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Molecular and morphological identification of some oligochaete species as indicator for ecological diversity of the Hilla River in Babylon Province-Iraq

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Abstract

The present study was carried out in Hilla river for the identification of Oligochaetes using morphological and molecular methods. The morphological study confirms the identification of five species (*Aulodrilus pigueti*, *Branchiura sowerbyi*, *Eiseniella tetraedra*, *Limnodrilus hoffmeisteri*, *Stylaria lacustris*) using scanning electron microscope (SEM) technique. Out of all species, *Eiseniella tetraedra* received the greatest percentage of density (55.19 %). Their diversity was assessed using several indices. Value of Shannon-Wiener index ranged between 0.23-0.93 and that of, Simpson index ranged between 0.32-0.91. The results of these and other biodiversity indicate the presence of environmental stress. 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 (*Paranais litoralis, Paranais frici, Stylaria lacustris, Monopylephorus rubroniveus, Tubifex tubifex*) and all sequence and strains matched to the complete genetic content of the samples at the NCBI to ensure the results. This study is considered the first study for the diagnosis of oligochaetes in the middle Euphrates region by using the molecular technique to confirm the identification of numerous unrecorded species that share morphological characteristics with other species, making it challenging to categorize them morphologically.

Keywords

biodiversity indices; DNA barcoding; Hilla river, oligochaetes

Introduction

Biodiversity is studied at three levels, genetic diversity, species diversity and ecosystem diversity (1). Mostly, morphological species richness is used as the mine scale for measuring biodiversity (2). 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 (3). Other studies found that genetic diversity, especially freshwater organisms, has given new concepts to diversity and recording species through their genetic content and species delimitation (4). This raises many concerns about the true number of species on earth and the true diversity of living organisms (5), as well as the influence of geology and geography on the spread and diversity of species (6). Few researchers have reported cryptic species, which are similar morphologically but different genetically using molecular techniques (7).

Recent studies of environmental pollutants have confirmed that measures of biodiversity and its indices are indicators for pollution monitoring and environmental changes (8). Some diversity indices, such as Shannon

diversity index, Dominance index (Simpson index), Relative abundance and Uniformity index, were used in this study (36). Since the beginning of interest in measuring biodiversity, the number of species and its changes have been considered biodiversity (9), which consists of two components, species richness and evenness (10) and can be regarded as factors of sustainability and healthiness for most ecosystems (11). Diversity is affected by many factors, including human activity, sampling size, farming, rivers, underground waters and the aquatic environment (12). 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 (13). 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 (14). Modern techniques such as gene sequencing are very successful in the genetic diagnosis of many species of aquatic invertebrates (15). In addition, through genetic biodiversity, it is possible to evaluate freshwater quality through biomonitoring of freshwater invertebrates (16).

Oligochaetes are the most diverse and abundant group in freshwater habitats (17). 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 (18). More than 1700 species of aquatic oligochaetes have been identified and 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 species of megadriles are also considered aquatic habitats (19).

Limited studies in Iraq concerning the annelid fauna, especially oligochaeta, have been conducted. Three new tubificid species (*Limnodrilus profundicola*, *Embolacephalus velutinus* and *Aulodrilus pigueti*) were identified in the marsh in southern of Iraq (20). Tubificid worms such as *Tubifex tubifex*, *Limnodrilus claparedianus*, *Limnodrilus profundicola*, *Branchiura sowerbyi*, *Limnodrilus maumeensis* and *Limnodrilus hoffmeisteri* have also been recorded in various aquatic habitats (21-26). Most studies on the oligochaetes have been focused on Naididae fauna, 37 naidid species have been previously reported (27-30). The aquatic oligochaetes community in AL-Abbasyia River (Al-Najaf province) identified three families Naididae, Lumbriculdae and Enchytraaeidae (31). Also, the studies of genetic variation between and within species at a molecular level are very few; The genetic techniques for molecular identification and classification of species (Annelida; Oligochaeta) by using DNA barcoding in Kurdistan-Iraq. By using morphological and molecular methods, *Limnodrilus claparedianus* was identified (32).

Finally, the morphological identification of oligochaetes to the species level depends on the arrangement of the chaetae, located dorsally on the prepatellar segments, but identification of some species needs to be done at mature stage, as their classification depends on the reproductive system. Therefore, our study focused on molecular analyses and Scanning Electron Microscope (SEM) as modern techniques for identification of the oligochaete species.

Materials and Methods

Collection of samples

The study samples were collected seasonally (Spring 2021 to Winter 2022) from Hilla river. Five sites were selected, including three stations from Hilla river and two streams branching from the river. The first site (St1) represents 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 site (St2) represents the Hilla river before entering the city center (Betta bridge) (32°31'1.35"N; 44°25'37.40"E). The third site (St3) depicts the Hilla river immediately following its departure from the governorate's center in Al-Farsi region (32°28'7.31"N; 44°26'23.79"E). The fourth site (St4) is a stream 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 site (St5) is a stream 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) (Fig. 1). An Ekman Birge grab sampler was used to collect sediment samples during the study period. Three replicates for each sample were brought to the laboratory and formalin (4 %) and ethanol (75 %) were used to fix and preserve the worms (34).

Analysis of diversity indices

Analysis of diversity includes the calculation of Relative abundance (35), Shannon index, Uniformity index and Simpson index (36).

Morphological identification

Scanning Electron Microscope (SEM) type (INSPECT S50) was used for identification of the specimens; the sample was coated with gold. Species identification was conducted using appropriate keys (37-42).

Molecular identification

DNA extraction: Cetyl trimethyl ammonium bromide "CTAB" Kit was used to extract genomic DNA from samples following manufacturers protocol provided by Macrogene company. Since the study was conducted to obtain genetic diversity among oligochaetes, 18s rRNA were selected for this study (32).

Preparation of primers: Primers were obtained from https://www.ncbi.nlm.nih.gov/ and designed using use free online tool https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/. The lyophilized primers were sourced from Macrogene company, List of primers used in this study are shown in Table 1.

Polymerase chain reaction (PCR): Conventional PCR was used to amplify the target DNA using specific primer pairs. It includes three consecutive steps that are repeated for specific number of cycles to get PCR product which could be finally visualized after agarose gel electrophoresis. The thermal cycling conditions are mentioned in Table 2. PCR products were sent to Macrogene company in Korea to be matched to the complete genetic content of the samples at NCBI to ensure the sequencing results.

Fig. 1. Maps of the Hilla river show the studied sites.

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

Primer	Sequence 5' to 3'	Product Size (base pairs)	Annealing temperature
Primer- F	GGGCGTAATGAAAGTGAAGG	500	56 °C
Primer- R	TAACCGGACGTTTGGTTCAT		

Results and Discussion

A total of 683 oligochaeta individuals belonging to five species were reported. The five identified species include *Aulodrilus pigueti* (5), *Branchuira sowerbyi* (178), *Limnodrilus hoffmeisteri* (9), *Stylaria lacustris* (114) and *Eiseniella tetraedra* (377). These represented 0.73 %, 26.06 %, 1.3 %, 16.78 % and 55.19 % of the total sample respectively. These results represent a new record for Hilla river (Fig. 2, Table 3). Regarding their distribution throughout the study sites and periods, the highest number (88) of individuals recorded in St1 during spring, while the lowest number (5) recorded in St5 during summer. As can be seen from (Table 3), 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 (43 & 44). *Eiseniella tetrahedra* was the most abundant species in majority of the sites across seasons (45).

Previously, reliance was placed on shape and phenotypic characteristics to identify and classify species. However, now the combined use of molecular techniques and morphology has been preferred for the identification of the species. Also, species identification using DNA technology has helped diagnose the young individuals of these species at different ages, which was previously possible only at adult stages. This is the first DNA molecular study in Babylon province to 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 is used for nucleotide sequence comparison. In addition, the information obtained from GenBank was used to compare sequences of different species and draw a phylogenetic tree (Fig. 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 (46).

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 (St4) recorded the highest value (0.93) in the summer and the (St5) recorded the lowest value (0.17) in the autumn (Table 4). This index's values dipped in the autumn, perhaps as a result of increased turbidity and suspended debris that affects the variety of food (47).

The Shannon-Wiener index estimates the diversity of species within a community, its high values are evidence of high diversity (48) and a value higher than 3 refers to high diversity of a healthy community living in a good

Table 2. PCR conditions for the current study

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

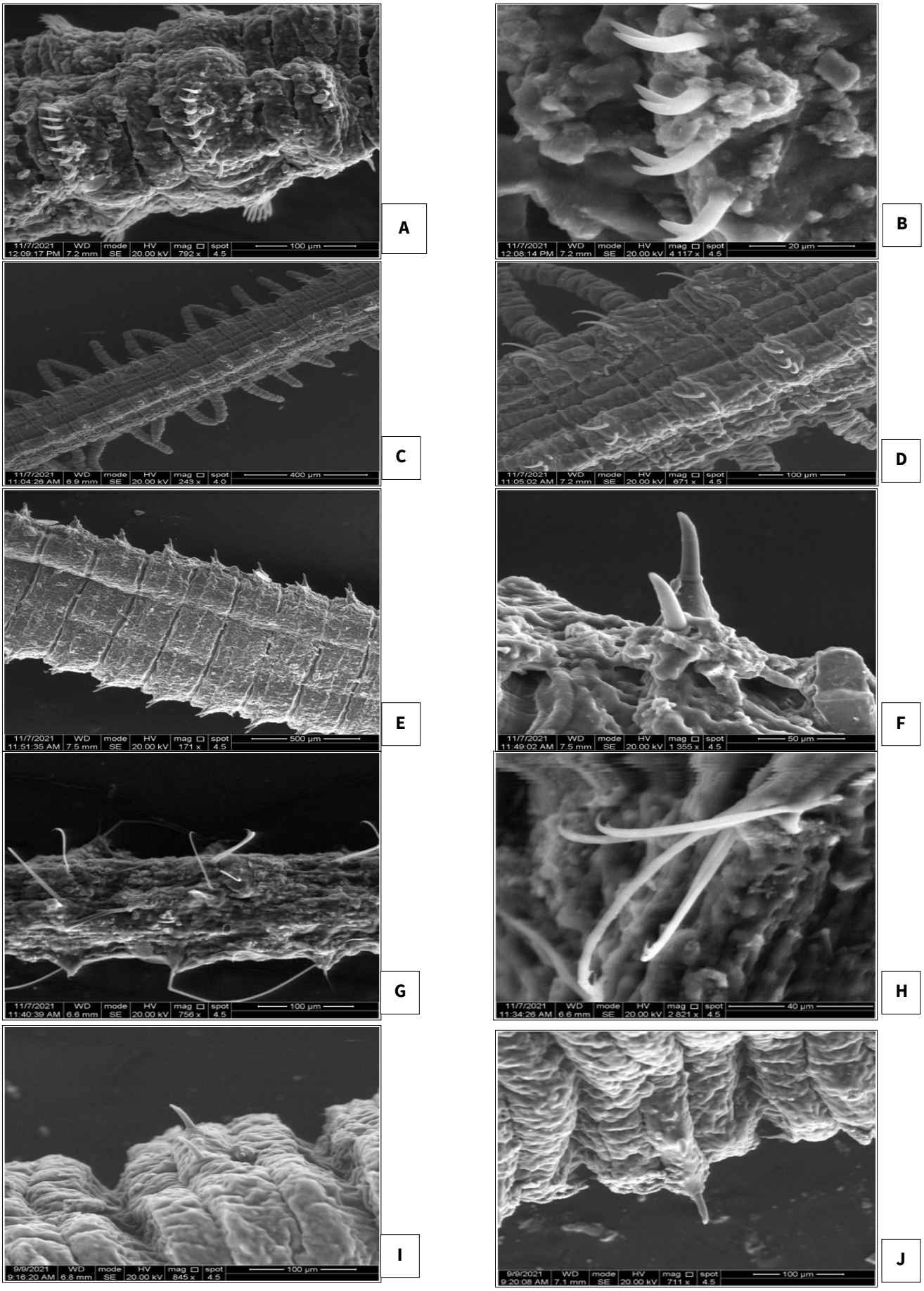

Fig. 2. SEM pictures of the indentified species : A-B, *Aulodrilus pigueti ; C-D, Branchuira sowerbyi; E-F, Limnodrilus hoffmeisteri; G-H, Stylaria lacustris; I-J, Eiseniella tetraedra* .

Table 3. Seasonal changes in the 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		
Aulodrilus pigueti	0	0	0	0	0	0	0	0	3	0	0	0	0	2	0	0	0	0	0	0	5	0.73
Branchiura sowerbyi	28	5	8	0	15	4	0	10	26	10	9	4	19	6	0	4	12	5	0	13	178	26.06
Limnodrilus hoffmeisteri	0	0	0	3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	5	9	1.3
Stylaria lacustris	17	8	9	3	0	7	9	5	0	11	12	0	0	0	8	6	5	4	2	8	114	16.78
Eiseniella tetraedra	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		

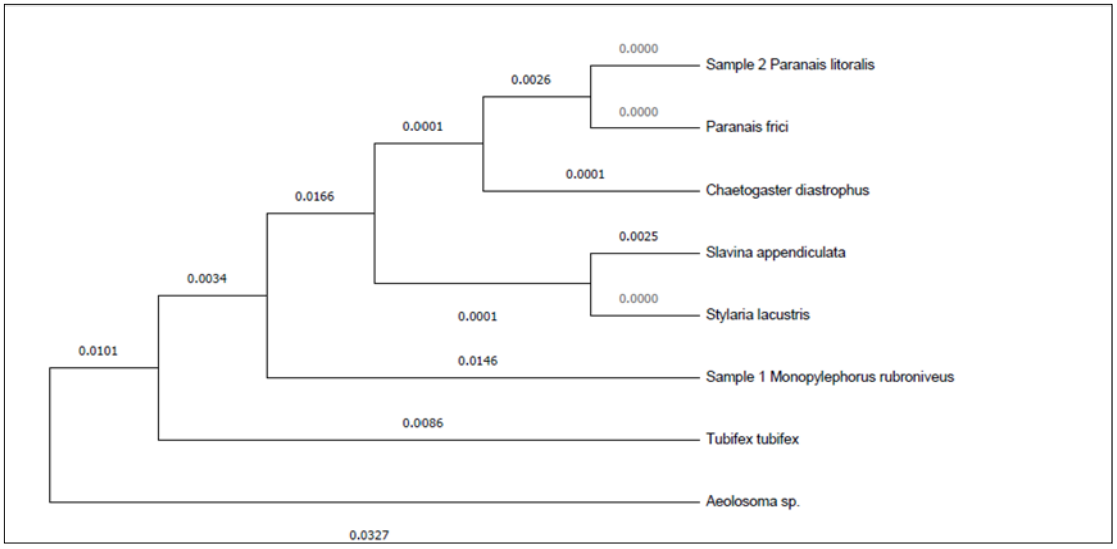

Fig. 3. Phylogenetic tree (according to BLAST) of the studied species.

Table 4. 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

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 (44), so, worms cannot grow in the current unstable environment due to stresses that may be brought on by pollution. The seasonal changes lead to changes in diverse values, which in turn affects the nature of the life cycle of each species and our results agree with previous reports (49).

The dominance (Simpson index) was measured in the current study and shows a high value (0.91) in (St5) during autumn and a low value (0.32) in (St3) during winter (Table 4). These findings showed that the communities are stable and mature because many different species share dominance. It is acknowledged that low diversity, which typically displays values near zero, indicates that the communities are under stress (50). The current study's findings demonstrated that the values of the Uniformity index fluctuated between a low value (0.24) in (St5) in autumn and a high value (0.85) in (St4) during summer, as shown in Table 4. The decrease in the values of the Uniformity index indicates the predominance of a few species with high densities, may be due to the presence of environmental pressure or some species being more competitive (51, 53).

The relative abundance index's value varied from a low value (0.73) in (St5) during summer to a high value (12.8) in St1 (Table 4). The results indicate that the current species were rare in all stations and seasons because their relative abundance was less than 10 % (52).

Conclusion

The biodiversity of oligochaetes fluctuated throughout the Hilla river among all seasons and we noticed there were little species richness and evenness of oligochaetes according to biodiversity indices values. Regarding the recorded species, there were four species (*Aulodrilus pigueti*, *Branchuira sowerbyi*, *Limnodrilus hoffmeisteri*, *Stylaria lacustris*) with low density and individual numbers while *Eiseniella tetraedra* had a good density during the study period may be more tolerant to environmental conditions. Ultimately, there was a decrease in diversity, which suggests that the river water may be under environmental stress due to changes in its chemical and physical characteristics which lead to the presence of pollution. We recommend reliance on molecular methods and SEM to prove the identification of the oligochaetes.

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Authors' contributions

AJAAR and WAKAY designed and performed the experiment. AJAAR analyzed data and wrote the first draft of manuscript; WAKAY performed statistical analysis and revised the manuscript. Both authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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