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Short communication

Morphological and molecular identification of some Oligochaetes species in the Hilla River, Babylon province, Iraq

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Abstract

Our comprehensive study navigates the Hilla River, employing both morphological and molecular methods to identify Oligochaetes. The morphological study confirms the identification of five species (*Aulodrilus pigueti*, *Branchiura sowerbyi*, *Eiseniella tetraedra*, *Limnodrilus hoffmeisteri*, *Stylaria lacustris*) using the SEM technique. Out of all species, *Eiseniella tetrahedra* received the greatest percentage (55.19 %). We also assessed their diversity using Shannon-Wiener (ranging between 0.23 – 0.93), Simpson (ranging between 0.32 – 0.91) and other biodiversity indices, indicating environmental stress. Oligochaetes DNA isolation and extraction. Mitochondrial cytochrome c oxidase subunit I (COI) region was amplified and sequenced to find the similarity and variations among species and confirm the diagnosis of species (*Paranaislitoralis*, *Paranaisfrici*, *Stylarialacustris*, *Monopylephorus rubroniveus*, *Tubifextubifex*) and all sequence and strains matched to the complete genetic content of the samples at the NCBI to ensure the results. This study is the first to diagnose Oligochaetes in the middle Euphrates region using the molecular technique, confirming the identification of numerous unrecorded species that share morphological characteristics with other species, making it challenging to categorize them morphologically.

Keywords: Hilla river, Oligochaetes, Biodiversity indices, DNA barcoding

Introduction

Biodiversity depends on three levels: genetic diversity, species diversity, and ecosystem diversity (Spangenberg, 2007). Morphological species richness is mostly used as the mine scale for measuring biodiversity (De Queiroz, 2007).Despite morphological importance, many studies and research have clarified that the species concepts dependent on the shape and morphology have miscalculated how crucial biodiversity is to ecological systems (Monro & Mayo, 2022). Other studies found that genetic diversity, especially in freshwater organisms, has given new concepts to diversity and recording species through their genetic content and species delimitation (White *et al.*,2014). This gives cause for many worries about the true numbers of species on Earth and the true diversity of living organisms (Zachos *et al.*,2013) And the influence of geology and geography on the spread and diversity of species (Spitzer, 2014). Other researchers have discovered evidence of cryptic species, which are similar morphologically but different genetically when using genetic techniques to determine their species based on genetic material (Bickford *et al.*,2007).

Recent studies of environmental pollutants have confirmed that measures of biodiversity and its indicators are indicators for pollution monitoring and environmental changes. (Zaghloul *et al.*,2020). Some diversity indices, such as the Shannon Diversity Index, Dominance index (Simpson Diversity Index), Relative abundance, and Uniformity index, were used in this study. Since the beginning of interest in measuring biodiversity, the number of species and its changes have been considered Biodiversity (Williams,2004), which consists of two components, species richness and evenness (Magurran,2004), and can be regarded as factors of sustainability and healthiness for most ecosystems (Simba,2024). The community's diversity affects many factors, including human activity, sampling size, farming, rivers, underground waters, and the aquatic environment (Afshani *et al.*,2009). Most freshwater invertebrates are characterized by the small size and complexity of their life cycle and contain several life stages, so it is often difficult to rely on morphological identification in biomonitoring, classification, and biodiversity (Strachan *et al.*,2015). Furthermore, when classifying to the species level, the adult stage is required, and it is impossible to rely on the larval or immature stages to identification (Stein *et al.*,2014). Modern techniques such as gene sequencing are very successful in the genetic diagnosis of many species of aquatic invertebrates (Webb *et al.*,2012). In addition, through genetic biodiversity, it is possible to evaluate freshwater quality through biomonitoring for freshwater invertebrates (Carter & Resh,2013).

Oligochaetes are the most diverse and abundant group in freshwater habitats (Atanacković *et al.*,2023). Aquatic Oligochaetes are very small, ranging from less than 0.5 mm up to 28 centimeters in length, although most are smaller than 3 cm (Krieger &Stearns, 2010). More than 1700 species of aquatic oligochaetes have been identified, most of which about 1100 species inhabit freshwater. The Tubificidae is the most diverse group, with more than 1000 species described; most of these species inhabit freshwater, and about 60 megadriles are also considered aquatic habitats (Martin *et al.*,2008).

Limited studies in Iraq concerning the Annelid fauna, especially Oligochaeta, have been conducted. Jaweir (2011)found three new tubificid species (*Limnodrilus profundicola, Embolacephalus velutinus,* and *Aulodrilus pigueti*) in the Marsh in southern Iraq. Tubificid worms have also been recorded by other researchers in various aquatic habitats, which included *Tubifextubifex*, *L. claparedianus*, *L. profundicola*, *Branchiura sowerbyi*, *L. maumeensis,* and *Limnodrilus hoffmeisteri*. (Jaweir*et al.*,2002; Al-Khafaji,2002; Al-Rubaiy,2002; Farman,2005; Ali,2007; Sebtie,2009). Most studies on the Oligochaetes have been focused on Naididae fauna, such as recording 37 naidid species previously by several workers (Nashaat, 2010; Al-Abbad, 2012; Jaweir*et al.*, 2012; Radhi, 2012 and Jaweir and Al-Janabi,2012). Recently, Al-Ameen and Jawair,2020 studied the aquatic Oligochaetes community in the AL-Abbasyia River (Al-Najaf province) and identified three families: Naididae, Lumbriculdae, and Enchytraaeidae. Also, the studies of genetic variation between and within species at a molecular level are very few; Othman & Ahmad (2020) from Kurdistan-Iraq use Genetic techniques for Molecular identification and classification of species (Annelida; Oligochaeta) by using DNA barcoding. Zaar and Jaweir (2021) also identified the Limnodrilus Claparede species using morphological and molecular methods.

Finally, the morphological identification of Oligochaetes to the species level depends on the arrangement of the chaetae, located dorsally on the prepatellar segments. Identifying some species requires a mature stage of prepatellar segments, but their classification depends on the reproductive system. That is why, in our study, we focused on molecular analyses and Scanning electron microscopy (SEM) as modern techniques for identification.

Material and methods

The study samples were collected seasonally (Spring 2021 to Winter 2022) from Hilla River, one of the main rivers branching off the Euphrates River. Five stations were selected, including three from the Hilla River and two drainages branching from the river (Figure 1). The first station (S1) is a representation of the Hilla River; It is situated at the river's entrance to the village of Anana (32°33'6.61"N; 44°25'14.22"E). The second station (S2) represents the Hilla River before entering the city center (Betta Bridge) (32°31'1.35"N; 44°25'37.40"E). The third station (S3) depicts the Hilla River immediately following its departure from the governorate's center in the Al-Farsi region (32°28'7.31"N; 44°26'23.79"E). The fourth station (S4) is a drain that arises from the Hilla River and flows through the village of Al-Ghalis (32°26'15.59"N; 44°26'53.56"E). The fifth station (S5) is a drain that splits off from the Hilla River near Nazim Dora and travels through the region between Al-Dolab and Al-Dabla (32°25'4.08"N; 44°29'14.67"E).

Figure 1. Maps of the Hilla River show the studied stations

An Ekman Birge grab sampler was used to collect sediment samples during the study period (Spring 2021 and Winter 2022). Three replicates for each sample were brought to the laboratory, and formalin (4 %) and ethanol (75%) were used to kill and preserve the worms(Anderson,2013).

Analysis of the diversity index

Diversity analysis includes calculating relative abundance (Salahi*et al.*, 2017), diversity and uniformity indexes, and dominance indexes(Magurran,2013).

Morphological part

The specimens were photographed using a Scanning Electron Microscope (SEM) type (INSPECT S50); the sample was coated with gold. Species identification was conducted using appropriate keys (Brinkhurst & Jamieson,1971; Brinkhurst,1971; Stimpson *et al*.,1982; Brinkhurst,1986; Timm,2009; Pinder,2010).

Molecular part

A cetyltrimethyl ammonium bromide "CTAB" Kit for DNA Extraction was used to extract genomic DNA from samples according to protocol. Since the study was conducted to obtain genetic diversity among Oligochaetes, 18s rRNA were selected for this study. Pairs of primers were chosen to amplify parts of studied samples (Bioneer/ Korea) (Table 1), and the sequences were matched to the complete genetic content of the samples at the NCBI to ensure the results of sequencing results.

Table 1. Primer was used for the characterization of oligochaetes species in the current study.

Type of Primer	Sequence 5' to 3'	Product Size(base pairs)	Conditions steps (temperature, time)
Oligochaetes,F	GGGCGTAATGAAAGTGAAGG	500	1: 95 Cº, 2 min. 2: 95 Cº, 30 sec. 3: 56 Cº, 30 sec. 4: 72 Cº , 20.0 sec. 6: 72 Cº, 5 min. 7: 4 Cº, forever
Oligochaetes,R	TAACCGGACGTTTGGTTCAT		

Results and discussion

A total of 683 Oligochaeta individuals and five species were identified. They were *Aulodrilus pigueti*, 178 were *Branchuira sowerbyi*, 9 were *Limnodrilus hoffmeisteri*, 114 were *Stylaria lacustris* and 377 were *Eiseniella tetraedra*. These represented 0.73 %, 26.06 %, 1.3 %, 16.78 % and 55.19 % of the total sample respectively. This result is a new record for the Al-Hilla River (Fig. 2, Table 2). Regarding their distribution through the study sites and period, the highest number of individuals, 88, was recorded in S1 during Spring, while the lowest number, 5, was recorded in S5 during Summer. As can be seen from Table 2, it turned out that the decline in the numbers of benthic invertebrates coincided with the rise in temperatures during the hottest season of the year, and this agrees with some studies (Jaweir&Radhi,2013Jaweir&Al-Seria,2015). *Eiseniella tetrahedra* was the most abundant species, consistent with Javidkar *et al*.(2020).

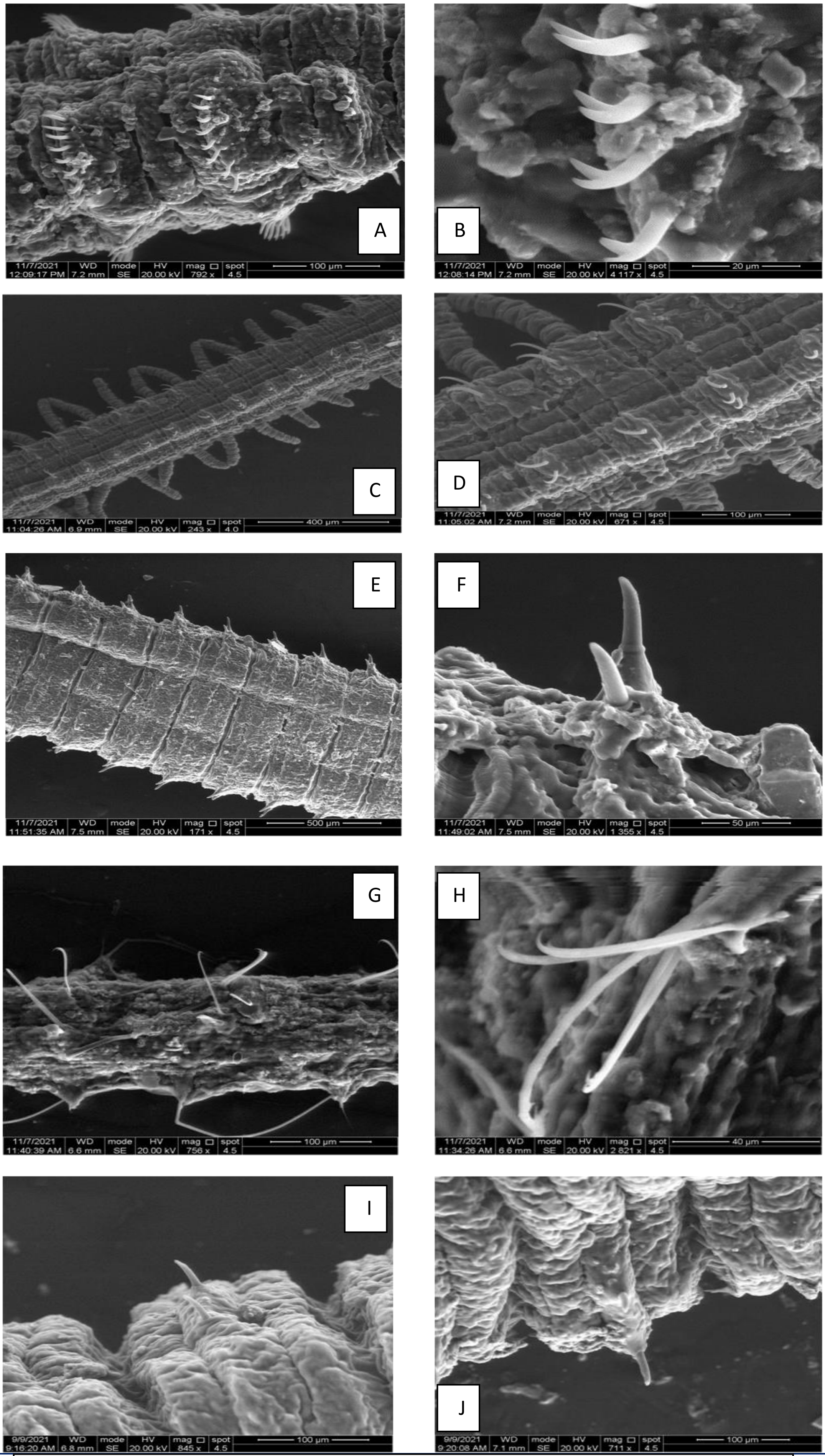

Figure 2. SEM pictures of the indentified species : A-B, *Aulodrilus pigueti ; C-D, Branchuira sowerbyi; E-F, Limnodrilus hoffmeisteri; G-H, Stylaria lacustris; I-J, Eiseniella tetraedra*

Table 2. Seasonal changes in numbers and percentage of the recorded species during the study period

Species	St 1 (village of Anana)				St 2(Betta Bridge)				St 3 (Al-Farsi region)				St 4 (village of Al-Ghalis)				St 5 (Nazim Dora)				Total No.	Percentage %
	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter		
Aulodriluspigueti	0	0	0	0	0	0	0	0	3	0	0	0	0	2	0	0	0	0	0	0	5	0.73
Branchiurasowerbyi	28	5	8	0	15	4	0	10	26	10	9	4	19	6	0	4	12	5	0	13	178	26.06
Limnodrilushoffmeisteri	0	0	0	3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	5	9	1.3
Stylarialacustris	17	8	9	3	0	7	9	5	0	11	12	0	0	0	8	6	5	4	2	8	114	16.78
Eiseniellatetraedra	43	15	17	20	46	24	19	13	43	23	12	3	35	10	6	7	39	0	22	0	377	55.19
Total number of Individuals	88	28	34	26	61	35	28	28	72	44	33	8	54	18	14	17	56	5	24	26	683	
number of Species	3	3	3	3	2	3	2	3	3	3	3	3	2	3	2	3	3	2	2	3		

Previously, shape and phenotypic characteristics were relied on to identify and classify species. However, molecular techniques and morphology have now developed the classification process for species in different environments. Also, species identification using DNA technology has helped diagnose the young individuals of these species at various ages, which were previously identified only by adults. This is the first DNA molecular study in Babylon provinceto identify and classify the Oligochaetes species by DNA barcoding using the cox1 gene and find the phylogenetic relationship among species. The Basic Local Alignment Search Tool (BLAST) in NCBI compares nucleotide sequences. In addition, the information obtained from Gen Bank was used to compare sequences of different species and draw a phylogenetic tree (Figure 3). According to the phylogenic tree, the results of sample 1 were two species belonging to the Naididae family, and sample 2 had five species belonging to the Naididae family, too. BLAST similarity analysis showed that the obtained sequences have 99 – 100% bootstrapping with the sequences in GenBank.The current study focused on the molecular identification of Oligochaetes for many reasons, frequently has intricate life cycles, dispersal abilities, and quick local adaptations, all of which may promote intraspecific divergence and interspecific gene flow (Cristescu,2014).

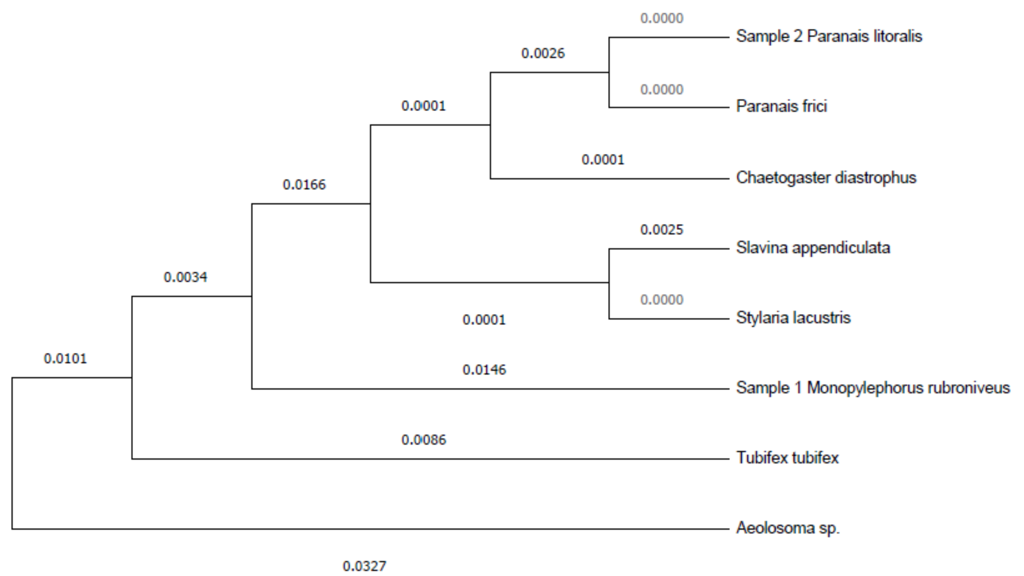

Figure 3. Phylogenetic tree (according to BLAST) of the studied species.

The values of the diversity indexes were calculated for the diagnosed species isolated from the stations. According to the Shannon-Wiener index, The present study indicated that the (S4) recorded the highest value (0.93) in the summer and the (S5)recorded the lowest value (0.17) in the Autumn (Table 3). This index's values dipped in the Autumn, perhaps due to increased turbidity and suspended debris that affects the variety of food, as mentioned (Abdulwahab&Rabee,2015).

The Shannon-Wiener index estimates the diversity of species within a community; high values of it are evidence of high diversity (Burton *et al.*,1999), and A value higher than three refers to a high diversity of a healthy community living in a good environment, while when the Shannon-Wiener value lower than 1 meaning the presence of environmental change which leads to the disappearance of sensitive species and their migration (Jaweir&Al-Seria,2015) so in this study indicate that the current unstable environment suffers from pressures possibly caused by pollution and is unsuitable for the growth of worms. The seasonal changes lead tochangesin diversity values, which in turn affects the nature of the life cycle of each species, and our results agree with Ibrahim (2005).

The dominance (Simpson index) was measured in the current study and showed a high value (0.91) in (S5) during autumn and a low value (0.32) in (S3) during winter (table 3). These findings showed that the communities are stable and mature because many species share dominance. It is acknowledged that low diversity, which typically displays values near zero, indicates that the communities are stressed, which agrees with Dash (2003). These results showed that the communities are stable and mature because many species share dominance. It is acknowledged that low diversity, which typically displays values near zero, indicates that the communities are stressed. The current study's findings demonstrated that the values of the Uniformity index fluctuated between a low value (0.24) in (S5) during Autumn and a high value (0.85) in (S4) during summer, as shown in Table 3. The decrease in the values of the Uniformity index indicates the predominance of a few species with high densities, which suggests the presence of environmental pressure, and our results agree with Proto-Neto (2003).

According to our findings, the relative abundance index's values varied from a low value (0.73) in (S5) during summer to a high value (12.8) in (S1) during Spring, as shown in table (3). The results indicate that the current species were rare in all stations and seasons because their relative abundance was less than 10% (Omori & Ikeda,1984).

Table 3. Seasonal changes in biodiversity indices for the study stations during the study period

Stations	Seasons	Simpson Index	Shannon Wiener index	Relative abundance (%)	Uniformity index

St1	Spring	0.5	0.73	12.8	0.66
	Summer	0.54	0.72	4.09	0.66
	Autumn	0.5	0.76	4. 97	0.69
	Winter	0.82	0.36	3.8	0.33
St2	Spring	0.87	0.23	8.93	0.33
	Summer	0.69	0.54	5.12	0.49
	Autumn	0.8	0.32	4.09	0.46
	Winter	0.44	0.82	4.09	0.75
St3	Spring	0.48	0.8	10.54	0.73
	Summer	0.53	0.72	6.44	0.66
	Autumn	0.44	0.83	4.83	0.76
	Winter	0.32	0.69	1.17	0.63
St4	Spring	0.9	0.19	7.9	0.27
	Summer	0.39	0.93	2.63	0.85
	Autumn	0.71	0.41	2.04	0.59
	Winter	0.4	0.88	2.48	0.8
St5	Spring	0.6	0.63	8.19	0.57
	Summer	0.66	0.45	0.73	0.65
	Autumn	0.91	0.17	3.51	0.24
	Winter	0.74	0.48	3.8	0.44

Conclusion

The biodiversity of oligochaetes fluctuated through the Hilla River during all seasons, and we noticed there was little species richness and evenness of oligochaetes according to biodiversity indices values. Regarding the recorded species, there were five species with low density and individual numbers. They were *Aulodrilus pigueti (5)*, *Branchuira sowerbyi (*178), *Limnodrilus hoffmeisteri* (9), *Stylaria lacustris* (114) and *Eiseniella tetraedra* (377).Ultimately, there was a decrease in genetic diversity, which suggests that the river water may be under environmental stress due to changes in its chemical and physical characteristics, leading to pollution. We recommend relying on genetic and molecular methods to prove the identification of the oligochaetes in addition to using the SEM technology for the morphological identification.

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