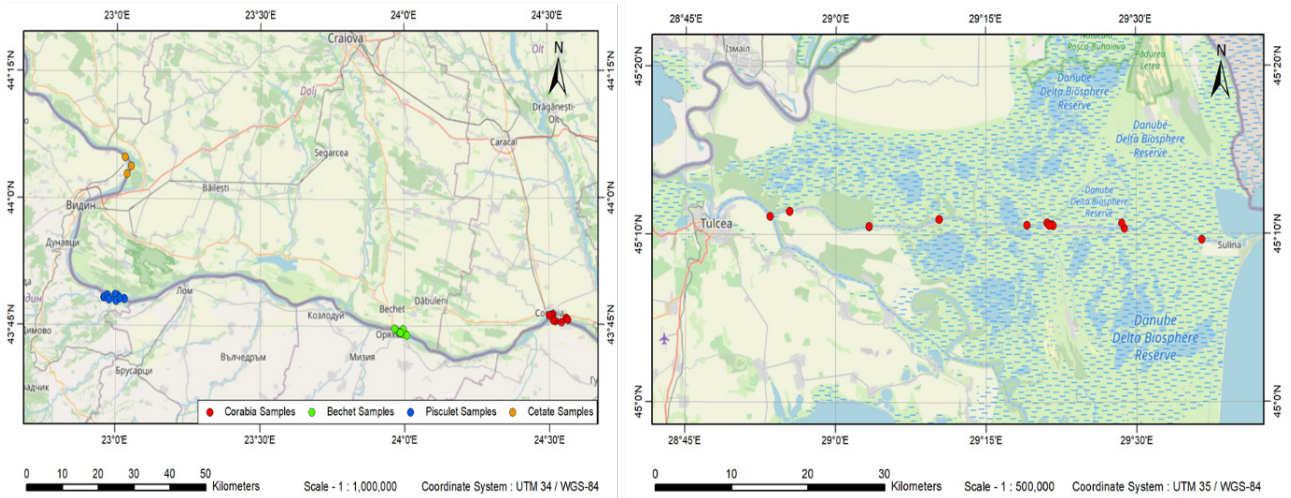

Figure 1

Map of the sampling areas in the Lower Danube River (A) and the Danube Delta (B)

Table 1

Sampling locations in the Lower Danube and density of larvae

Crt. No	Sites	Coordinates		Number of individuals/m²						
		Lat. (α)	Long. (λ)	*G.f.*	*H.f.*	*E.v.*	*A.e.*	*H.b.*	*T.b.*	*S.m.*
1.	*km 811	44°4'53.2"	23°1'48.8"	0	0	7.4			0	0
2.	**km 808	44°3'49.7"	23°3'7.4"	0	0	0	0	50.4	0	0
3.	**km 806	44°2'55.3"	23°2'14.5"	0	0	0	0	226.8	0	0
4.	**km 765	43°48'18.2"	22°57'34.3"	0	0	0	0	403.2	0	0
5.				0	0	0	0	0	25.2	0
6.	**km 764.5	43°48'36.8"	22°58'12.0"	0	0	0	0	0	1008	0
7.	**km 764	43°48'5.7"	22°58'35.5"	0	0	0	0	25.2	25.2	0
8.	*km 762.5	43°48'37.7"	22°59'52.7"	7.4	0	0	0	162.8		0
9.	*km 762	43°47'54.0"	23°0'2.4"	0	29.6	0	0	0	0	0
10.				0	0	0	0	4388.2	7.4	0
11.	*km 761	43°48'14.3"	23°0'54.4"	0	0	0	0	0	7.4	0
12.	**km 760	43°48'9.8"	23°1'51.1"	0	0	0	0	201.6	0	0
13.	*km 678	43°44'40,6"	23°57'52.9"	0	0	7.4	0	7.4	0	0
14.	*km 676.5	43°44'18.0"	23°58'42.9"	0	0	0	0	96.2	0	0
15.	*km 676	43°44'13.0"	23°59'4.3"	0	0	0		1813	0	0
16.				0	0	0	29.6	0	0	0
17.	*km 675.5	43°44'38,7"	23°59'42,9"	0	7.4	0	0	7.4	7.4	0
18.	*km 675	43°44'5.5"	23°59'45.1"	0	0	7.4	0	0	0	0
19.	*km 674.5	43°44'3.0"	24°0'8.1"	0	66.6	0	7.4	488.4	0	0
20.	**km 674	43°43'52.9"	24°0'27.3"	0	0	0	0	25.2	0	0
21.	*km 631	43°46'8.3"	24°29'58.8"	0	96.2	0	0	0	0	0
22.	*km 630	43°45'32.0"	24°30'49.5"	0	429.2	14.8	0	0	0	0
23.				0	0	0	0	1894.4	0	0
24.	*km 629	43°45'25.4"	24°31'18.5"	0	0	0	0	7.4	0	0
25.	**km 627.5	43°45'17.2"	24°32'34.2"	0	25.2	0	0	252	0	0
26.	**km 626.5	43°45'46.6"	24°33'30.4"	0	0	0	0	100.8	0	0
27.	*km 626	43°45'37.5"	24°33'48.9"	29.6	7.4	0	0	310.8	0	0
28.	*km 108(a) Ceatal Sf. Gheorghe	45°11'04.9"	28°53'26.7"	0	0	0	0	214.6	0	0
29.	*km 108(b) Ceatal Sf. Ghoerghe	45°11'04.39"	28°53'26.7"	0	0	0	0	7.4	0	0
30.	*NM 33 Sulina branch	45°11'22.8"	28°55'22.3 "	0	0	0	0	74	0	0
31.	*NM 24 (a) Sulina branch	45°10'28.5"	29°03'19.4"	0	0	0	0	384.8	0	0
32.	*NM 24 (b) Sulina branch	45°10'29.9"	29°03'19.3"	0	0	0	0	629	0	0
33.	*NM 21 Sulina channel	45°10'54.2"	29°10'19.1"	0	0	0	0	1420.8	0	0
34.	*NM 16 Sulina branch	45°10'33.3 "	29°19'05.5"	0	0	0	0	651.2	0	0
35.	*NM 14 (a) Sulina branch	45°10'33.9 "	29°21'34.8"	0	0	0	0	370	0	0
36.	*NM14 (b) Sulina branch	45°10'32.4 "	29°21'17.0"	0	0	0	0	1206.2	0	0
37.	*NM 14 (c) Sulina branch (Crisan)	45°10'30.9"	29°21'39.6"	0	0	0	0	518	0	0
38.	*Old Danube (Meander)	45°10'41.4"	29°21'05.1"	0	0	0	0	0	7.4	0
39.	*NM 8 (a) Sulina branch	45°10'20.3"	29°28'47.6"	0	0	0	0	148	0	0
40.	*NM 8 (b) Sulina branch	45°10'39.4"	29°28'33.2"	0	0	0	0	0	0	14.8
41.	*NM 2 Sulina branch	45°09'39.6"	29°36'31.6 "	0	0	0	0	296	0	0

G. f. – G. flavipes, H.f. – H. flava, E.v. – E. viridulum, A.e. – A. ephemerella, H.b. – H. bulgaromanorum, T.b. – T. bicolor, S.m. – S. morio, * – samples collected with the large grab, ** – samples collected with the small grab, NM – nautical miles (for the Sulina branch, as officially used location units)

2.2. Granulometric analysis and oxygen records

Granulometric analysis of sediment samples was performed using a Mastersizer 2000E Ver 5.20 Malvern diffractometer (Malvern Instrument Ltd). Grain size classes (sand, silt, clay) and fractions within each class were determined according to the Udden–Wentworth logarithmic scale. Sediment classification was carried out based on the Folk diagram (Folk 1954).

The dissolved oxygen content in each water sample was measured in situ using an oximeter Oxi 320/ Set (WTW Germany).

Table 2

Year of sampling, depth, substrate type, dissolved oxygen and presence of macrophytes

Crt. No.	Sites	Year of sampling	Depth (m)	Type of substrate	Dissolved oxygen mg/L		Macrophytes presence (+/-)
					Mean values/ area	Value/ site	
Corabia area					8.58		
1.	km 626	2019	3.0	sand		8.90	-
2.	km 626.5	2020	7.3	gravelly sand		8.10	-
3.	km 627.5	2020	5.0	sand		8.81	-
4.	km 629	2019	9.2	gravelly sand		8.82	-
5.	km 630	2019	8.2	gravelly sand		8.04	+
		2020				8.67	
6.	km 631	2019	5.3	sand		8.77	-
Bechet area					8.77		
7.	km 674	2019	3.3	sand		8.75	-
8.	km 674.5	2019	3.2	sand		8.75	+
9.	km 675	2019	4.0	sand		8.67	-
10.	km 675.5	2019	6.3	sand		8.67	+
11.	km 676	2019	3.7	gravelly sand		9.31	+
		2020				8.10	
12.	km 676.5	2020	3.0	sand		8.15	-
13.	km 678	2019	5.9	sand		9.78	+
Pisculeţ area					8.85		
14.	km 760	2019	3.9	sand		9.36	-
15.	km 761	2019	4.2	sand		9.61	+
16.	km 762	2019	5.0	sand		8.95	+
		2020				8.99	
17.	km 762.5	2019	4.8	gravelly sand		8.96	-
18.	km 764	2019	3.5	sand		8.55	+
19.	km 764.5	2019	4.9	sand		8.55	+
20.	km 765	2019	2.8	gravelly sand		8.38	+
		2020				8.31	
Cetate area					7.80		
21.	km 806	2019	3.8	gravelly sand		8.13	-
22.	km 808	2019	5.0	sand		7.25	-
23.	km 811	2019	7.8	gravelly sand		8.02	-
Sulina area					8.25		
24.	Old Danube meander	2019	5.0	sandy mud		7.04	-
25.	NM 24(a) Sulina branch	2019	16.5	sand		7.93	-
26.	NM 16 Sulina branch	2019	5.2	muddy sandy gravel		7.90	-
27.	NM 8(a) Sulina branch	2019	14.4	sandy mud		7.95	-
28.	NM 2 Sulina branch	2019	17.5	gravelly mud		7.96	-
29.	NM 33 Sulina branch	2019	12.5	gravelly mud		9.38	-
30.	NM 24(b) Sulina branch	2019	12.5	sand		9.01	-
31.	NM 14(b) Sulina branch	2019	13.5	sandy mud		9.55	-
32.	NM 8(b) Sulina branch (Meander)	2019	4.3	sandy mud		9.07	-
33.	NM 14(c) Sulina branch (Crisan)	2019	11.5	sand		7.95	-
34.	NM 14(a) Sulina branch (Crisan)	2019	10.7	gravelly mud		7.95	-
35.	NM 21 Sulina channel	2019	6.5	sandy mud		7.97	-
36.	Km 108(a) Ceatal Sf. Gheorghe	2019	6.4	sandy mud		7.97	-
37.	Km 108(b) Ceatal Sf. Gheorghe	2019	6.4	gravelly mud		7.97	-

NM – nautical miles

2.3. Morphological identification

Taxonomic identification was performed by examining the morphological characters of different body parts (antennae, head, thorax, abdomen, annal region, caudal cerci, external gills, legs) according to the identification keys provided by Heidemann & Seidenbusch (2002) for Odonata, Hickin (1967), Lecureuil et al. (1983), Wallace et al. (2003) for Trichoptera, Baurenfeind & Soldán (2012) for Ephemeroptera, Vallenduuk & Cuppen (2004) for Lepidoptera and Kaiser (1977) for Megaloptera, using a SteREO Discovery V8 (Carl Zeiss) microscope and an Axiostar (Carl Zeiss) microscope.

2.4. Molecular identification by COI barcoding

Total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen), following an optimized protocol that includes an initial stage of cell disruption (Iancu et al. 2015). Organisms introduced into Tris-EDTA pH 8 buffer were homogenized for 12 min at 20°C, 50 Hz, in a SpeedMill PLUS Cell Homogenizer (Analitik Jena, Germany) in the presence of 5 ZR BashingBead lysis matrix 0.2 mm (Zymo Research), and then processed following the manufacturer's instructions.

Genetic identification of insect larvae was performed by DNA barcoding. Fragments of the mitochondrial COI gene were PCR amplified using the Mastercycler ProS System (Eppendorf, Austria). The PCR mixture contained 50 ng/µ genomic DNA, 1 x Taq buffer of 2.5 mM MgCl2 (ThermoFisher Scientific), 0.1 mM dNTP (ThermoFisher Scientific), 1 x BSA (New England Biolab), 1 unit of Taq DNA polymerase (ThermoFisher Scientific) and 10 pmol/µl of COI invertebrate universal forward (LCO1490: 5'-GGTCAACAAATCAAA-GATATTGG-3') and reverse (HCO2198: 5'-TAAACTTCAGGGTGAC-CAAAAAATCA-3') primers (Folmer et al. 1994) in a 50 µl final volume. The reaction was conducted for an initial denaturation step for 2 min at 95°C, followed by 5 cycles of 30 s at 94°C, 1.5 min at 45°C and 1 min at 72°C, 35 cycles of 30 s at 94°C, 1.5 min at 50°C, 1 min at 72°C, with a final extension step of 5 min at 72°C. Each PCR reaction contained a negative control without insect DNA. The PCR products were analyzed on 1% agarose gel (Cleaver Scientific, Ltd, England) by electrophoresis and amplicons were visualized via a UV transilluminator (Syngene International Ltd.). Further, the PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced on both strands (Macrogen, the Netherlands).

2.5. Nucleotide sequences analysis

Nucleotide sequences were analyzed using Sequence Assembly and Alignment – CodonCode Aligner Software (CodonCode Corporation 2003). Sequence identification was performed using the BLAST-NCBI platform (https://blast.ncbi.nlm.nih.gov).

The identified insect larvae COI sequences were deposited in GenBank under accession numbers [MW139674] for *E. viridulum*, [MW139673] for *G. flavipes*, [MW139670] for *T. bicolor*, [MW139677] for *H. bulgaromanorum*, [MW139671] for *H. flava*, [MW139675] for *A. ephemerella*, and [MW139669] for *S. morio*.

3. Results

3.1. Environmental parameters

Considering the varying grain size of the studied sediments along the Danube River sections (Opreanu et al. 2007; Tiron & Provansal 2010; Dutu et al. 2018; Tiron Dutu et al. 2019), the presence of identified insect larvae was correlated with the substrate type, including sand, gravelly sand, muddy sandy gravel, gravelly mud, and sandy mud sediments (Table 1). All analyzed species were found in sandy and gravelly sandy substrates, except for *S. morio,* while only *H. bulgaromanorum*, *T. bicolor* and *S. morio* were encountered in areas dominated by sandy muddy sediments, and a single species (*H. bulgaromanorum*) occurred in both gravelly mud and muddy sandy gravel sediments (Tables 2, 3).

The water depth in the study area ranged from 2.8 m to 17.5 m (Table 2), depending on the sampling location. Thus, all the investigated species were present in the bathymetric range of 2.8–5 m. However, only *E. viridulum*, *T. bicolor*, *H. flava* and *H. bulgaromanorum* were found at water depths between 5 m and 8.2 m, and only *H. bulgaromanorum* was identified in waters deeper than 8.2 m (Fig. 2).

No major differences in dissolved oxygen concentrations were observed between the surveyed areas, with mean values ranging from 7.80 mg l^{-1} (Cetate) to 8.85 mg l^{-1} (Bechet; Table 2). In general, the obtained values indicate relatively unpolluted waters conducive to the development of larvae belonging to these taxonomic orders of insects (Jacob et al. 1984).

Macrophytes, represented by submerged plants (*Myriophyllum* sp., *Ceratophyllum* sp., *Potamogeton* sp.), were identified at 10 sampling sites (Table 2). Larvae of *E. viridulum*, *T. bicolor* and *A. ephemerella* were found only at sites overgrown with aquatic vegetation.

Table 3

Association of insect larvae with different types of substrate

Insect species	Order	Type of substrate				
		Sand	Gravelly sand	Muddy sandy gravel	Gravelly mud	Sandy mud
Erythromma viridulum	Odonata	+	+	-	-	-
Gomphus flavipes		+	+	-	-	-
Triaenodes bicolor	Trichoptera	+	+	-	-	+
Hydropsyche bulgaromanorum		+	+	+	+	+
Heptagenia flava	Ephemeroptera	+	+	-	-	-
Acentria ephemerella	Lepidoptera	+	+	-	-	-
Sialis morio	Megaloptera	-	-	-	-	+

(+) presence; (-) absence of insect larvae

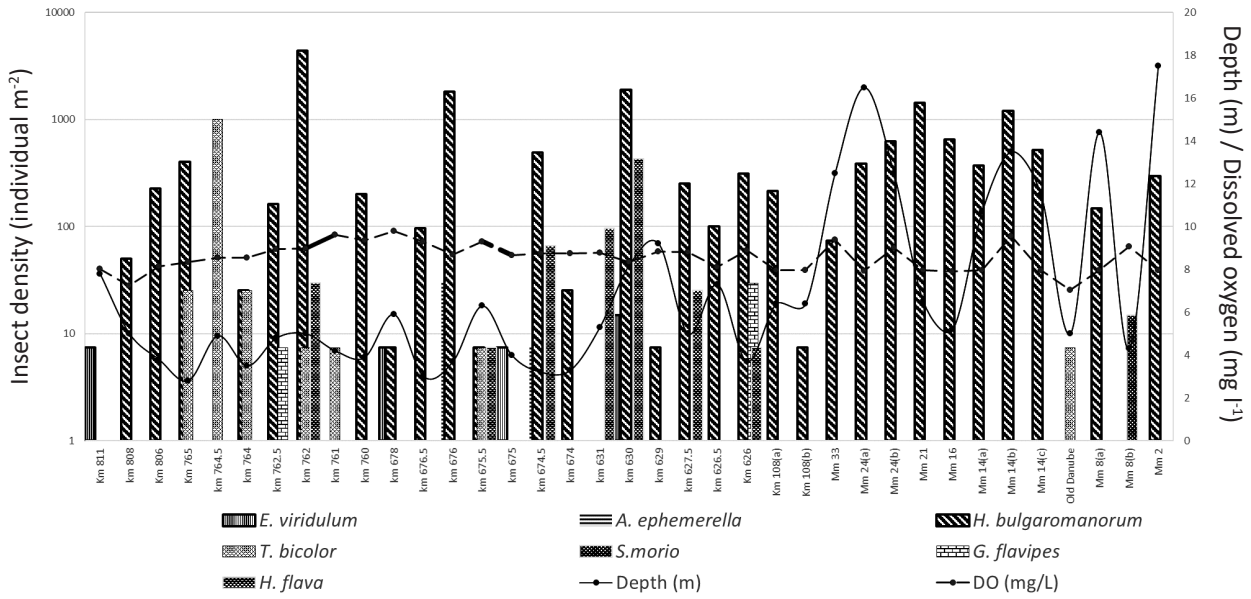

Figure 2

Densities and distribution of insect larvae, depth (m) and oxygen records in the Lower Danube River

3.2. Identification and distribution of insect larvae

Genetic identification of isolated insect larvae from all locations along the lower Danube section (Table 4) was based on sequence identity of the COI amplicon using a 97% threshold for BLAST sequence screening of the NCBI GenBank database (Hebert et al. 2003). All specimens belonging to *E. viridulum*, *G. flavipes, T. bicolor, H. bulgaromanorum, H. flava*, *A. ephemerella* and *S. morio* were identified to the species level, with an identity percentage of > 97% with homologous COI gene sequences (Table 4).

In addition to molecular identification, all collected larvae species were morphologically confirmed (data not shown).

The presence and density of the seven insect species along the Danube sections showed a varying profile in relation to the type of sediment in the two consecutive years (Figs 2 & 3; Tables 1 & 2).

In 2019, *E. viridulum* was recorded at three sites (km 630, km 631, km 675), and in 2020 at two sites (km

Table 4

Best match COI gene sequence of insect larvae species from the Danube River

Insect species	Identity (%)	Cover (%)	GenBank reference
E. viridulum	99.84	100.00	MT298455.1
G. flavipes	99.36	98.00	MT298633.1
T. bicolor	99.83	99.00	KX143573.1
H. bulgaromanorum	100.00	98.00	KX104210.1
H. flava	99.84	98.00	KY262534.1
A. ephemerella	98.66	97.00	LR135742.1
S. morio	98.41	97.00	JX438311.1

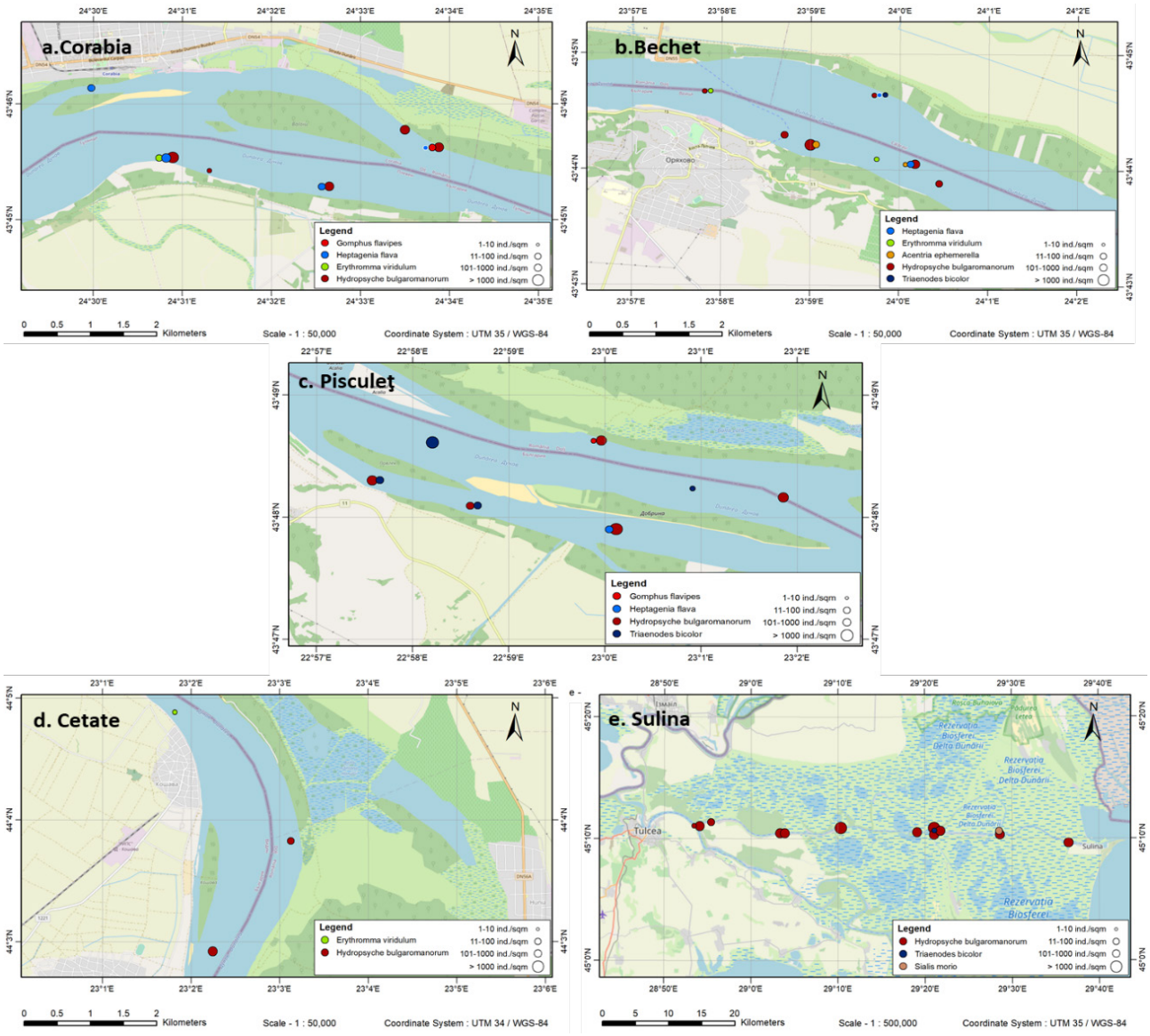

Figure 2

Densities and distribution of insect larvae, depth (m) and oxygen records in the Lower Danube River

678 and km 811). Low densities of the species were recorded at all sites of its occurrence, with the highest density detected at km 630 (14.8 ind. m^{-2}) in a gravelly sandy bed.

Larvae of *G. flavipes* were identified in 2019 at only two sites, at km 626 (29.6 ind. m^{-2}) and at km 762.5, (7.4 ind. m^{-2}) in areas characterized by sandy and gravelly sandy substrates, respectively.

T. bicolor was recorded in 2019 at four sites located between km 675.5 and km 764.5 (sandy substrate) and at the Old Danube (sandy muddy sediments). In 2020, it was found at only two sites (km 762, km 765), on sand and gravelly sand. The highest densities of the species were recorded at km 764.5, amounting to 1008 ind. m^{-2}, in areas characterized by a sandy substrate.

In 2019, larvae of *H. bulgaromanorum* were observed at 14 sites located between km 626 and km 808, 12 sites in the Sulina branch (including two sites at Ceatal Sf. George, where the Tulcea branch divides into two main distributaries, the Sulina and the St. George), in a variety of sediments consisting of sand, gravelly sand, sandy mud, muddy sandy gravel and gravelly mud. In 2020, they were encountered at only four sites (km 627.5 to km 762), in sandy and gravelly sandy bottom sediments. Their highest densities were found at km 762 (4388.2 ind. m^{-2}), followed by km 630 (1894.4 ind. m^{-2}) and km 676 (1813 ind. m^{-2}), in gravelly sandy bottom sediments.

H. flava was found in 2019 at six sites (km 626 to km 762); in 2020 it was encountered at only one site, at km 627.5 in sandy and gravelly sandy substrates. The highest abundance of the species was recorded in the gravelly sandy substrate, at km 630 (429.2 ind. m^{-2}).

Larvae of *A. ephemerella* were encountered at only two sites, at km 674.5, recording only 7.4 ind. m^{-2} (in 2019) and at km 676, reaching 29.6 ind. m^{-2} (in 2020). The species was detected in sandy and gravelly sandy bottom sediments, respectively.

S. morio larvae were found in 2019, at only one site, on the Sulina branch, reaching 14.2 ind. m^{-2}, in sandy mud.

4. Discussion

Due to their sensitivity to several abiotic and biotic factors, immature stages of aquatic insects are particularly useful for monitoring the water quality in freshwater ecosystems (Arimoro & Ikomi 2008; Barman & Gupta 2015). As such, our study addressed the identification and distribution of several biological indicators in the Danube section that crosses the Romanian territory to highlight their presence at different depths and in different substrates.

In Romania, the first detailed taxonomic, biological, and ecological studies of Odonata were performed by Cîrdei & Bulimar (1969), and by Bulimar (1973, 1976, 1993). Odonata larvae were used as bioindicators of anthropogenic disturbances, as shown for *E. viridulum* (Buczyńska & Buczyński 2019). Therefore, the results of a survey performed in two areas of the El-Kebir-East hydrosystem with different degrees of human impact showed that the species was only found at less disturbed sites characterized by good water quality with high concentrations of dissolved oxygen (Benchalel et al. 2017), as in our case. As a cosmopolitan species, it is widespread in most European countries (de Jong. et al. 2014). It was previously reported in the Romanian reaches of the Danube near the city of Galați (Șerban & Cristescu 2013), Timiş (Manci 2016), and Sf. Gheorghe (de Vries et al. 2017). Furthermore, small populations were reported from the Danube Delta (Mahmudia, Murighiol and Caraorman areas; de Vries et al. 2017). Our current study revealed its presence in only two of the Romanian Danube sectors, Bechet and Cetate. Although previous studies (Carchini et al. 2003; Benchalel et al. 2013) identified this species at water depths < 1m, the current study revealed its presence at greater depths (4–8.2 m). Similar to our results, Heidemann & Seidenbusch (2002) found this species in areas with aquatic vegetation, represented mainly by species from the genera *Ceratophyllum* and *Myriophyllum*.

G. flavipes, an indicator species of less polluted sections of large rivers, listed in Annex IV of the Habitats Directive, is currently no longer considered a threatened species in Europe and its populations are expanding (Kalkman et al. 2010). The species was reported from the Danube Delta, Banat, Galați and Suceava counties (Cîrdei & Bulimar 1965; Bulánková et al. 2013), on the banks of the Danube, and the access pier to the meander of the Danube Zătuni-Cotul Pisicii (Șerban & Cristescu 2013). This species showed the highest prevalence among the European species of the genus *Gomphus*, spreading from France to eastern Siberia in the Sava, Tisza, Danube and Morava rivers (Anđus 1992; Rajkov & Šćiban 2012), with very abundant populations identified in the Small Danube and the Danube Delta (Šibl et al. 2003; Bulánková et al. 2013). In contrast, only one individual of this species was detected in the Slovak section of the Danube during a 20-year survey (Dolny & Bulánková 2006). Large and stable populations of *G. flavipes* were encountered along the entire Romanian section of the Danube, in areas characterized by a silty substrate, within a wide range of water depths, from shallow (< 3 m) to 30 m deep waters (Pavel et al. 2019). However, our survey revealed the presence of this species at only two sites, at km 626 and 762.5 at a depth of 3.7 m and 4.8 m, respectively, in the Corabia and Pisculeţ sections. It was shown that both *G. flavipes* and *E. viridulum* prefer areas with reduced hydrological connectivity and dynamics and high rates of sedimentation, dominated by sandy substrates (Schultz et al. 2003; Bulánková et al. 2013). Our results were consistent with these findings, as samples were collected from a similar substrate.

Another cosmopolitan species recorded during our survey was *T. bicolor*. The species was recorded in the Danube Delta area (Ciubuc 2004), in the German and Austrian sections of the Danube River (Moog et al. 1994; Graf et al. 2006), and in the Prut River (Munjiu 2014). The current study revealed the presence of this species in the Bechet and Pisculet sections, as well as in the Sulina branch. Furthermore, our data confirmed the presence of larvae in sandy and gravelly sandy substrates, as previously suggested by van den Brink et al. (2013). These larvae feed on aquatic plants and build cases from macrophytes tissues, developing abundant populations in areas rich in aquatic vegetation (Graf et al. 2008), suggesting their preference for areas dominated by submerged vegetation, as confirmed by our survey. This Trichoptera species was reported as a marker for oxygen-rich waters of unpolluted environments (Nijboer & Goedhart 2006). While a recent study by Buczyńska et al. (2018) reported its presence at depths of up to 2 m in waters

characterized by low oxygen concentrations, our report indicates that the depth for *T. bicolor* in the Danube River is up to 6.3 m.

Larvae of the caddisfly *H. bulgaromanorum* were found during our investigation in all analyzed Romanian sections of the Danube River, including the Sulina branch. It is a widespread species in Europe (Malicky 1984), colonizing the Austrian, German, Slovak and Serbian sections of the Danube (Moog et al. 1994; Elexová & Nemethová 2003; Paunovic et al. 2007), inhabiting areas with a hard benthic substrate containing stones and gravel (Pîrvu et al. 2012). Our data indicating the presence of this species in several types of substrates (sand, mud, gravel) have expanded the knowledge of the types of aquatic habitats of this insect's larvae. Moreover, although previous studies considered the distribution of *H. bulgaromanorum* as correlated with the stream velocity (van den Brink et al. 2013), being restricted to the lotic habitat (eupotamon), Ciubuc (2004) found this species in the Danube Delta (including the Razelm Sinoe Lagoon System).

Furthermore, the reported eurybathic character of this species (Savić et al. 2013) appeared to be consistent with our findings that identified an abundant population in both shallow (< 5 m) and deep (> 10 m) Danube locations, suggesting that depth variation is not a limiting factor for its distribution.

So far, the eurybiontic mayfly *H. flava* has been reported along the Bulgarian, Serbian and Austrian Danube sections (Russev 1992; Moog et al. 1994; Vidinova & Russev 1997; Petrovic et al. 2015). In Romania, this species was observed in the Vişeu and Tejeajen rivers, establishing abundant populations in unpolluted environments (Curtean-Bănăduc 2009; Sava 2016–2017). However, Vidinova & Russev (1997) found larvae in heavily polluted areas with low oxygen concentrations, as well as living in fragments of decaying vegetation, under stones and gravel. The preference for such living substrates was also confirmed in our study, showing high abundance of larvae in substrates characterized by a significant amount of gravel. During an investigation conducted at 46 lotic slow-flowing study sites located in the Pannonian lowland ecoregion in Croatia, a single individual of *H. flava* was identified in fine sediments at a depth of 1 m (Vilenica et al. 2020). Our findings along the Pisculeţ, Bechet and Corabia sections of the Danube showed its presence at different depths, ranging from 3.2 m to 8.2 m.

The herbivorous caterpillar *A. ephemerella* is one of the few insect species that can live underwater throughout its entire life cycle (Miller & Bargsten 2016). It is commonly found in lakes and coastal waters throughout Europe (Berg 1941; Gross et al. 2001), including the Austrian part of the Danube (Moog et al. 1994). In our study, this caterpillar was found at only two sites in the Bechet section. Similar to our results, where *A. ephemerella* was found in areas with macrophytes and a significant amount of organic matter, the study conducted by Gross et al. (2002) revealed that this species occurs in high abundance, feeding also on many submerged macrophytes, especially in areas with *Potamogeton* sp., *Ceratophyllum demersum* (Linnaeus, 1753) and *Myriophyllum spicatum* (Linnaeus 1753). This aquatic moth is an important herbivore insect feeding on aquatic macrophytes (Gross et al. 2002). Therefore, biomonitoring of this species has demonstrated its potential as a biological control agent limiting the growth of the Eurasian watermilfoil *M. spicatum* (Johnson et al. 1998; Newman 2004).

The alderfly *S. morio* is also a widespread species in Europe, previously found in Norway, Sweden, Finland, Slovakia, countries of former Yugoslavia, and Romania (Aspöck et al. 1980; Meinander 1996; Ábrahám & Kovács 1999; Tierno del Figueroa et al. 2002). *S. morio* larvae were found in both lentic and lotic ecosystems (Kaiser et al. 1977; Aspöck et al. 1980). Even though several studies (Aspöck et al. 1980; Ábrahám & Kovács 1999) highlighted the eurybiontic character of this species, the tolerance to hostile environmental conditions and the vertical distribution ranging from lowlands to higher altitudes, Yakovlev (2009) indicated that the species is mostly associated with lowland lakes. This could explain the absence of this alderfly in most of the study areas, being found only at one site in the Sulina branch (Meander) characterized by a muddy substrate and slow water flow.

The dissolved oxygen content in water appeared to be one of the most important environmental factors for the survival, development, and reproduction of aquatic insects, which generally require high concentrations of dissolved oxygen to complete their life cycle (Hossain et al. 2015; Prommi & Payakka 2015). In our study, relatively high concentrations of dissolved oxygen were recorded in all analyzed Danube areas. Despite this, only *H. bulgaromanorum* was encountered at most sites, showing a wide distribution along the Romanian sections of the river. This could be attributed to its high ecological plasticity, as indicated by both previously published results and the current study.

Given the importance of these organisms as biological indicators, their accurate taxonomic identification is essential. Thereby, DNA barcoding was used during our survey as the main method for taxonomic identification of species, being a successfully applied technique in biodiversity

assessment studies (Cordero et al. 2017). The morphological investigation of larvae failed to identify the taxonomic characters of *S. morio*. As such, DNA barcoding proved to be effective in resolving this intricacy, given that, in addition to this species, two others were reported in Romania, *S. fuliginosa* (Pictet 1836) and *S. lutaria* (Linnaeus 1758) (Aspöck et al. 2001; Devetak et al. 2016). Moreover, Ábrahám & Kovács (1999) showed difficulties during taxonomic identification based on morphological characters due to high degree of phenotypic similarities between *S. morio* and *S. lutaria*. Several studies (Doskocil et al. 2008) focused on the species-level resolution for insects, showing that conventional taxonomic methods can be ambiguous and may lead to inaccurate results. In this regard, Jalalizand et al. (2012) reported challenges in identifying aphids, while Talbalachi & Shaikevich (2010) and Chan et al. (2014) in identifying Diptera species based on morphology. Nevertheless, the COI gene fragment amplification for identification of aquatic insect species was used as a sensible tool in recent studies (Molina et al. 2017; Pramual et al. 2020). For most of the currently examined species, only a limited number of nucleotide sequences are available in the reference databases for DNA barcodes (Benson et al. 2007; Ratnasingham & Hebert 2007). Also, few studies focused on the molecular identification and characterization of these taxa. In some of these studies, *E. viridulum* and other damselflies and dragonflies populating the water bodies of Vienna were identified using DNA barcoding and the eDNA approach (Fisher et al. 2018). Studies on the distribution of Odonata and Trichoptera species in Italian environments using molecular methods also focused on *E. viridulum* and *G. flavipes* (Galimberti et al. 2020) and *H. bulgaromanorum* (Geraci et al. 2011; Kučinić et al. 2018), respectively.

The current data, advancing the knowledge about the spatial distribution and abundance of Odonata, Trichoptera, Ephemeroptera, Lepidoptera and Megaloptera larvae in the lower Danube River, provide a novel insight into their role as freshwater biological indicators. Aquatic insects are among the most sensitive organisms to changes in environmental conditions, therefore further research should be undertaken to assess their ecological role in relation to various environmental parameters that may affect their distribution.

Acknowledgements

The authors thank Victoria Ioana Paun and Mihaela Muresan for technical support. The study was financially supported by the Romanian Ministry of Research as part of the CORE Programme projects PN 19200302, PN 19200204, PN19200401, and PN 19200203, RO1567/2021, and the ERANET-MARTERA-MOBILTOX-2-224 H2020 project.

References

Ábrahám, L. & Kováks, T. (1999). A report on the Hungarian alderfly fauna (Megaloptera: Sialidae). A Janus Pannonius Múzeum Évkönyve. 43(1998), 49–56.

Andem, A B., Okorafor, K. A., Udofia, U., Okete, J. A., & Ugwumba, A. A. (2012). Composition, distribution and diversity of benthic macroinvertebrates of Ona River, South-west, Nigeria. European Journal of Zoological Research., 1(2), 47–53.

Anđus, Lj. (1992). New data on the distribution of Odonata in Serbia. Bulletin of the Natural History Museum in Belgrade. Ser. B., 47, 149–170.

Arimoro, F. O., & Ikomi, R. B. (2009). Ecological integrity of upper Warri River, Niger Delta using aquatic insects as bioindicators. *Ecological Indicators, 9*(3), 455–461. https://doi.org/10.1016/j.ecolind.2008.06.006

Aspöck, H., Aspöck, U., & Hölzel:, H. (1980). The neuropterans of Europe I-II. Goecke and Evers. (In German)

Aspöck, H., Hölzel, H., & Aspöck, U. (2001). Annotated catalog of the Neuropterida (Insecta: Raphidioptera, Megaloptera, Neuroptera) of the Western Palearctic. Austria [In German]. Denisia, 2, 1–606

Baird, D. J., & Hajibabaei, M. (2012). Biomonitoring 2.0: a A new paradigm in ecosystem assessment made possible by next-generation DNA sequencing. Molecular Ecology, 21(8), 2039–2044. https://doi.org/10.1111/j.1365-294X.2012.05519.x PMID:22590728

Balian, E. V., Segers, H, Lévèque, C.,., & Martens, K. (2008). The freshwater animal diversity assessment:An overview of the results. Hydrobiologia, 595(1), 627–637. 10.1007/s10750-007-9246-3

Barman, B., & Gupta, S. (2015). Aquatic insects as bio-indicator of water quality - A study on Bakuamari stream, Chakras hila Wildlife Sanctuary, Assam, North East India. Journal of Entomology and Zoology Studies, 3(3), 178–186.

Baurenfeind, E., & Soldán, T. (2012). The Mayflies of Europe (Ephemeroptera). Apollo Books. https://doi.org/10.1163/9789004260887

Barrett, R. D. H., & Hebert, P. D. N. (2005, March 01). Identifying spiders through DNA barcodes. *Canadian Journal of Zoology, 83*(3), 481–491. https://doi.org/10.1139/z05-024

Begun, T., Teaca, A., Muresan, M., & Pavel, A. B. (2020). Current state of mollusc populations in the Razim-Sinoe Lagoon System. Scientific Papers. Series D. Animal Science (Penicuik, Scotland), LXIII(1), 553–561.

Benchalel, S., Merah, S., Bouslama, Z., Ramdani, M., Elmsellem,

H. & Flower, R., (2017). Odonata as indicators of environmental impacts in rivers, case of wadi El-Kébir-East (northeastern Algeria). Moroccan Journal of Chemistry., 5, 610–621. 10.48317/IMIST.PRSM/morjchem-v5i4.9762

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Wheeler, D. L. (2007). GenBank. Nucleic Acids Research, 35(Database), D21–D25.

Berg, K. (1941). Contributions to the biology of the aquatic moth Acentropus niveus (Oliv.). Videnskabelige meddelelser fra den Naturhistoriske forening i Kjöbenhavn. 105, 59–139.

Bondar, C., & Panin, N. (2001). The Danube Delta Hydrologic Database and Modeling. Geo-Eco-Marina, 5, 5–52.

Bouchard, R. W. Jr. (2004). Guide to aquatic macroinvertebrates of the Upper Midwest. Minnesota: Water Resources Center, University of Minnesota, St. Paul. 208 pp.

Buczyńska, E., Szlauer-Łukaszewska, A., Czachorowski, S., & Buczyński, P. (2018). Human impact on large rivers: The influence of groynes of the River Oder on larval assemblages of caddisflies (Trichoptera). *Hydrobiologia*, 819(1), 177–195. https://doi.org/10.1007/s10750-018-3636-6

Buczyńska, E., & Buczyński, P. (2019). Aquatic Insects of Man-Made Habitats: Environmental Factors Determining the Distribution of Caddisflies (Trichoptera), Dragonflies (Odonata), and Beetles (Coleoptera) in Acidic Peat Pools. *Journal of Insect Science*, 19(1), 17. 10.1093/jisesa/iez005

Buczyński, P., Zawal, A., Stępień, E., Buczyńska, E. & Pešić V. (2013). Contribution to the knowledge of dragonflies (Odonata) of Montenegro, with the first record of Ophiogomphus cecilia (Fourcroy, 1785). Annales Universitatis Mariae Curie-Skłodowska Lublin-Polonia. Vol. LXVIII, 2. Sectio C: 57-71. DOI: http://dx.doi.org/10.17951/c.2013.68.2.57.

Buczyńska, E., & Buczyński, P. (2019). Survival under anthropogenic impact: The response of dragonflies (Odonata), beetles (Coleoptera) and caddisflies (Trichoptera) to environmental disturbances in a two-way industrial canal system (central Poland). PeerJ, 6, e6215. https://doi.org/10.7717/peerj.6215 PMID:30643685

Bulánková, B. E., Beracko, P., & Derka, T. (2013). Occurrence of protected species (Gomphus flavipes, Odonata and Palingenia longicauda, Ephemeroptera) in the Danube River and its deltas (Romania, Slovakia). *Sci. Ann. Danube Delta Inst.*, 19, 21–24. 10.7427/DDI.19.03

Boardman, J. (2013). Soil erosion in Britain: Updating the record. Agriculture, 3(3), 418–442. . https://doi.org/10.3390/agriculture3030418

Bulimar, F. (1973). Ecology of Odonata larvae (Odonata Ord., Insecta Cl.) from Danube Delta. [In Romanian]. Annals of the "Alexandru Ioan Cuza" University of Iaşi 19, 171–178

Bulimar, F. (1976). Comparative study of the buccal apparatus of Odonata larvae (Insecta Cl.). [In Romanian]. *Nymphaea.*, 4, 145–159

Bulimar, F. (1993). Study of the Odonata fauna from the Danube Delta and neighboring areas. Scientific Annals of the Danube Delta Institute: 87–91. (In Romanian).

Van den Brink, F. W. B., Van der Velde, G., & Wijnhoven, S. (2013). Seasonal changes in caddis larvae assemblages in river-floodplain habitats along a hydrological connectivity gradient. *Hydrobiologia*, 716(1), 75–85. https://doi.org/10.1007/s10750-013-1545-2

Carchini, G., Di Domenico, M., Pacione, T., Solimini, A. G., & Tanzilli, C. (2003). Species distribution and habitat features in lentic Odonata. *The Italian Journal of Zoology, 70*(1), 39–46. https://doi.org/10.1080/11250000309356494

Ciubuc, C. (2004). Trichoptera (Insecta) of the Danube Delta Reserve and Razim–Sinoe Lagoon System. Travaux du Muséum d'Histoire Naturelle „Grigore Antipa". 47, 211–231. (In Romanian).

Ciutti, F., Cappelletti, C., Monauni, C., & Siligardi, M. (2004). Influence of substrate composition and current velocity of macroinvertebrates in a semi-artificial system. *Journal of Freshwater Ecology*, 19(3), 455–460. 10.1080/02705060.2004.9664919

Cîrdei, F., & Bulimar, F. (1965). Fauna of the Romanian people's repuplic. Insecta – Odonata Ord. 7(5). Bucharest, Romania: Ed. Academiei. 247 pp. (In Romanian).

Cîrdei, F., & Bulimar, F. (1969). Contributions to the study of Odonata fauna (Odonata Ord.) on the territory of the future accumulation lake "Iron Gates". Annals of the "Alexandru Ioan Cuza" [In Romanian]. University of Iaşi., 15, 329–334

Chan, A., Chiang, L. P., Hapuarachchi, H. C., Tan, C. H., Pang, S. C., Lee, R., Lee, K. S., Ng, L. C., & Lam-Phua, S. G. (2014). DNA barcoding: Complementing morphological identification of mosquito species in Singapore. *Parasites & Vectors, 7*(1), 569. https://doi.org/10.1186/s13071-014-0569-4 PMID:25498759

Chiriac, G. (2004). The communities of macro-invertebrates in the Romanian part of the Danube, which were identified along the JDS – 2001 Expedition. *Eco Terra (Cluj-Napoca)*, 3, 23–24.

CodonCode Corporation. (2003). Codoncode aligner version 9.0. https://www.codoncode.com/

Cordero, R. D., Sánchez-Ramírez, S., & Currie, D. C. (2017). DNA barcoding of aquatic insects reveals unforeseen diversity and recurrent population divergence patterns through broad-scale sampling in northern Canada. *Polar Biology, 40*(8), 1687–1695. https://doi.org/10.1007/s00300-016-2062-3

Cretescu, I., Kovacs, Z., Lazar, L., Burada, A., Sbarcea, M., Teodorof, L., Padure, D., & Soreanu, G. (2021). Danube Delta: Water Management on the Sulina Channel in the Frame of Environmental Sustainability. IntechOpen. https://doi.org/10.5772/intechopen.97877. Available from: https://www.intechopen.com/online-first/77075

Curtean-Bănăduc, A. (2009). The Maramureş Mountains Nature Park (Romania) mayfly (Insecta, Ephemeroptera) communities diveristy analyse. *Transylvanian Review of*

Systematical and Ecological Research, 8(8), 95–104.

Crisci-Bispo, V. L., Bispo, P. C., & Froehlich, C. G. (2007). Ephemeroptera, plecoptera and trichoptera assemblages in two Atlantic Rainforest streams, Southeastern Brazil. *Revista Brasileira de Zoologia*, 24(2), 312–318. https://doi.org/10.1590/S0101-81752007000200007

Czachorowski, S., & Serafin, E. (2004). The distribution and ecology of Hydropsyche bulgaromanorum and Hydropsyche contubernalis (Trichoptera: Hydropsychidae) in Poland and Belarus. *Lauterbornia*, 50, 85–98.

Davidson, T. A., Reid, M. A., Sayer, C. D., & Chilcott, S. (2013). Palaeolimnological records of shallow lake biodiversity change: Exploring the merits of single versus multi-proxy approaches. *Journal of Paleolimnology*, 49(3), 431–446. https://doi.org/10.1007/s10933-013-9696-8

Devetak, D., Slavevska-Stamenković, V., & Sivec, I. (2016). Alderflies (Insecta: Megaloptera: Sialidae) from Serbia and Macedonia, with Notes on their Occurrence in the Neighbouring Balkan Countries. *Acta Zoologica Bulgarica, 68*(1), 39–42.

Dincă, V., Zakharov, E. V., Hebert, P. D. N., & Vila, R. (2011). Complete DNA barcode reference library for a country's butterfly fauna reveals high performance for temperate Europe. Proceedings. Biological Sciences, 278(1704), 347–355. https://doi.org/10.1098/rspb.2010.1089

Dincă, V., & Székely, L. (2018). First record of *Scopula orientalis* (Alphéraky, 1876) (Lepidoptera, Geometridae) in Romania, at the northern limit of the Balkans. *Nota Lepidopterologica*, 41(2), 189–197. https://doi.org/10.3897/nl.41.24316

Dolny, A. & Bulánková, E. (2006). Distribution and ecology of priority dragonfly species (Odonata) in Czech and Slovak Republics. NUA-Heft. 45–45.

Duţu, F., Panin, N., Ion, G., & Tiron Duţu, L. (2018). Multibeam Bathymetric Investigations of the Morphology and Associated Bedforms, Sulina Channel, Danube Delta. *Geosciences*, 8(1), 7. https://doi.org/10.3390/geosciences8010007

Elexová, E., & Némethová, D. (2003). The effect of abiotic environmental variables on the Danube macrozoobenthic communities. *Limnologica*, 33(4), 340–354. https://doi.org/10.1016/S0075-9511(03)80028-2

Farkas, A., Mérő, T. O., Móra, A., & Dévai, G. Y. (2014). Urban dragonflies: Data on the odonata fauna of the Danube at Budapest. *Acta Biologica Debrecina Supplementum Oecologica Hungarica*., 32, 23–29.

Fischer, I., Sittenthaler, M., Traugott, M., Thalinger, B., Zangl, L., Koblmüller, S., Kunz, G., Chivanec, A., Sattmann, H., Randolf, S., & Haring, E. (2018). Monitoring and DNA barcoding of dragonflies (Insecta: Odonata) in Vienna, Austria. *Acta ZooBot Austria, 155*(2), 39–42.

Folk, R. L. (1954). The distinction between grain size and mineral composition in sedimentary rock nomenclature. *The Journal of Geology*, 62(4), 344–359. https://doi.org/10.1086/626171

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology, 3*(5), 294–299. PMID:7881515

Galimberti, A., Assandri, G., Maggioni, D., Ramazzotti, F., Baroni, D., Bazzi, G.,– Chiandetti, I., Corso, A., Ferri, V., Galuppi, M., Ilahiane, L., La Porta, G., Laddaga, L., Landi, F., Mastropasqua, F., Ramellini, S., Santinelli, R., Soldato, G., Surdo, S., & Casiraghi, M. (2021). Italian odonates in the Pandora's box: A comprehensive DNA barcoding inventory shows taxonomic warnings at the Holarctic scale. *Molecular Ecology Resources*, 21(1), 183–200. https://doi.org/10.1111/1755-0998.13235 PMID:32755053

Geraci, C. J., Al-Saffar, M. A., & Zhou, X. (2011). DNA barcoding facilitates description of unknown faunas: A case study on Trichoptera in the headwaters of the Tigris River, Iraq. *Journal of the North American Benthological Society*, 30(1), 163–173. https://doi.org/10.1899/10-011.1

Gómez-Baggethun, E., Tudor, M., Doroftei, M., Covaliov, S., Năstase, A., Onără, D. F.,- Mierlă, M., Marinov, M., Doroșencu, A.-C., Lupu, G., Teodorof, L., Tudor, I.-M., Köhler, B., Museth, J., Aronsen, E., Ivar Johnsen, S., Ibram, O., Marin, E., Crăciun, A., & Cioacă, E. (2019). Changes in ecosystem services from wetland loss and restoration: An ecosystem assessment of the Danube Delta (1960–2010). *Ecosystem Services*, 39, 100965. https://doi.org/10.1016/j.ecoser.2019.100965

Graf, W. J., Waringer, P., Chovanec, P. W., & Moog, O. (2006). The river Danube – biodiversity and habitat assessment based on Trichoptera assemblages. In Proceedings of the 36th International Conference of the International Association of the Danube, 241-245. Vienna: Austrian Communications of the Danube Research.

Graf, W. J., Murphy, J., Dahl, J., Zamora-Muñoz, C., & López-Rodríguez, M. J. (2008). Distribution and ecological preferences of European freshwater organisms. In A. Schmidt-Kloiber & D. Hering (Eds.), Trichoptera (Vol. 1). Pensoft Publishers.

Gross, E. M., Johnson, R. L., & Hairston, N. G., Jr. (2001). Experimental evidence for changes in submersed macrophyte species composition caused by the herbivore Acentria ephemerella (Lepidoptera). *Oecologia, 127*(1), 105–114. https://doi.org/10.1007/s004420000568 PMID:28547160

Gross, E. M., Feldbaum, C., & Choi, C. (2002). High abundance of herbivorous Lepidoptera larvae (*Acentria ephemerella* DENIS & SCHIFFERMÜLLER) on submersed macrophytes in Lake Constance (Germany). *Fundamental and Applied Limnology*, 155(1), 1–21. https://doi.org/10.1127/archiv-hydrobiol/155/2002/1

Hamsi, N. A., Norazlimi, N. A., & Abd-Latip, N. F. (2021). Abundance of Aquatic Insects in Spatial Variation of Two Rivers in Perak, Malaysia. In IOP Conf. Ser.: Earth Environ. Sci. 736 01201.Hebert, P. D. N., Cywinska, A., Ball, S. L., &

deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, 270(1512), 313–321.

Heidemann, H. & Seidenbusch, R. (2002). Larves et exuvies des libellules de France et d'Allemagne (sauf de Corse). France: Société française d'odonatologie. (In French).

Hershey, A. E., & Lamberti, G. A. (2001). Aquatic insect ecology. In J. P. Thorp & A. P. Covich (Eds.), Ecology and Classification of North American Freshwater Invertebrates (2nd- ed., pp. 733–775. Academic Press. https://doi.org/10.1016/B978-012690647-9/50019-3

Hickin, N. E. (1967). Caddis Larvae: Larvae of the British Trichoptera. Associated University Presses, Inc.

Hossain, S., Aslam, A. F. M., Saha, B., & Howlader, A. J. (2015). Abundance of aquatic insects in relation to physico-chemical parameters of two highly polluted Rivers Sitalakkhya and the Buriganga. *Bangladesh Journal of Zoology*, 43(1), 63–72. https://doi.org/10.3329/bjz.v43i1.26138

Iancu, L., Carter, D. O., Junkins, E. N., & Purcarea, C. (2015). Using bacterial and necrophagous insect dynamics for post-mortem interval estimation during cold season: Novel case study in Romania. *Forensic Science International*, 254, 106–117. https://doi.org/10.1016/j.forsciint.2015.07.024 PMID:26217916

Jalalizand, A. R., Mirhendi, H., Karimi, A., Modaresi, M., & Mahmoodi, E. (2012). Morphological and Molecular Identification Aphids of Rosae. *APCBEE Procedia, 4*, 12–15. https://doi.org/10.1016/j.apcbee.2012.11.003

Johnson, R. L., Gross, E. M., & Hairston, N. G., Jr. (1997). Decline of the invasive submerged macrophyte Myriophyllum spicatum (Haloragaceae) associated with herbivory by larvae of Acentria ephemerella (Lepidoptera). *Aquatic Ecology, 31*(3), 273–282. https://doi.org/10.1023/A:1009960131857

de Jong, Y., Verbeek, M., Michelsen, V., Bjørn, P. P., Los, W., Steeman, F., Bailly, N., Basire, C., Chylarecki, P., Stloukal, E., Hagedorn, G., Wetzel, F. T., Glöckler, F., Kroupa, A., Korb, G., Hoffmann, A., Häuser, C., Kohlbecker, A., Müller, A., . . . Penev, L. (2014). Fauna Europaea - all European animal species on the web. *Biodiversity Data Journal, 2*, e4034. https://doi.org/10.3897/BDJ.2.e4034 PMID:25349527

Kaiser, E. W. (1977). Eggs and larvae of six Sialis species from Scandinavia and Finland (Megaloptera, Sialidae). *Flora Fauna Arhus*., 83, 65–79.

Kamsia, B., Amran, A., Noraini, A., & Maryam, D. (2007). Correlation analysis on water quality parameter with aquatic insects' abundance in Telipok River, Sabah, Malaysia. In 12th WSEAS International Conference on Applied Mathematics 12, 29–31.

Kalkman, V. J., Boudot, J. P., Bernard, R., Conze, K. J., De Knijf, G., ... (2010). European Red List of Dragonflies. Publications Office of the European Union., https://doi.org/10.2779/84650

Kristensen, N. P. (1981). Phylogeny of insect orders. *Annual Review of Entomology, 26*, 135–157. https://doi.org/10.1146/annurev.en.26.010181.001031

Krno, I., Beracko, P., Navara, T., Šporka, F., & Mišíková Elexová, E. (2018). Changes in species composition of water insects during 25-year monitoring of the Danube floodplains affected by the Gabčíkovo waterworks. *Environmental Monitoring and Assessment, 190*(7), 412. https://doi.org/10.1007/s10661-018-6773-5 PMID:29926194

Kučinić, M., Čukušić, A., Pdnar, M., Žalac, S., Ćuk, R., Cerjanec, D., & Vučković, I. (2018). DNA barcoding of Croatian faunal biodiversity with notice of caddisflies (Insecta, Trichoptera). Poster In. 16th International Symposium on Trichoptera. 3-9 June 2018. Surat Thani, Thailand.

Lecureuil, J. Y., Chovet, M., Bournaud, M., & Tachet, H. (1983). Description, répartition et cycle biologique de la larve d'Hydropsyche bulgaromanorum Malicky 1977 (Trichoptera, Hydropsychidae) dans la Basse Loire. *Annales de Limnologie, 19*(1), 17–24. https://doi.org/10.1051/limn/1983001

Lilly, C. K., Ashley, D. L., & Tarter, D. C. (1978). Observations on a population of Sialis Itasca Ross in West Virginia. *Psyche, 85*, 209–217. https://doi.org/10.1155/1978/96295

Malicky, M. (1984). The distribution of Hydropsyche guttata Pictet and H. bulgaromanorum Malicky (Trichoptera: Hydropsychidae), with notes on their bionomics. *Entomologist's Gazette*, 35, 257–264.

Manci, C. O. (2016). Dragonflies (Insecta, Odonata) in Timiş County (Banat, Romania), A general view of distribution. *Acta Oecologica Carpatica*., IX, 85–120.

Mazzucco, R., & Nguyen, T. V., Dong-Hwan Kim, Chon T.S. & Dieckmann, U. (2015). Adaptation of aquatic insects to the current flow in streams. Ecological Modelling. 309-310: 143-152. https://doi.org/10.1016/j.ecolmodel.2015.04.019

Meinander, M. (1996). Megaloptera Sialidae, Alder Flies: 105-110, In: Nilsson, A.N. (Eds.), Aquatic Insects of North Europea Taxonomic Handbook. Vol. 1. (pp. 105-110). Denmark: Apollo Books.

Miller, K. B., & Bergsten, J. (2016). Diving beetles of the World. Systematics and Biology of the Dytiscidae. Johns Hopkins University Press.

Moog, O., Konar, M., & Humpesch, U. H. (1994). The macrozoobenthos of the River Danube in Austria. *Lauterbonia H*., 15, 25-51.

Molina, C. I., Gibon, F. M., Domingue, E., Pape, T., & Rønsted, N. (2017). Associating immatures and adults of aquatic insects using DNA barcoding in high Andean streams. *Ecología en Bolivia*, 52(2), 88–99.

Munjiu, O., Toderaş, I., Zubcov, E., Bietchi, L., & Subernetk, I. (2014). Composition and distribution of benthic macroinvertebrates in the Pruth River (2012-2013). Annals of the "Alexandru Ioan Cuza" University of Iaşi, s. Animal biology. Tom, LX, 27–34.

Mureşan, M., Teacă, A., Popa, A., & Begun, T. (2019). Free-living

marine nematodes community structural changes within a post-dredging site at the Romanian shelf. *Journal of Environmental Protection and Ecology*, 20(2), 753–760.

Nasirian, H., & Irvine, K. N. (2017). Odonata larvae as a bioindicator of metal contamination in aquatic environments: Application to ecologically important wetlands in Iran. *Environmental Monitoring and Assessment*, 189(9), 436. https://doi.org/10.1007/s10661-017-6145-6 PMID:28779428

Navajas, M., & Fenton, B. (2000). The application of molecular markers in the study of diversity in acarology: A review. *Experimental & Applied Acarology, 24*(10-11), 751–774. https://doi.org/10.1023/A:1006497906793 PMID:11345314

Navara, T., Kokavec, I., Cíbik, J., Lukáš, J., & Chvojka, P. (2020, December). Adicella syriaca (Trichoptera: Leptoceridae) in Slovakia – the northernmost record from Central Europe. Spreading of an aquatic insect in the period of climate change? *Biologia*, 75(12), 2321–2326. https://doi.org/10.2478/s11756-020-00486-y

Newman, R. M. (2004). Invited review: Biological control of Eurasian watermilfoil by aquatic insects: basic insights from an applied problem. *Archiv für Hydrobiologie*, 159(2), 145–184. https://doi.org/10.1127/0003-9136/2004/0159-0145

Nijboer, R. C., Park, Y. S., Lek, S., & Verdonschot, P. F. M. (2005). Comparison of clustering and ordination methods implemented to the full and partial data of benthic macroinvertebrate communities in streams and channels. In S. Lek, M. Scardi, F. M. Verdonschot, J.-P. Descy, & Y. S. Park (Eds.), Modelling Community Structure in Freshwater Ecosystems (pp. 167–188). Springer., 10.1007/3-540-26894-4_16

Opreanu, G., Oaie, G., & Păun, F. (2007). The dynamic significance of the grain size of sediments transported and deposited by the Danube. *Geo-Eco-Marina, 13/2007*, 111–119. https://doi.org/10.5281/zenodo.57352

Paunovic, M. M., Jakovcev-Todorovic, D. G., Simic, V. M., Stojanovic, B. D., & Cakic, P. D. (2007). Macroinvertebrates along the Serbian section of the Danube River (stream km 1429–925). *Biologia*, 62(2), 214–221. https://doi.org/10.2478/s11756-007-0032-5

Pavel, A. B., Menabit, S., Skolka, M., Lupaşcu, N., Pop, I. C., Opreanu, G., Stănescu, I., & Scrieciu, A. (2019). New data regarding the presence of two insect larvae species – *Gomphus* (Stylurus) *flavipes* (Odonata) and *Palingenia longicauda* (Ephemeroptera) in the Lower Sector of the Danube River. *Geo-Eco-Marina, 25/2019*, 253–264. https://doi.org/10.5281/zenodo.3611187

Petrović, A., Milošević, D., Paunović, M., Simić, S., Đorđević, N., Stojković, M., & Simić, V. (2015). New data on the distribution and ecology of the mayfly larvae (Insecta: Ephemeroptera) of Serbia (central part of the Balkan Peninsula). *Turkish Journal of Zoology*, 39, 195–209. https://doi.org/10.3906/zoo-1304-2

Pîrvu, M., Ciubuc, C., & Petrovici, M. (2012). Caddisflies (Insecta, Trichoptera) community structure in Cefa Nature Park (Crişana, Romania). *Transylvanian Review of Systematical and Ecological Research*, 13, 121–132.

Pramual, P., Thanee, I., Uttaruk, Y., Thaijarern, J., & Wongpakam, K. (2020). Efficiency of DNA Barcodes for Identification and Documenting Aquatic Insect Diversity in Rice Fields. Tropical Natural History. 20(2), 169-181. Retrieved from https://li01.tci-thaijo.org/index.php/tnh/article/view/169512

Prisecaru, F.S., Tabacaru, I., Prisecaru, M., Stoica, I. & Călin, M. (2014). Contributions to a revised species conspect of the Ephemeroptera Fauna from Romania (mayfliesyst). Studii şi Cercetări, Biologie, Universitatea "Vasile Alecsandri" din Bacau. 23(2), 20–30.

Prommi, T., & Payakka, A. (2015). Aquatic Insect Biodiversity and Water Quality Parameters of Streams in Northern Thailand. *Sains Malaysiana*, 44(5), 707–717. https://doi.org/10.17576/jsm-2015-4405-10

Rajkov, S. & Šćiban, M. (2012). Contribution to the Knowledgde on Odonata of "Zasavica" SNR. Pokret gorana Sremska Mitrovica: 154-161. (In Serbian).

Ratnasingham, S., & Hebert, P. D. N. (2007). bold: The Barcode of Life Data System (http://www.barcodinglife.org). *Molecular Ecology Notes, 7*(3), 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x PMID:18784790

Mauricio da Rocha, J. R., De Almeida, J. R., Lins, G. A., & Durval, A. (2010). Insects as indicators of environmental changing and pollution: A review of appropriate species and their monitoring. *Holos Environment*, 10(2), 250–262. https://doi.org/10.14295/holos.v10i2.2996

Ross, K. S., Haites, N. E., & Kelly, K. F. (1990). Repeated freezing and thawing of peripheral blood and DNA in suspension: Effects on DNA yield and integrity. *Journal of Medical Genetics*, 27(9), 569–570. https://doi.org/10.1136/jmg.27.9.569 PMID:2231649

Rosenberg, D. M., & Resh, V. H. (1993). Freshwater Biomonitoring and Benthic Macroinvertebrates. In D. M. Rosenberg & V. H. Resh (Eds.), Freshwater Biomonitoring and Benthic Macroinvertebrates (pp. 1–9). Chapman and Hall.

Russev, B. K. (1992). Threatened species of Ephemeroptera (Insecta) from Bulgaria. *Lauterbomia H.*, 9, 13–17.

Samways, M. J., & Deacon, Ch. (2021). Extinction Reprieve for the Ancient and Imperiled Dragonflies at the Southern Tip of Africa. Reference Module in Earth Systems and Environmental Sciences., https://doi.org/10.1016/B978-0-12-821139-7.00047-7

Sava, M. (2016-2017). Composition and diversity of Ephemeroptera (Larval communities) in seven sections (Lotic environments) from Telejean River. Complexul Muzeal de Ştiinţele Naturii „Ion Borcea" Bacău, Studii şi Comunicari. Vol. 26, 37–43.

Schultz, H., Waringer, J. A., & Chovanec, A. (2003). Assessment

of the ecological status of Danubian floodplains at Tulln (Lower Austria) based on the Odonata Habitat Index (OHI). *Odonatologica*, 32(4), 355–370.

Šibl, J., Seginkova, A., & Bulánková, E. (2003). Dragonflies (Odonata) of Little Danube, Klátovské branch and Važskeho Dunaja'. [In Slovak]. *Entomofauna Carpathica*, 15, 55–58.

Sivaramakrishnan, K. G., Janarthanan, S., Selvakumar, C., & Arumugam, M. (2014). Aquatic insect conservation: A molecular genetic approach. *Conservation Genetics Resources*, 6(4), 849–855. https://doi.org/10.1007/s12686-014-0250-4

Şerban, C., & Cristescu, M. (2013). Rare species of insects in anthropogenic ecosystems located in southeastern Romania. *Natura Montenegrina Podgorica., 12*(3-4), 673–686.

Tachet, H., Richoux, P., Bourneau, M. & Usseliopolatera, P. (2003). Freshwater Invertebrates: Systematic, Biology, Ecology. Paris: CSRS edition. (In French).

Talbalaghi, A., & Shaikevich, E. (2011). Molecular approach for identification of mosquito species (Diptera: Culicidae) in Province of Alessandria, Piedmont, Italy. *European Journal of Entomology*, 108(1), 35–40. https://doi.org/10.14411/eje.2011.004

Tierno de Figueroa, J. M., Derka, T., & Krno, I. (2002). First records of Sialis morio Klingstedt, 1932 (Megaloptera, Sialidae) in Slovakia. *Biologia*, 57(5), 572.

Teaca, A., Muresan, M., Begun, T., Popa, A., & Ion, G. (2019). Marine benthic habitats within a physical disturbed site from the Romanian Coast of the Black Sea. *Journal of Environmental Protection and Ecology*, 20(2), 723–732.

Vilenica, M., Kerovec, M., Pozojević, I., & Mihaljević, Z. (2020). Odonata assemblages in anthropogenically impacted lotic habitats. *Journal of Limnology, 80*(1). https://doi.org/10.4081/jlimnol.2020.1968

Vallenduuk, H. J., & Cuppen, H. M. J. (2004). The aquatic living caterpillars (Lepidoptera: Pyraloidea: Crambidae) of Central Europe. A key to the larvae and autecology. *Lauterbornia*, 49, 1–17.

Tiron Dutu, L., Dutu, F., Secrieru, D., & Opreanu, G. (2019). Sediments grain size and geo-chemical interpretation of three successive cutoff meanders of the Danube Delta, Romania. *Geochemistry*, 79(2), 399–407. https://doi.org/10.1016/j.chemer.2019.03.001

Tiron, L., & Provansal, M. (2010). Dynamique sedimentaire dans un milieu deltaique: Le Bras de St. George dans le delta du Danube. *Zeitschrift für Geomorphologie*, 54(4), 417–441. https://doi.org/10.1127/0372-8854/2010/0054-0014

Tubić, B. T., Simic, V. M., Zoric, Z. S., Gačić, Z. M., Atanacković, A. D., Csányi, B. J., & Paunović, M. M. (2013). Stream section types of the Danube River in Serbia according to the distribution of macroinvertebrates. *Biologia*, 68, 294–302. https://doi.org/10.2478/s11756-013-0152-z

Vidinova, Y., & Russev, B. (1997). Distribution and ecology of the representatives of some Ephemeropteran families in Bulgaria. In P. Landolt & M. Sartori (Eds.), Ephemeroptera & Plecoptera: Biology-Ecology-Systematics (pp. 139–146). MTL Fribourg.

de Vries, J. P. R., Buesink, R., van Leeuwen, J., & Baller, G. (2017). Odonata observations in the Danube Delta, Romania. *Brachytron*, 19(1), 35–43.

Wallace, I. D., Wallace, B., & Philipson, G. N. (2003). Keys to the Case-bearing Caddis Larvae of Britain and Ireland. Freshwater Biological Association.

Woodrum, J. E., & Tarter, D. C. (1973). The life history of the alderfly, *Sialis aequalis* Banks, in an acid mine stream. *American Midland Naturalist*, 89(2), 360–368. https://doi.org/10.2307/2424040

Zahrádková, S., Soldán, T., Bojková, J., Helešic, J., Janovská, H., & Sroka, P. (2009). Distribution and biology of mayflies (Ephemeroptera) of the Czech Republic: present status and perspectives. Aquatic Insects, 31(sup1, Supplement 1), 629–652.

Yakovlev, V. A. (2009). The fauna and distribution of alderfly larvae (Sialidae, Megaloptera) in Northern Fennoscandia and its dependence on natural and anthropogenous factors. *Inland Water Biology, 2*(3), 193–198. https://doi.org/10.1134/S1995082909030018

ICPDR (2009). Danube River Basin District Management Plan.

SR EN ISO 5661-1 (2008) Water quality. Part 1: General guidelines for establishing sampling programs and techniques.

SR EN ISO 10870 (2012). Water quality - Guidelines for the selection of sampling methods and devices for benthic macroinvertebrates in fresh waters. https://blast.ncbi.nlm.nih.gov