



ROMANIAN ACADEMY
School of Advanced Studies of the Romanian Academy
Institute of Biology Bucharest

PhD THESIS - SUMMARY

**GENETIC DIVERSITY OF BENTHIC COMMUNITIES
IN THE DANUBE RIVER – DANUBE DELTA – BLACK SEA
SYSTEM**

PhD COORDINATOR:
Senior Researcher Dr. Cristina Ligia PURCĂREA

PhD STUDENT:
Selma MENABIT

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INTRODUCTION

Biodiversity is essential for human well-being, the health of the planet, and global economic prosperity, facilitating a balanced and harmonious life with the natural environment. Ecosystems provide indispensable resources such as food, medicine, energy, air, and water, while also offering protection against natural disasters. Additionally, they contribute significantly to cultural enrichment, supporting the integrity of all vital systems on Earth (CBD, 2024).

In the Danube - Danube Delta - Black Sea system, before the adoption of molecular techniques, biodiversity assessment relied on traditional methods. Thus, for many decades, studies focused on the distribution and ecology of species rather than on molecular analyses. However, in recent years, molecular techniques such as DNA barcoding and eDNA (environmental DNA) have started to become increasingly utilized tools in biodiversity studies.

In recent years, the distribution, ecology, and diversity of benthic invertebrates, including aquatic insect larvae, gastropod species, and crustaceans inhabiting the Danube River, have been the subject of numerous studies (Graf et al., 2006; Tubić et al., 2013; Farkas et al., 2014; Krno et al., 2018; Navara et al., 2020). Many research efforts have also evaluated these aspects in the section of the Danube that crosses the Romanian territory (Chiriac, 2004; Glöer and Sîrbu, 2005; Gomoiu et al., 2008; Paraschiv et al., 2010; Graf et al., 2014; Pavel et al., 2018; Begun et al., 2020; Pavel et al., 2021; Pavel et al., 2023).

In contrast, molecular identification using DNA barcoding, based on the analysis of the cytochrome oxidase 1 (CO1) gene fragments of insects colonizing terrestrial habitats in Romania, has been scarcely addressed, for example, a study targeting 180 species of butterflies (Dincă et al., 2011). Meanwhile, this technique has not yet been used for the identification of aquatic insects.

Moreover, information on Ponto-Caspian amphipods and mysids in the Lower Danube is limited (Begun, 2006; Paraschiv et al., 2007; Graf et al., 2008; Borza et al., 2010; Stoica et al., 2014), and the molecular identification of amphipods (Cristescu et al., 2003; Rewicz et al., 2015; Jażdżewska et al., 2020) and mysids (Audzijonyte et al., 2006; Cristescu and Hebert, 2005) in the lower Danube and Danube Delta has only targeted a few species. Furthermore, the studies conducted by Audzijonyte et al. (2006); Jażdżewska et al. (2020); Cristescu and Hebert (2005) included specimens collected from unspecified locations in the

Danube Delta. Moreover, to date, no molecular studies have been conducted on the gastropod fauna inhabiting the Lower Danube.

Due to the anoxic conditions of the Black Sea, this basin represents a favourable environment for the development of anaerobic microorganisms and extremophile eukaryotes, which hold great potential for new products that can be used in various fields (Black Sea SRIA Implementation Plan, 2023). Since much of the microbial diversity of the Black Sea is insufficiently documented, one of the strategic actions of the Black Sea Strategic Research and Innovation Agenda Implementation Plan emphasizes establishing a knowledge system for species (including microorganisms) with the potential to generate bioactive compounds, such as new pharmaceutical products, biofuels, enzymes, and biopolymers (Black Sea SRIA Implementation Plan, 2023).

Research in the Black Sea has primarily focused on microbial populations in sediments (Schulz et al., 1999; Thamdrup et al., 2000; Leloup et al., 2007; Schäfer et al., 2007; Coolen and Shtereva, 2009; Schippers et al., 2012) and pelagic habitats (Jørgensen et al., 1991; Sorokin et al., 1995; Glaubitz et al., 2010; Bryukhanov et al., 2015; Ruginescu et al., 2022). However, only one study has examined the bacteria associated with the bivalve *Mytilaster lineatus* (Onyshechenko et al., 2005), while no research has been conducted so far on bacteria colonizing other benthic organisms.

Given these aspects, the studies conducted within this thesis aimed to assess the distribution and ecology of benthic invertebrates inhabiting the Danube sector that crosses Romania by integrating DNA barcoding molecular technique, thus providing new insights into the diversity and ecology of invertebrates in the Danube River. Moreover, the research focused on evaluating the bacterial communities colonizing two benthic invertebrates and the adjacent sediments in the Romanian Black Sea shelf, using Illumina sequencing of the 16S rRNA gene. This study was the first to explore the microbiome of the species *Melinna palmata* and offered the first analysis of the compositional variation of the microbiome colonizing the bivalve *Mya arenaria* at the organ level.

The main objectives of the thesis include:

- Molecular identification of aquatic insect larvae using DNA barcoding technique and assessment of their spatial distribution in the Romanian part of the Danube River, in relation to substrate type, dissolved oxygen, and water depth;

- Molecular identification using DNA barcoding and spatial distribution assessment of crustacean species (amphipods and mysids) and gastropods in the Lower Danube River (including the Danube Delta), in relation to substrate type and water depth;

- Characterization of the microbiome associated with the marine invertebrates *M. palmata* and *M. arenaria* and their surrounding sediments in the Romanian Black Sea shelf

The thesis is structured into two main parts. The first part provides an overview of current data on macrozoobenthic communities, their associated microbiomes, and the particularities of macrozoobenthic communities in the studied areas, as well as the techniques used in aquatic biodiversity assessment. The second part of the thesis is dedicated to personal contributions, including a chapter on the methods employed and four chapters of original results with discussions and conclusions for each study case. The four original studies conducted are: (1) Identification and distribution of aquatic insect larvae in the lower Danube, (2) Identification and distribution of gastropod and crustacean species in the lower Danube, (3) Assessment of bacterial communities associated with the polychaete species *M. palmata* and adjacent sediments in the Black Sea, and (4) Assessment of bacterial communities associated with different organs of the bivalve *M. arenaria* and adjacent sediments in the Black Sea. The thesis concludes with a chapter summarizing the general conclusions and providing perspectives for future research. Additionally, appendices with supplementary data, a list of published articles related to the thesis topic, and references are included.

1. CURRENT STATE OF KNOWLEDGE

1.1 Benthic invertebrates

Benthic invertebrates are organisms that live in or on the sediments at the bottom of seas, rivers, streams, and lakes. Their distribution is strongly influenced by their environment, including sediment composition and quality, water quality, and hydrological factors. Benthic invertebrates are considered among the most important components of aquatic ecosystems (Castella et al., 1984), providing essential ecosystem services in nutrient cycling and energy flow (Sivaramakrishnan et al., 2014).

Aquatic insects

Aquatic insects are a diverse and complex group derived from various terrestrial ancestors that have repopulated aquatic environments, encompassing approximately 76,000 species adapted to various freshwater habitats (Samways and Deacon, 2021). These organisms are a crucial component of aquatic (and sometimes terrestrial) food webs, playing an essential role in nutrient recycling processes (Balian et al., 2008).

Gastropods

Gastropods represent one of the most important groups of benthic organisms in terms of species diversity and abundance (Gomes et al., 2004; Tubić et al., 2013). They play a critical role in riparian systems by regulating the development of algal communities (Rosemond et al., 1993) and serve as a significant food source for some fish species (Brown et al., 1998).

Melinna palmata Grube, 1870

The tube-dwelling polychaete *M. palmata* has a Boreal-Mediterranean distribution (Grehan, 1991). In the Black Sea, it was relatively uncommon in the Romanian shelf region until the 1970s (Gomoiu, 1982). Currently, it is one of the engineering species of the Circalittoral mud habitat with *M. palmata* and/or *Spisula subtruncata*, reaching an average density of 3,450 ind. m⁻² in the Danube influence area (Teacă et al., 2020).

Mya arenaria Linnaeus, 1758

The Boreal-Atlantic bivalve *M. arenaria* originates from the North Atlantic, particularly along the coasts of America and Canada (Strasser, 1998). It was first recorded in the Black Sea in 1966 near Odessa (Beshevli and Kolyagin, 1967; Gomoiu and Porumb,

1969). By the early 1980s, *M. arenaria* had become established on the Romanian continental shelf, extending to depths of up to 35 meters (Skolka and Gomoiu, 2004).

1.2. Microbiome of benthic invertebrates

Benthic organisms are naturally colonized by microorganisms that are involved in their host biology (Kelman et al., 2009; Ketchum et al., 2018). These microorganisms play a crucial role in immunity, metabolism, and physiology (McFall-Ngai et al., 2013; Bordenstein and Theis, 2015; Theis et al., 2016), as well as in the development, adaptation, and evolution of these invertebrates (Rosenberg et al., 2007; Zilber-Rosenberg and Rosenberg, 2008). Filter and deposit feeders can concentrate bacteria from water and sediments, thus serving as bioindicators of microbial diversity in aquatic environments (Burkhardt et al., 1992; Graczyk et al., 2002; Marino et al., 2005).

Bacterial communities associated with tube-dwelling polychaetes could play a significant role in the biogeochemical processes occurring at the interface between benthic organisms and their habitat (Konhauser et al., 2020). Moreover, the microbiome of bivalves is crucial for the health and disease of the host, with the bacterial component known to be involved in various functions, including digestion, nutrient cycling, and immune defence (Pierce and Ward, 2018; Timmins-Schiffman et al., 2021).

1.3. Biodiversity of benthic communities in the Danube - Danube Delta - Black Sea system

In the Lower Danube River, the composition of macrozoobenthos is primarily determined by the type of habitat. Overall, organic habitats provide the highest number of indicator species, with the greatest diversity found in areas with plant debris, where communities are largely composed of dipterans, gastropods, oligochaetes, bivalves, and amphipods (ICPDR, 2015). In the Romanian Black Sea part, the habitats including the investigated areas in this thesis are represented by: 1. terrigenous circalittoral muds with *M. palmata*, and 2. circalittoral muds with varied fauna and *Mytilus galloprovincialis* reefs.

1.4. Techniques for assessing aquatic biodiversity

Traditional taxonomic techniques

Traditional or classical taxonomy, also known as the Linnaean system, involves using the morphological and anatomical characteristics of organisms for their classification and identification (Uno et al., 2001).

DNA Barcoding

DNA barcoding is an important tool in biodiversity assessment (Hebert et al., 2003). For the animal kingdom, efforts have primarily focused on the "Folmer" region, a ~650 base pair segment of the cytochrome c oxidase subunit I (CO1) gene (Folmer et al., 1994). This technique offers an effective solution for overcoming the challenges associated with traditional taxonomic identification, providing a rapid, precise, and reliable method for species identification (Baird et al., 2012).

Next-Generation Sequencing (NGS) (DNA Metabarcoding)

In this method, a specific region of DNA from all genomes present in an environmental sample is amplified and sequenced to generate a community profile (Kelly, 2016). This technique, known as DNA metabarcoding, involves amplifying DNA using PCR with primers targeting more or less specific genetic features. Following the PCR amplification, the products are sequenced, and the sequences are processed bioinformatically, which allows for the taxonomic assignment of the sequences against a reference database (Pilloni et al., 2012).

PERSONAL CONTRIBUTIONS

2. THESIS AIM OBJECTIVES

Considering the current knowledge of macrozoobenthic communities in the Danube – Danube Delta – Black Sea system and the associated microbiome of these species, the research undertaken within this thesis aimed to assess the distribution and ecology of benthic invertebrates in the Lower Danube by integrating the molecular biology technique DNA barcoding. This provided new information about the biodiversity and ecology of invertebrate species in the Danube River. Moreover, the research focused on characterizing the structural and functional diversity of bacterial communities associated with two benthic invertebrates (the tube-dwelling polychaete *Melinna palmata* and the bivalve *Mya arenaria*) and their surrounding sediments in the Romanian Black Sea shelf, based on Illumina sequencing of the 16S rRNA gene. The study made significant contributions by being the first to investigate the microbiome of *M. palmata* and provided new insights by investigating, for the first time, the compositional variation of the microbiome associated with the bivalve *M. arenaria* at the organ level.

The primary objectives of the doctoral thesis were:

- To identify insect larvae using DNA barcoding techniques, specifically through sequencing the mitochondrial CO1 gene, and to evaluate their distribution in the Romanian sector of the Danube, in relation to substrate type, dissolved oxygen, and water depth.
- To perform molecular identification using CO1 gene sequencing and assess the spatial distribution of Pontic-Caspian amphipod and mysid species, as well as gastropods, in the Lower Danube Sector (including the Danube Delta), in relation to substrate type and water depth.
- To characterize the microbiome associated with two marine invertebrate species (the polychaete *M. palmata* and the bivalve *M. arenaria*) and the surrounding sediments from the Romanian Black Sea shelf.

3. MATERIAL AND METHODS

3.1. Description of the study areas

The investigated areas belong to riverine and marine zones located in the Lower Danube River, the Danube Delta, and the Romanian Black Sea shelf.

3.2. Benthos sample collection

For the insect larvae, gastropod species, and crustaceans populating the Lower Danube River and Danube Delta, the samples were collected using two Van Veen grabs. Additionally, to collect phytophilous organisms, the vegetation was washed using a limnological net. For *Melinna palmata* specimens, sediment samples were collected using a Box Corer, while for *Mya arenaria*, the samples were collected using a Van Veen grab.

3.3. Assessment of physicochemical parameters of water samples

For samples collected from the Lower Danube River and the Sulina branch, dissolved oxygen content was measured using an Oxi 320 oxygen meter (WTW, Germany). For samples collected from the Black Sea, temperature, salinity, dissolved oxygen, and pH were determined using an EXO2 multiparameter probe (YSI Incorporated, Yellow Springs, USA).

3.4. Macrozoobenthos and sediment samples processing

Individuals selected for genetic analyses were washed with ultrapure water, placed in 200 µl of Tris-EDTA buffer solution (pH 8), and stored at -20°C (Ross et al., 1990). For morphological identification, specimens were preserved in a 4% formaldehyde solution and stored in 250 mL plastic containers.

3.5. Quantitative and taxonomic analysis of freshwater benthic communities

For samples collected using Van Veen devices, the number of individuals per unit area (1 m²) was calculated based on the total number of individuals in each sample. For samples collected with the limnological net, abundance was expressed as the total number of individuals collected. Taxonomic identification, based on morphological criteria, was performed using the identification keys specific to each organism group.

3.6. Molecular identification of benthic organisms

Total DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Molecular identification of benthic specimens was carried out using DNA

barcoding, based on PCR amplification of the mitochondrial gene cytochrome c oxidase 1 (CO1) (Hebert et al., 2003). CO1 gene sequences of identified individuals (insect larvae, gastropods, and crustaceans) were deposited in the international GenBank database.

3.7. Phylogenetic analysis and calculation of intra- and interspecific genetic distances

Phylogenetic analysis based on CO1 sequences for both gastropod and crustacean species was conducted using a maximum likelihood statistical method. Intra- and interspecific genetic distances were calculated using the Kimura 2-parameter (K2P) model.

3.8. Assessment of bacterial diversity associated with invertebrate species and adjacent sediments

The bacterial diversity associated with aquatic invertebrates *M. palmata* and *M. arenaria*, as well as their adjacent sediments, was investigated through Illumina sequencing of the 16S rRNA gene. For organisms, total DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). For sediment samples, DNA was isolated using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). Sequencing of the V3-V4 regions of the 16S rRNA gene was performed using an Illumina MiSeq 300PE platform (Macrogen, Seoul, South Korea). The obtained sequences were analysed using bioinformatic and statistical methods.

3.9. Functional diversity of bacterial communities

The functional diversity of bacterial communities identified through 16S rRNA gene sequencing was estimated using the Tax4Fun conversion tool (Aßhauer et al., 2015).

4. RESULTS: IDENTIFICATION AND DISTRIBUTION OF AQUATIC INSECT LARVAE IN THE LOWER DANUBE RIVER

4.1. Collection of macrozoobenthos samples and habitat characteristics

The samples were collected in late spring (May) 2019 and 2020 from 36 locations along the Lower Course of the Danube River, specifically between km 2 and km 811, including the Sulina Branch.

4.2. Taxonomic diversity and distribution of insect larvae

Genetic identification of insect larvae isolated from all locations along the Lower Danube River, based on the sequence identity of the mitochondrial cytochrome oxidase CO1 amplicon, revealed the presence of larvae from the following species: *Erythromma viridulum*, *Gomphus flavipes*, *Triaenodes bicolor*, *Hydropsyche bulgaromanorum*, *Heptagenia flava*, *Acentria ephemerella*, and *Sialis morio*, belonging to the orders Odonata, Trichoptera, Ephemeroptera, Lepidoptera, and Megaloptera, respectively.

The CO1 sequences for each identified individual were submitted to GenBank with the 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*.

The presence and densities of the seven insect species varied depending on the type of sediment from which they were collected. The investigations showed that all analysed species were present in sandy sediments and gravelly sand, except for *Sialis morio*. Moreover, areas dominated by sandy mud were inhabited only by *Hydropsyche bulgaromanorum*, *Triaenodes bicolor*, and *Sialis morio*, while regions characterized by gravelly mud and sandy gravel were colonized by only one species, *H. bulgaromanorum*.

The spatial distribution of these aquatic larval species across different sections of the Lower Danube River was uneven (Figure IV.2), likely due to environmental factors. For instance, *H. bulgaromanorum* larvae were found throughout all areas, including the Sulina branch (Figure IV.2). The Bechet sector was populated by most species (except for *G. flavipes* and *S. morio* larvae), whereas the Cetate sector was inhabited by only two species, *E. viridulum* and *H. bulgaromanorum* (Figure IV.2).

The density of the seven aquatic insect species varied widely across river sites, influenced by the location, the year of collection, and the presence of macrophytes.

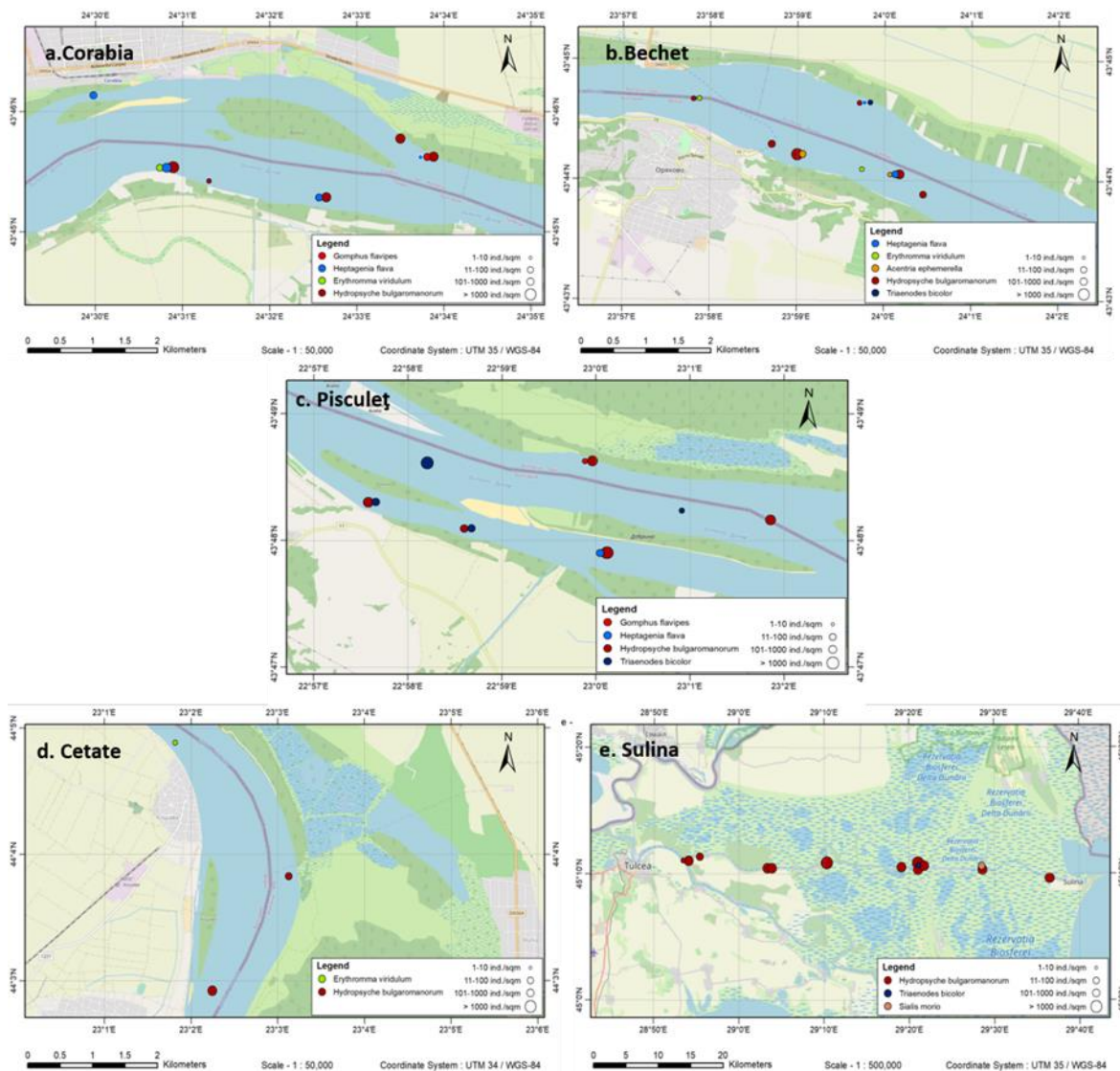


Figure IV.2. Distribution of insect larvae in the Corabia, Bechet, Pisculeț, Cetate sectors and Sulina branch (Menabit et al., 2022)

The variables considered to elucidate the factors influencing the presence and distribution of the seven studied aquatic insect species (Figure IV.3) indicates a predominance of *H. bulgaromanorum* and *H. flava* in extensive but non-overlapping sectors of the Lower Danube River.

The distribution profile of these insect larvae revealed their presence at depths between 2.8 and 5 m. *E. viridulum*, *T. bicolor*, *H. flava*, and *H. bulgaromanorum* were found at depths ranging from 5 to 8.2 meters, with *H. bulgaromanorum* also inhabiting waters up to 17.5 meters deep (Figure IV.3). These findings suggest that ecological factors and specific habitat conditions, such as substrate type and water depth, influence the presence of these insect larvae in the Lower Danube sector. Furthermore, the data imply that the measured

concentration of dissolved oxygen did not impact the distribution of these larvae (Figure IV.3).

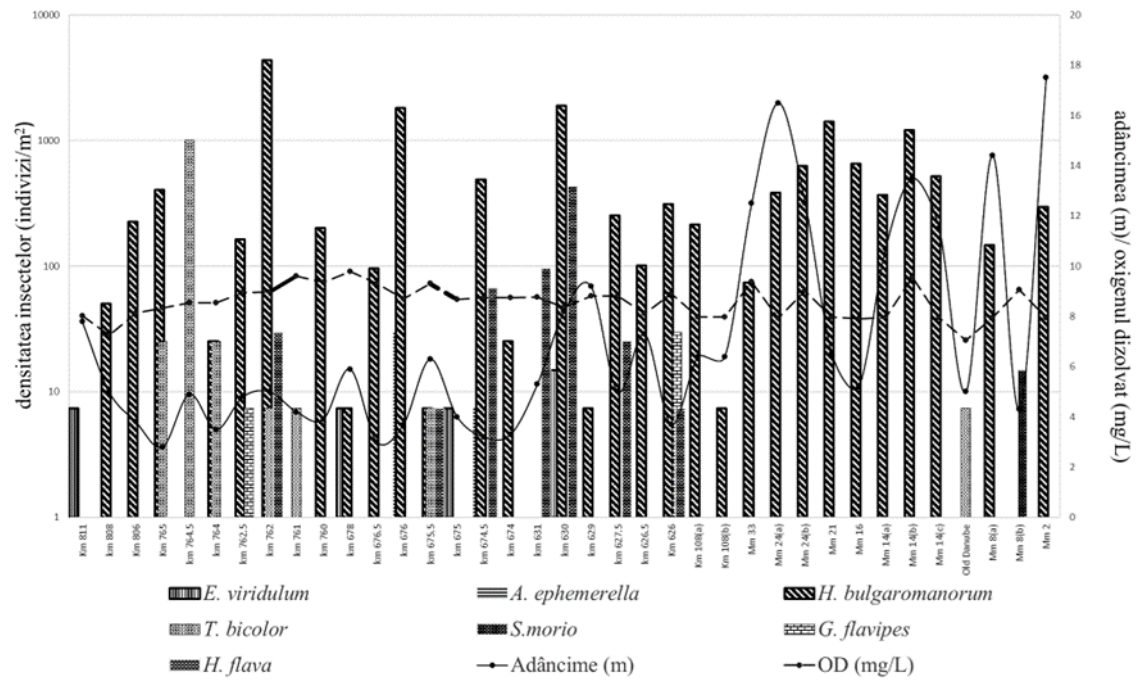


Figure IV.3. Multiparametric variation of insect larvae distribution in the Lower Danube River (Menabit et al., 2022)

5. RESULTS: IDENTIFICATION AND DISTRIBUTION OF GASTROPOD AND CRUSTACEAN SPECIES IN THE LOWER DANUBE

5.1. Sample collection and habitat characteristics

The samples were collected from 8 stations along the Lower Danube River over a period of 3 years (2019–2021). This work was carried out during 3 field campaigns conducted in May and June.

5.2. Taxonomic diversity and phylogenetic analysis of gastropod and crustacean Species in the Lower Danube

All gastropod and crustacean specimens collected from the 8 sampling stations were identified using DNA barcoding technique, including 16 gastropods and 12 crustaceans.

The CO1 sequences corresponding to each individual were submitted to the GenBank database with the following accession numbers: [ON000192] and [ON000184] for *Holandriana holandrii*, [MW139680] for *Microcolpia daudebartii acicularus*, [ON000180], [ON000191], and [ON000197] for *Viviparus viviparus*, [MW139681] and [MW139682] for *Bithynia tentaculata*, [MW139678] for *Theodoxus transversalis*, [MW600073], [ON000193], and [ON036036] for *Ampullaceana lagotis*, [MW600069] for *Physa fontinalis*, [MW600083] for *Planorbarius corneus*, [ON000188] and [ON000194] for *Gyraulus parvus*, [MW139683] and [ON000195] for *Chelicorophium robustum*, [MW600074], [MW600089], [ON000187], and [ON000189] for *Dikerogammarus haemobaphes*, [MW600090] for *D. villosus*, [MW600070], [MW600071], [MW600072], and [ON000186] for *Limnomysis benedeni*, and [MW600085] for *Paramysis (Serrapalpis) lacustris*.

The 9 gastropod species identified in this study belong to the orders Caenogastropoda, Architaenioglossa, Littorinimorpha, Cycloneritida, and Hygrophila, and the families Amphimelaniidae, Melanopsidae, Viviparidae, Bithyniidae, Neritidae, Lymnidae, Physidae, and Planorbidae. The 5 crustacean species from the orders Amphipoda and Mysida are classified into three families: Corophiidae, Gammaridae, and Mysidae.

The phylogenetic analysis of the identified gastropod and crustacean species revealed that the collected individuals belong to monophyletic groups, highlighting the accuracy of DNA barcoding for taxonomic identification.

For gastropod species, the phylogenetic tree was constructed based on CO1 sequences from 28 individuals (Figure V.2). Of these, 16 sequences were obtained in the current study, while 10 additional sequences were retrieved from the NCBI GenBank database (Figure V.2). All specimens identified as the same species clustered into monophyletic groups on the phylogenetic tree (Figure V.2). Each group corresponded to a single branch and exhibited high "bootstrap" values (Figure V.2).

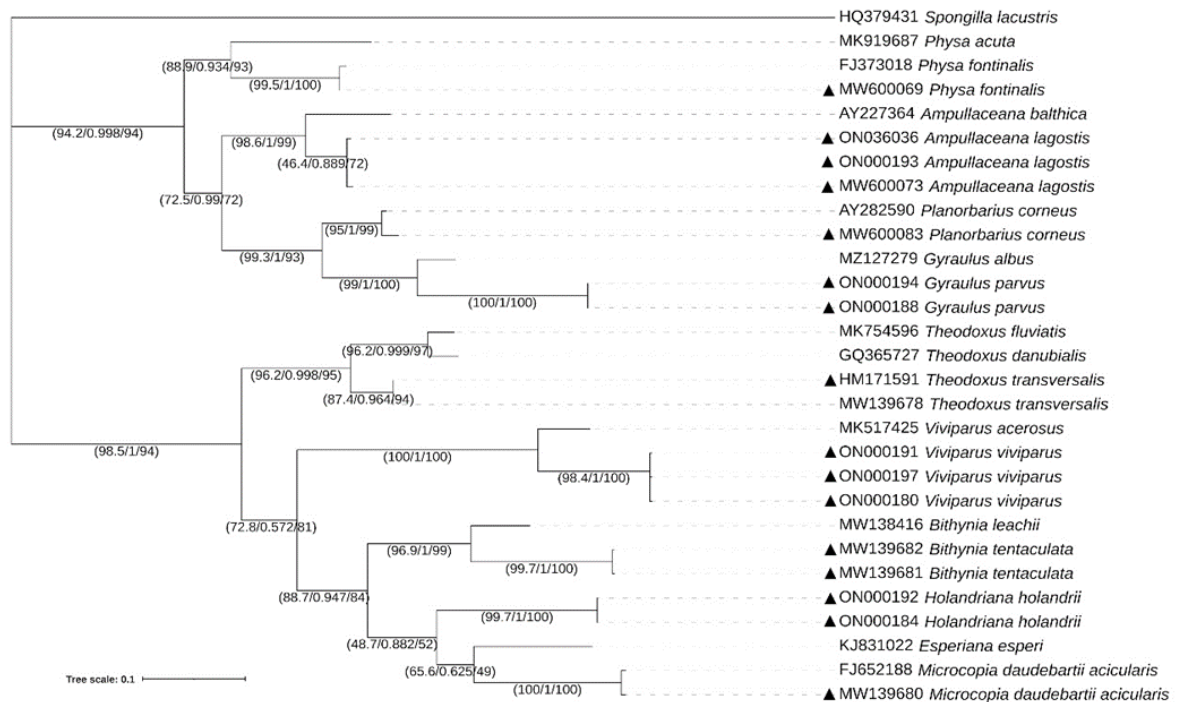


Figure V.2. Phylogenetic tree of gastropod species based on CO1 gene sequences. ▲: Sequences from the current study; CO1 of *Spongilla lacustris* (HQ379431) was used as the "outgroup"; values in parentheses represent SH-aLRT support (%)/aBayes support/ultrafast bootstrap support (%) (Menabit et al., 2022).

For the crustacean species, the phylogenetic tree was constructed based on CO1 sequences from 16 individuals, including 12 from this study and 4 sequences from homologous species retrieved from the GenBank database (Figure V.3). Similar to the gastropods, the phylogenetic reconstruction showed that all individuals of the same species clustered into distinct groups, with high "bootstrap" values (Figure V.3).

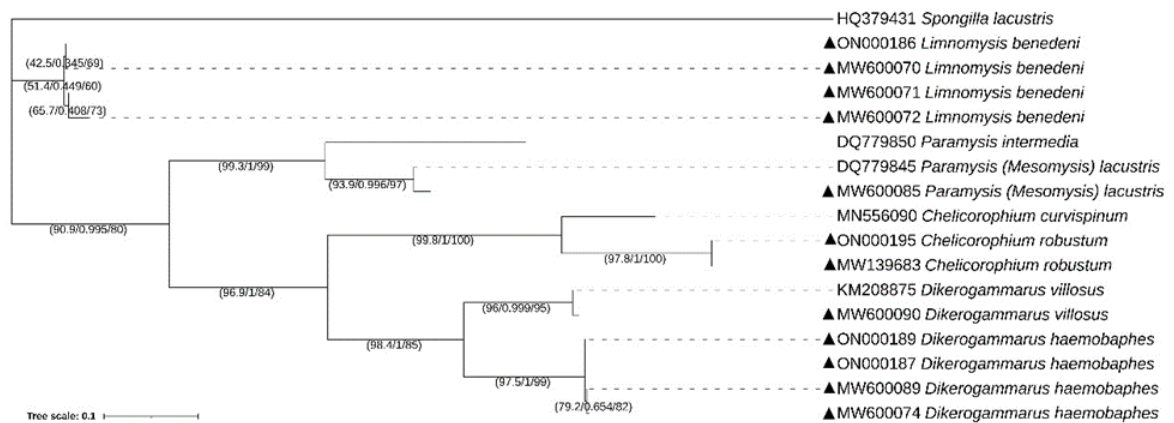


Figure V.3. Phylogenetic tree of crustacean species based on CO1 gene sequences. ▲: Sequences from the current study; CO1 of *Spongilla lacustris* (HQ379431) was used as the "outgroup"; values in parentheses represent SH-aLRT support (%)/aBayes support/ultrafast bootstrap support (%) (Menabit et al., 2022)

5.3. Spatial distribution and ecology of gastropod and crustacean species in the Lower Danube

In this study, gastropod species were identified in 4 out of the 7 sampling stations located along the three main Danube branches and at the Bechet station. In contrast, crustacean species were observed at all 7 stations across the Danube branches over the 3-year study period.

The gastropod species *M. daudebartii acicularis*, *V. viviparus*, *B. tentaculata*, *P. fontinalis*, and *P. corneus* were found only on the Chilia and Sulina branches of the Danube (Figure V.4). The Bechet area, on the other hand, was inhabited by phytophilous gastropod species *H. holandrii*, *T. transversalis*, and *G. parvus*.

Crustacean species (amphipods and mysids) were observed at stations along all three Danube branches: Chilia, Sulina, and Sf. Gheorghe, showing variable distribution. While *D. haemobaphes* was found on all three branches, *D. villosus* was identified only on the Sulina and Sf. Gheorghe branches, and *C. robustum* only on the Chilia and Sulina branches. The two identified mysid species, *L. benedeni* and *P. lacustris*, were present exclusively on the Sulina branch.

6. RESULTS: ASSESSMENT OF BACTERIAL COMMUNITIES ASSOCIATED WITH THE MARINE POLYCHAETE *MELINNA PALMATA* GRUBE, 1870 AND ADJACENT SEDIMENTS IN THE BLACK SEA

6.1. Sample collection and habitat characteristics of the investigated area

Sediment samples were collected from 4 stations located in the North-Western part of the Black Sea, with sampling conducted in June 2020.

The substrate of all 4 sampling stations consisted of muddy sediments composed of silt and clay particles. *In situ* measurements of the physicochemical parameters of the water at the 4 locations showed no significant variations.

6.2. Bacterial Diversity in *M. palmata* tissues and associated sediments

To assess the microbiome diversity, 16 microbial DNA samples were obtained and analysed from tissues of 8 *M. palmata* specimens and from 8 adjacent sediments.

The diversity and structure of the bacterial communities associated with this polychaete and the sediments representing the substrate of each investigated individual were determined by Illumina sequencing of the 16S rRNA gene.

The total number of DNA sequences obtained (134,975) corresponded to 1,444 unique amplicon sequence variants (ASVs).

Alpha diversity, calculated using the Chao1 index, revealed significant differences between the two analysed groups (tissues and sediments), with higher values observed in sediment samples (Figure VI.3). Consistent with this finding, the Fisher diversity index showed a similar trend. Regarding variations in Chao1 and Shannon diversity indices across the depth gradient, no significant differences were observed between the analysed samples. Minor variations in the three calculated diversity indices were noted both between different samples and among duplicates.

Beta diversity analysis of the bacterial communities in the examined samples revealed significant differences in diversity and relative abundance between tissues and sediments. The results indicated that these matrices formed predominantly distinct communities, with higher differences observed in the microbiome of tissue samples.

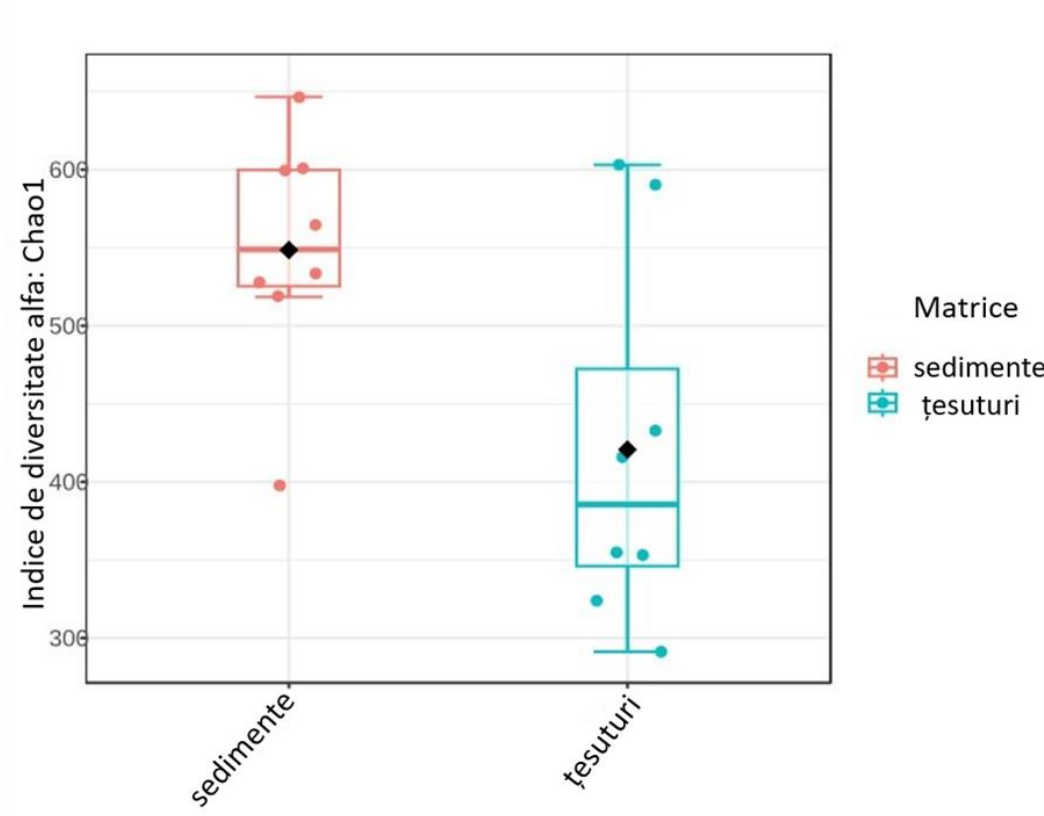


Figure VI.3. Alpha diversity (Chao1 index) of bacterial communities for *M. palmata* and sediment samples (Menabit et al., 2024).

6.3. Structure of bacterial communities in *M. palmata* tissues and adjacent sediments

Bioinformatic analyses of the taxonomic identification of microbial communities colonizing the marine polychaete *M. palmata*, based on Illumina 16S rRNA gene sequencing, identified 12 phyla, 14 classes, 26 orders, 26 families, and 30 genera of bacteria (Appendix 1). In contrast, the bacterial communities associated with sediments were classified into 12 phyla, 19 classes, 34 orders, 29 families, and 36 genera (Appendix 1).

The microbiome associated with *M. palmata* was mainly composed of Proteobacteria, Actinobacteriota, Cyanobacteria, and Chloroflexi phyla (Figure VI.6A). In comparison, bacteria inhabiting the sediments were primarily represented by Actinobacteriota, Chloroflexi, Proteobacteria, and Campylobacterota phyla (Figure VI.6A).

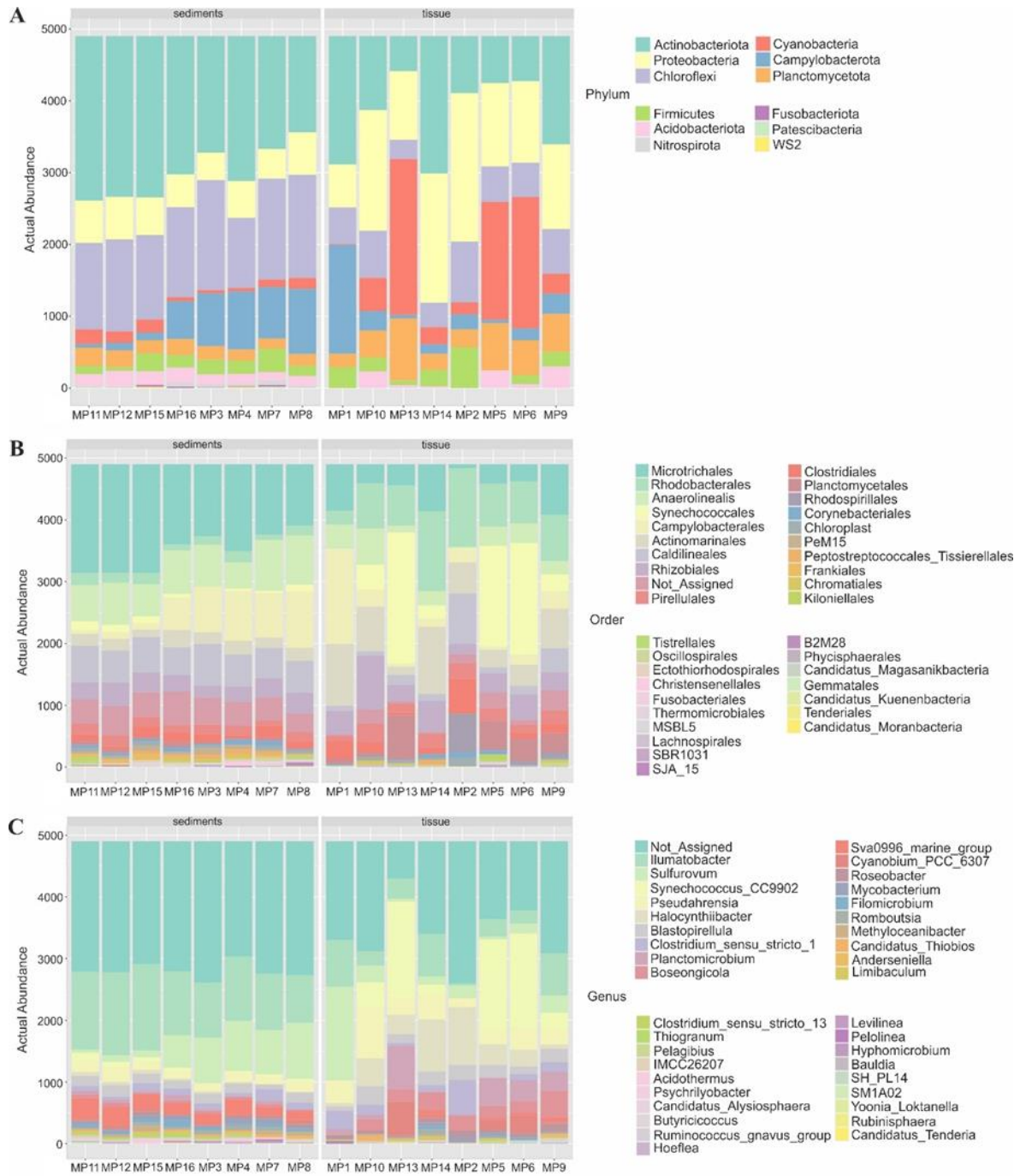


Figure VI.6. Abundance of bacterial communities colonizing tissues and sediments at the phylum (A), order (B), and genus (C) levels expressed in ASV (Menabit et al., 2024).

6.4. Functional profile of bacterial communities colonizing *M. palmata* and associated sediments

The estimated functional profile of these bacterial communities showed a high presence of genes involved in the metabolism of amino acids and carbohydrates, followed by those related to energy metabolism, cofactors, vitamins, and lipids ($p < 0.05$) (Figure

VI.11A). Additionally, a high number of genes responsible for the degradation of xenobiotics, nucleotide metabolism, terpenoids, polyketides, and the biosynthesis of glycans and other secondary metabolites were identified in all analysed samples (Figure VI.11A).

However, principal component analysis (PCA) of the functional genes revealed a clear segregation in the metabolic profiles of the microbiomes from the two analysed matrices (Figure VI.11B).

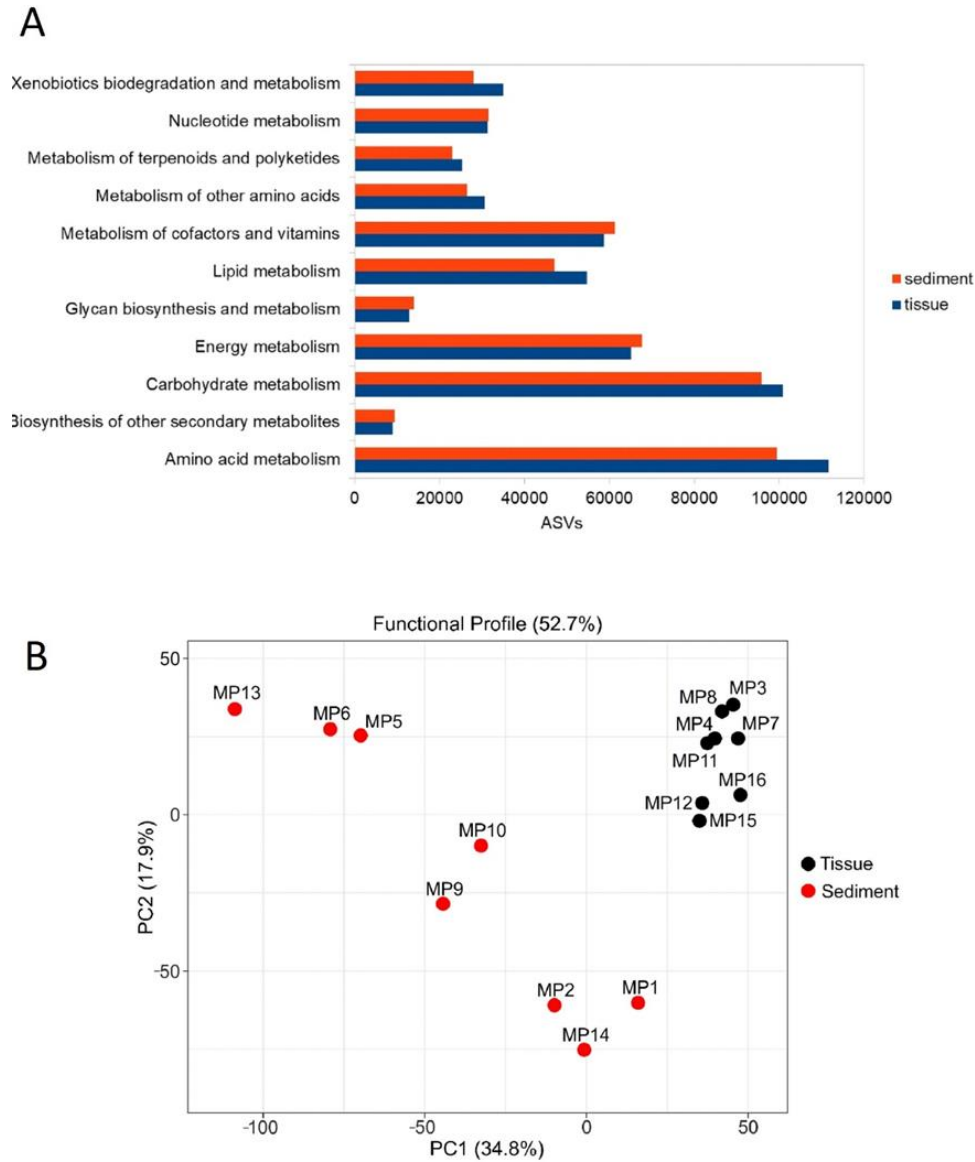


Figure VI.11. Predicted functional profile of bacterial communities from tissues and sediments based on the gene abundance from KEGG metabolic pathways. (A) Number of predicted functional genes based on amplicon sequence variants (ASVs) from the analysed matrices (B) diversity of functional genes in the analysed matrices based on PCA analysis (Menabit et al., 2024)

7. RESULTS: ASSESSMENT OF BACTERIAL COMMUNITIES ASSOCIATED WITH DIFFERENT ORGANS OF THE BIVALVE *MYA ARENARIA* LINNAEUS, 1758 AND ADJACENT SEDIMENTS IN THE BLACK SEA

7.1. Collection of macrozoobenthos samples and habitat characteristics

Sediment samples were collected from two stations located in the North- Western part of the Black Sea, in July 2020. The sedimentary material from both sites consisted of mud originating from alluvial deposits composed of silt and clay particles. *In situ* measurements of the physicochemical parameters of the water at both locations showed no significant variations.

7.2. Bacterial community in the organs of *M. arenaria* and associated sediments

To assess the microbial diversity associated with different organs of the marine bivalve *Mya arenaria* and the adjacent sediments, 14 samples of total DNA were obtained and analysed from the siphon (4 samples), gills (4 samples), and stomach (2 samples) of this invertebrate, as well as from sediments (4 samples) collected from the two sampling stations.

The diversity and structure of the microbiome associated with the three analysed organs of *M. arenaria* (siphon, gills, stomach) and the adjacent sediments were evaluated through Illumina sequencing of the 16S rRNA gene. The total number of DNA sequences obtained (1,340,099) corresponded to 11,223 unique bacterial amplicon sequence variants (ASVs).

Alpha diversity, calculated using the Chao1 index, revealed significant differences between the bacterial communities colonizing the analysed organs of the bivalve and the sediments (Figure VII.4A). The Shannon, Fisher, and ACE diversity indices showed a similar trend, confirming a lower diversity for bacteria in the organs compared to the adjacent sediments. Variability was observed both between samples and between replicates, with greater diversity variations among the communities associated with different organs of the invertebrate. However, no significant differences were noted among these bacterial communities based on water depth (Figure VII.4B). Beta diversity analysis of the bacterial communities relative to the sample type indicated significant differences in microbial diversity among the various organ types and sediments, with higher differences between the microbiomes in the gills and stomach compared to those in the siphon.

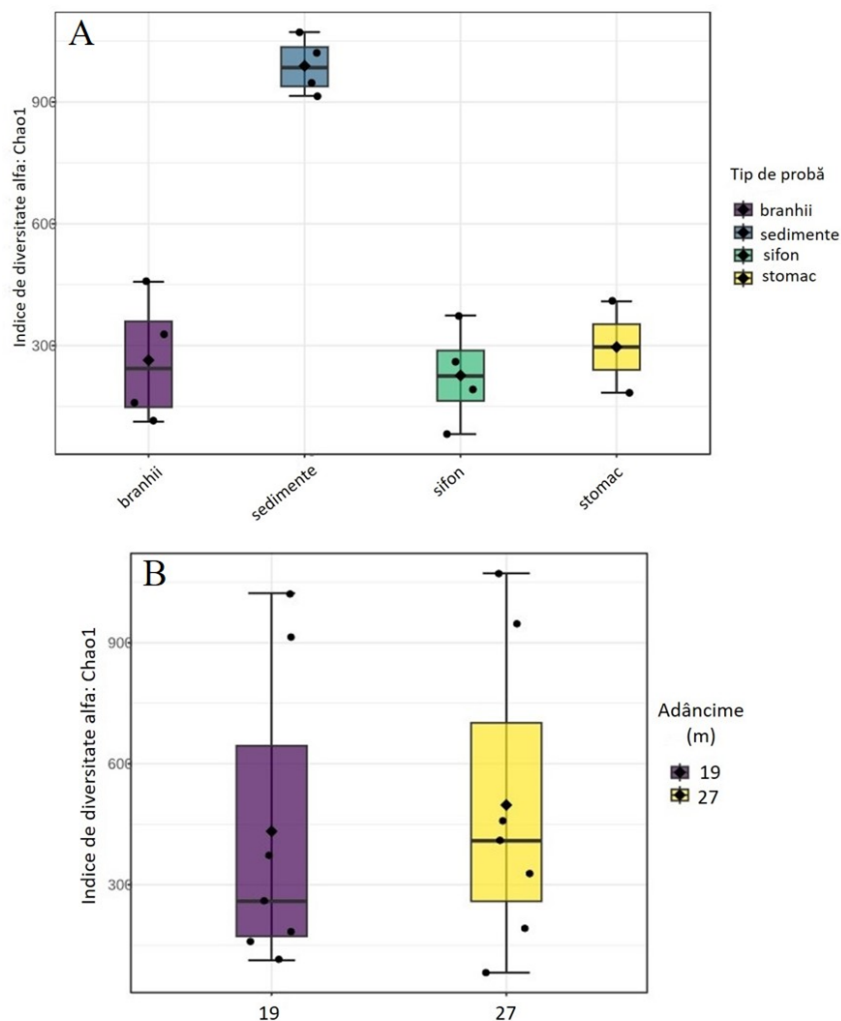


Figure VII.4. Alpha diversity of bacterial communities calculated based on the Chao1 index by: (A). sample type; (B). depth

7.3. Structure of bacterial communities associated with different organs (siphon, gills, and stomach) of *M. arenaria* and adjacent sediments

Taxonomic identification of bacterial communities from the tissues of *M. arenaria* revealed a variable structure. Specifically, the bacterial taxa associated with the siphon included 16 phyla, 23 classes, 34 orders, 41 families, and 43 genera (Annex 2). The microbiome of the gills was classified into 15 phyla, 21 classes, 33 orders, 39 families, and 35 genera (Annex 2), while the bacteria associated with the stomach comprised 14 phyla, 22 classes, 30 orders, 29 families, and 31 genera. In contrast, the microbiome of the adjacent sediments included 20 phyla, 38 classes, 50 orders, 48 families, and 54 genera.

Phylum-level distribution of the analysed samples highlighted a variable profile both among organ types and between the invertebrate and the adjacent sediments (Figure VII.6). Thus, bacterial communities in the siphon of *M. arenaria* were predominantly dominated by Spirochaetota, Verrucomicrobiota, Proteobacteria, and Bacteroidota phyla. The gills were mainly populated by Verrucomicrobiota, followed by Spirochaetota, Firmicutes, and Proteobacteria, with similar relative abundances (Figure VII.6). The stomach microbiome was primarily dominated by bacteria from the Firmicutes phylum, followed by Spirochaetota, Verrucomicrobiota, and Planctomycetota, all with comparable abundances. In contrast, sediment communities were mainly represented by Proteobacteria, followed by Desulfbacterota, Bacteroidota, and Chloroflexi (Figure VII.6).

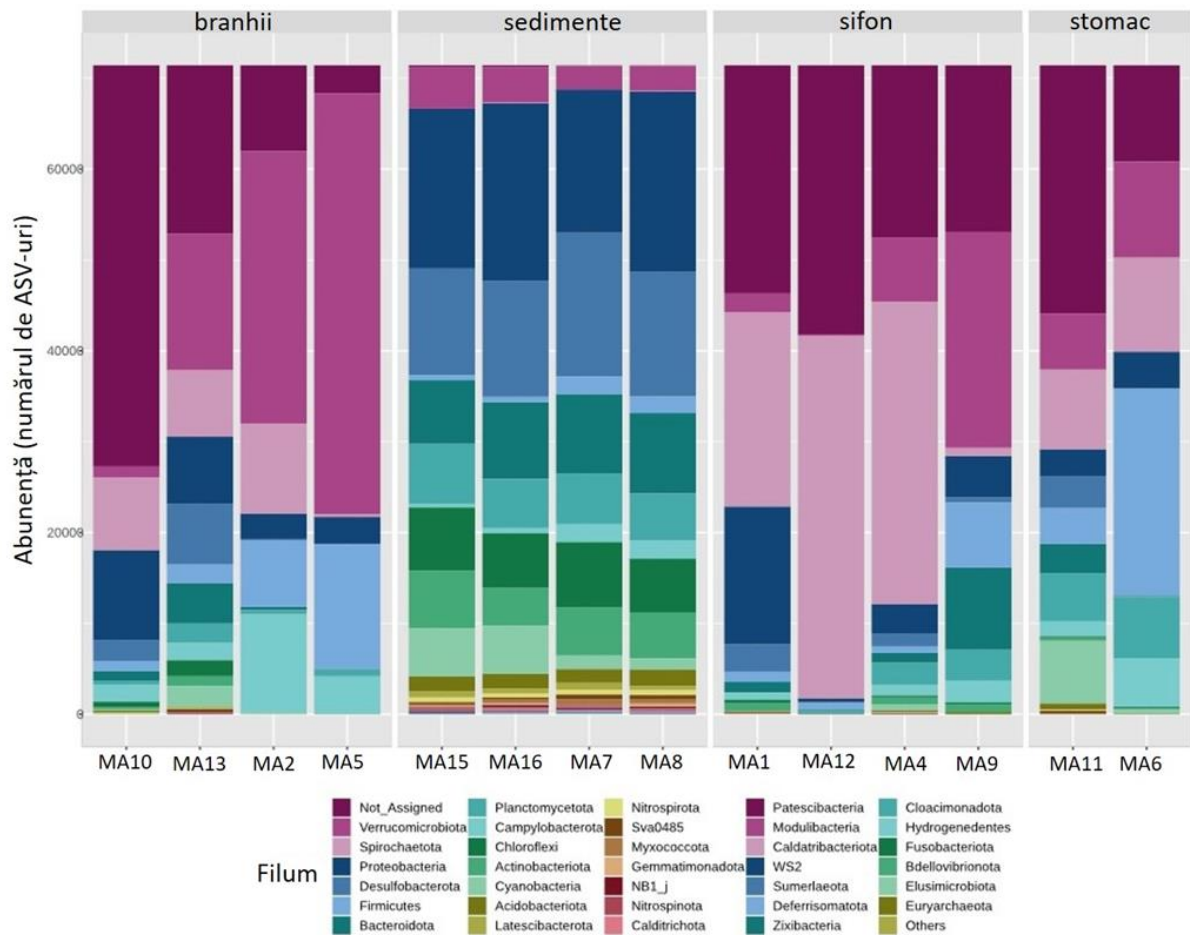


Figure VII.6. Taxonomic distribution of bacterial communities associated with the organs of *Mya arenaria* (gills, siphon, and stomach) and adjacent sediments (phylum level)

7.4. Functional diversity of bacterial communities colonizing *M. arenaria* organs and adjacent sediments

The 16S rRNA gene sequencing results were also used to estimate the various metabolic pathways in which the bacteria colonizing *M. arenaria* and the adjacent sediments are involved. This was achieved by comparing sequence similarities with organisms whose complete genomes have been sequenced and annotated, using the Tax4Fun application.

The estimated functional profile showed a relatively similar distribution of gene abundances across different KEGG metabolic pathways, with a prevalence of genes involved in carbohydrate, amino acid, and energy metabolism for all analysed samples (Figure VII.12). Although the Student's t-test revealed a significantly higher content of genes involved specifically in nucleotide metabolism, a slight increase in the number of genes related to lipid metabolism, energy, carbohydrate metabolism, and xenobiotic degradation in the tissue samples has been observed (Figure VII.12).

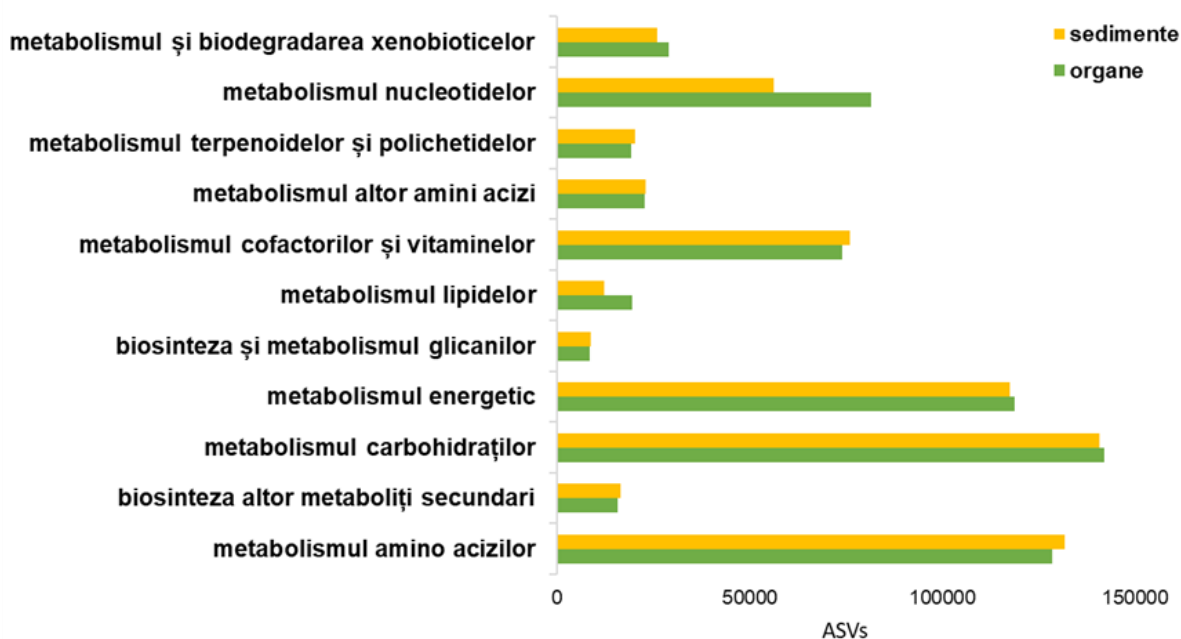


Figure VII.12. Predicted functional profile of bacterial communities from organs and sediments based on the gene abundance from KEGG metabolic pathways. The number of predicted functional genes is based on ASV

8. GENERAL CONCLUSIONS AND PERSPECTIVES

The four case studies presented in this doctoral thesis provide a detailed perspective on the distribution and ecology of invertebrates (insect larvae, gastropods, and crustaceans) inhabiting the Lower Danube River. Moreover, they examine the diversity and structure of bacterial communities associated with two marine invertebrate species (the tube-dwelling polychaete *Melinna palmata* and the bivalve *Mya arenaria*), as well as with the adjacent sediments from the Romanian Black Sea coast.

These studies further demonstrate the application of molecular biology techniques, such as DNA barcoding and Illumina 16S rRNA gene sequencing, to gain new insights into the distribution and genetic diversity of the aquatic organisms studied and their associated microbiomes, including their surrounding sediments.

The first study case aimed to identify seven species of insect larvae from the orders Odonata, Trichoptera, Ephemeroptera, Lepidoptera, and Megaloptera using DNA barcoding based on mitochondrial cytochrome c oxidase 1 (CO1) gene sequencing. It also assessed their distribution in the Romanian sector of the Danube River in relation to substrate type, dissolved oxygen, and water depth.

The results revealed significant differences in the distribution and density of these species across the different sectors of the Danube. Most species (including *Erythromma viridulum*, *Gomphus flavipes*, *Triaenodes bicolor*, *Hydropsyche bulgaromanorum*, *Heptagenia flava*, and *Acentria ephemerella*) were identified in the Corabia, Bechet, Pisculeț, and Cetate sectors, with the exception of *Sialis morio*, which was only present in the Sulina arm.

These findings provide new insights into the distribution of aquatic insects, suggesting that environmental factors such as substrate type and water depth influence the presence of these insect larvae in the Romanian sector of the Danube River.

The second study case focused on the molecular identification and spatial distribution of several amphipod crustacean species (*Chelicorophium robustum*, *Dikerogammarus villosus*, and *D. haemobaphes*) and mysids (*Limnomysis benedeni* and *Paramysis lacustris*) from the Ponto-Caspian region, as well as gastropods (*Viviparus viviparus*, *Bithynia tentaculata*, *Microcolpia daudebartii acicularis*, *Ampullaceana lagotis*,

Planorbarius corneus, *Holandriana holandrii*, *Theodoxus transversalis*, and *Gyraulus parvus*) in the lower Danube River, in relation to substrate type and water depth.

Using the CO1 gene sequencing, 16 gastropod and 12 crustacean individuals were identified at the species level, marking the first molecular identification of gastropod species in the Lower Danube River.

Phylogenetic reconstruction revealed that the gastropod and crustacean species belonged to monophyletic groups, with individuals of the same species clustering together in single clades.

Most of the identified gastropod species (*V. viviparus*, *B. tentaculata*, *M. daudebartii acicularis*, *A. lagotis*, and *P. corneus*) were found along the arms of the Danube, whereas the macrophyte-associated species (*H. holandrii*, *T. transversalis*, and *G. parvus*) were identified at the Bechet sector. Crustaceans (amphipods and mysids), on the other hand, were detected at stations across all three Danube arms, with a variable distribution.

The third study case aimed to evaluate the microbiome associated with the marine polychaete *Melinna palmata* and the adjacent sediments from the Romanian Black Sea coast, using Illumina sequencing of the 16S rRNA gene. This represents the first characterization of the bacterial communities colonizing this species.

The results identified 134,975 bacterial amplicon sequence variants (ASVs), with the bacterial communities of *M. palmata* belonging to 12 phyla, 14 classes, 26 orders, 26 families, and 30 genera, while those associated with the sediments were classified into 12 phyla, 19 classes, 34 orders, 29 families, and 36 genera.

Significant differences in diversity were observed between the two analysed matrices, with higher diversity indices in sediments. However, no significant differences in microbial diversity were found across the depth gradient. The composition of bacterial communities revealed a specific microbiota for *M. palmata*, distinct from its physical habitat, primarily dominated by bacteria from the phylum Proteobacteria, whereas the sediments were dominated by Actinobacteriota species.

Both bacterial communities are involved in various metabolic processes, showing a functional profile with the prevalence of amino acid and carbohydrate metabolism.

The fourth study case focused on evaluating the bacterial communities associated with different organs of the bivalve *Mya arenaria* (siphon, gills, and stomach) and the

surrounding sediments from the Romanian Black Sea coast, using Illumina sequencing of the 16S rRNA gene. This study represents the first characterization of the variation in the microbiome of *M. arenaria* at the organ level.

The results led to the identification of 1,340,099 bacterial amplicon sequence variants (ASVs). The bacterial communities associated with the siphon were classified into 16 phyla, 23 classes, 34 orders, 41 families, and 43 genera. The communities colonizing the gills were classified into 15 phyla, 21 classes, 33 orders, 39 families, and 35 genera, while the stomach-associated bacteria belonged to 14 phyla, 22 classes, 30 orders, 29 families, and 31 genera. The microbiome of the adjacent sediments comprised 20 phyla, 38 classes, 50 orders, 48 families, and 54 genera.

Significant differences in microbial diversity were observed between the sediment-associated communities and those of the three organs analysed, with higher diversity indices in the sediment microbiome. No notable differences in microbial diversity were observed based on water depth.

Taxonomically, the siphon microbiome was primarily represented by the phylum Spirochaetota, while Verrucomicrobiota species were predominant in the gills, and Firmicutes in the stomach. The bacteria colonizing the sediments were dominated by taxa from the phylum Proteobacteria.

The bacterial communities associated with both the bivalve organs and the sediments were involved in various metabolic processes, with the prevalence of carbohydrate, amino acid, and energy metabolism.

Perspectives

Understanding the taxonomic and functional diversity, distribution of invertebrates, and the role of bacterial communities associated with these organisms in the Danube - Danube Delta - Black Sea ecosystem is crucial for grasping the functioning and resilience of these aquatic systems.

In this context, continued monitoring of these organisms, as well as other species such as polychaetes, bivalves, crustaceans, sponges, and tunicates, is essential for understanding population dynamics and the impact of environmental factors on their distribution.

Implementing biodiversity management strategies based on genetic data can support the conservation of native species and the detection and control of invasive ones. Future studies should also encompass additional invertebrate groups, providing essential information for biodiversity conservation and sustainable management of natural resources in aquatic ecosystems.

For a detailed characterization of the functional profile of bacterial communities associated with these invertebrate species, molecular analyses using advanced metagenomic and metatranscriptomic techniques are needed. This will contribute to elucidating the role of microbiomes in the adaptation of aquatic invertebrates to environmental conditions in the Black Sea and Danube habitats.

Furthermore, exploring the potential applications of the bacteria identified in these studies for pollutant degradation or various biotechnologies — through extensive enzymatic activity testing and genomic analysis of these strains — could open new opportunities for discovering bioactive compounds with unique properties. This research could also expand into bioprospecting these marine and riverine ecosystems to find new microorganisms that could benefit the bioeconomy.

DISSEMINATION OF RESULTS

Published articles

Menabit, S., Lavin, P., Mureşan, M., Begun, T., Teacă, A., Purcarea, C. 2024. First screening of bacteria assemblages associated with the marine polychaete *Melinna pakmata* Grube, 1870 and adjacent sediments. *Front. Mar. Sci.*, 10, 1279849. <https://doi.org/10.3389/fmars.2023.1279849> (Q1)

Menabit, S., Begun, S., Teacă, A., Mureşan, M., Lavin, P., Purcarea, C. 2022. DNA barcoding and distribution of gastropods and malacostracans in the Lower Danube region. *Diversity*, 14, 533. <https://doi.org/10.3390/d14070533> (Q2)

Menabit, S., Iancu, L., Pavel, A. B., Popa, A., Lupascu, N., Purcarea, C.. 2022. Molecular identification and distribution of insect larvae in the Lower Danube River. *Oceanological and Hydrobiological Studies*, vol.51(1), pp. 74-89. <https://doi.org/10.26881/oahs.2022.1.07> (Q4)

Other published articles

Ruginescu, R., Lavin, P., Iancu, L., **Menabit, S.**, Purcarea, C. 2022. Bioprospecting for Novel Bacterial Sources of Hydrolytic Enzymes and Antimicrobials in the Romanian Littoral Zone of the Black Sea. *Microorganisms*, 10, 2468. <https://doi.org/10.3390/microorganisms10122468> (Q2)

Pavel, A. B., **Menabit, S.**, Lupaşcu, N., Kreuter, S. 2022. Role of environmental parameters in the distribution of meiobenthic fauna (Ostracoda and Foraminifera) in the Romanian Black Sea shelf. *AgroLife Scientific Journal*, 11(1). <https://doi.org/10.17930/AGL2022119> (Q4)

Begun, T., Teacă, A., Mureşan, M., Quijón, P. A., **Menabit, S.**, Surugiu, V. 2022. Habitat and Macrozoobenthic Diversity in Marine Protected Areas of the Southern Romanian Black Sea Coast. *Frontiers in Marine Science*. 9, 845507. doi: 10.3389/fmars.2022.845507 (Q1)

Mureşan, M., Motoc, R., **Menabit, S.**, Teacă, A., Begun, T. 2022. A New Species of Free-Living Nematodes (Desmodorida, Desmodoridae) in the Romanian Black Sea Waters. *Diversity*. 14, 933. <https://doi.org/10.3390/d14110933> (Q2)

Teacă, A., Begun, T., **Menabit, S.**, Mureșan, M. 2022. The First Record of *Marenzelleria neglecta* and the Spread of *Laonome xeprovala* in the Danube Delta–Black Sea Ecosystem. *Diversity*, 14, 423. <https://doi.org/10.3390/d14060423> (Q2)

Pavel, A. B., **Menabit, S.**, Pop, I. C. 2021. New records of soft-shelled monothalamous Foraminifera and gromiids on the Romanian Black Sea shelf. *Biologia* 76, 2241–2251. <https://doi.org/10.1007/s11756-021-00708-x> (Q3)

Pavel., A. B., **Menabit, S.**, Pop, I. C., Stanescu, I., Lupascu, N. 2021. The spatio-temporal distribution of the Ponto-Caspian polychaete in the Lower Sector of the Danube River and in Danube Delta. *Global Ecology and Conservation*, 28, e01623. DOI10.1016/j.gecco.2021.e01623 (Q1)

Teacă, A., Mureșan, M., **Menabit, S.**, Bucșe, A., Begun, T. 2020. Assessment of Romanian circalittoral soft bottom benthic habitats under Danube River influence. *Regional Studies in Marine Science*, 40, 101523. DOI10.1016/j.rsma.2020.101523 (Q3)

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