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Original Article

Morphological plasticity of *Nemoura Cinerea* (Arthropoda, Nemouridae) as a biological indicator for aquatic systems

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Abstract: Macrobenthos are indicators of the physical and chemical changes in aquatic ecosystems. In this research, the diversity of macrobenthos communities in the Ortkand River, Iran, was investigated during four consecutive seasons using the Shannon-Weiner, EPT richness indices, and the Hilsenhoff index for water quality at four sampling stations. In addition, sampling of *Nemoura cinerea* (Arthropoda, Nemouridae) was done to compare morphological differences between specimens from two upstream and downstream sites using a geometric morphometric approach. The physicochemical parameters of water were also recorded. The entry of fish farm wastewater significantly affects biotic and abiotic environmental factors according to the Shannon-Weiner and Hilsenhoff indices. The results showed a significant positive correlation between DO and Shannon-Wiener index, TDS and the Hilsenhoff index, and DO and EPT richness index. A significant negative correlation was observed between BOD and the Shannon-Wiener index, DO and Hilsenhoff index, and between BOD and EPT richness index. Geometric morphometrics analyses revealed that the two groups differed mainly in pronotum and metanotum morphology. According to the results, monitoring of macrobenthos can help assess rivers' water quality, and *N. cinerea* can be a proper bioindicator.

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Introduction

Benthic communities play important roles in the food chains of aquatic ecosystems (Fries and Bowles, 2002). The quantification of their changes using indices of diversity provides crucial information. The study of macrobenthos communities is also one of the common methods for evaluating stresses on aquatic ecosystems and water quality monitoring (Lydy et al., 2000). In addition, the physical and chemical status of the aquatic ecosystem can be recognized through the plasticity of the community structure of the benthic organisms, which is why benthic communities make the typical subjects for the biological assessment of water quality (Young et al., 2014a).

Although evaluating water quality includes measuring physicochemical and biological parameters, these methods are insufficient and often costly and time-consuming. The evaluation of diversity in the macrobenthos communities using biodiversity indices such as the Shannon-Wiener, Margalef, and EPT species richness (for Ephemeroptera, Plecoptera, and Trichoptera), and the assessment of water quality by indicators such as the Hilsenhoff biotic index (HBI), have already been recommended for the biological assessment of an ecosystem (Borja et al., 2003). Now becomes apparent that macrobenthos species play an effective role in improving and preserving water quality through the mineralization and recycling of organic

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materials (Venkateswarlu, 1986).

Environmental factors are potent forces in the development of organisms during evolutionary processes (Costa and Cataudella, 2006). Morphological plasticity in aquatic organisms such as fish and macrobenthos may reflect the physicochemical conditions of their habitats and food habits (Ostrand et al., 2001). In other words, morphotype reflects genotype and can also reflect an organism's habitat type (Sarker Md et al., 2016). Similarly, macrobenthos can be adapted to different environmental conditions and show morphological plasticity (Nacua et al., 2010). Shape analysis is crucial in many biological studies (Zelditch et al., 2004). Geometric morphometrics is a relatively new and efficient method for detecting similarities and differences between morphological structures and has been applied in biological sciences, e.g. evolutionary morphology investigations of fish and mammals (Tabatabaei Yazdi and Adriaens, 2013; Yazdi et al., 2015). This technique allows visualization of shape variations and quantitative comparison of morphological differences (Sheets et al., 2004; Zelditch et al., 2004). This type of morphometry can statistically represent the shape differences among species or populations of a species (Sheets et al., 2004).

Aquatic organisms are used as water quality indicators to determine the quality of streams and rivers (Eklöv and Jonsson, 2007). Aquatic organisms, especially fish and Macrobenthos, are good indicators of changes in aquatic habitats because their body shape and size are susceptible to physical changes (Hammer et al., 2001). *Nemoura cinerea* (Retzius) (Nemouridae) plays a vital role in the food chain but is sensitive to the environment (Franken et al., 2008). Thus, it can be used as a bio-indicator. The main objective of the present study is to investigate the effect of fish farm effluent on some of the biodiversity indices (Shannon-Wiener and EPT richness) for macrobenthos communities and the Hilsenhoff index of Ortkand River. We also evaluate the morphological plasticity of *N. cinerea* in response to the conditions of its environment in Ortkand River as a potential bio-indicator.

Material and Methods

Study area and sampling stations: Samples were collected during four seasons from four sampling stations along the Ortkand River, which is located 110 Km North of Mashhad in the Kalat, Khorasan Razavi Province (Fig. 1). The first station was located in the upstream area about two kilometers away from the point source of pollution (station A). Since no pollution entered this station, it was considered a control station. The second station is located at 1348 meters above sea level (36˚48′10. N, 59˚48′39.41″E), and it was considered the point source of pollution’s load station (Fig. 1B). This station is located just after the main source of pollution discharged into the river. The third and fourth stations are located two and four kilometers downstream from the point source, respectively (Fig. 1A-D). The type of substrate at the upstream station (station A) was rocky. The substrate was sandy at the fish farm wastewater entrance (station B), sandstone at the first downstream station (station C), and rocky at the second downstream station (station D).

Sampling of the macrobenthos communities was done randomly along a hypothetical line perpendicular to the flow of water, using a 40x40 cm-wide Sorber with two repetitions. The sampling was done in August, February, October, and April (in four seasons). The sampled materials were separated, washed, and finally fixed in 4% formalin in the fishery laboratory of Ferdowsi University of Mashhad, Iran. The specimens were identified using identification keys at the level of order, family, and genus (Holland, 1972; Edington and Hildrew, 1995). The specimens were saved in containers containing ethyl alcohol based on Armitage (1995).

Biological indicators: The Shannon-Wiener index, the EPT richness index, and the Hilsenhoff Family Biotic Index (HFBI) were calculated using R ver. 3.3.0. The Shannon index is one of the common indicators of species diversity that has applications in ecological assessment for measuring the pollution

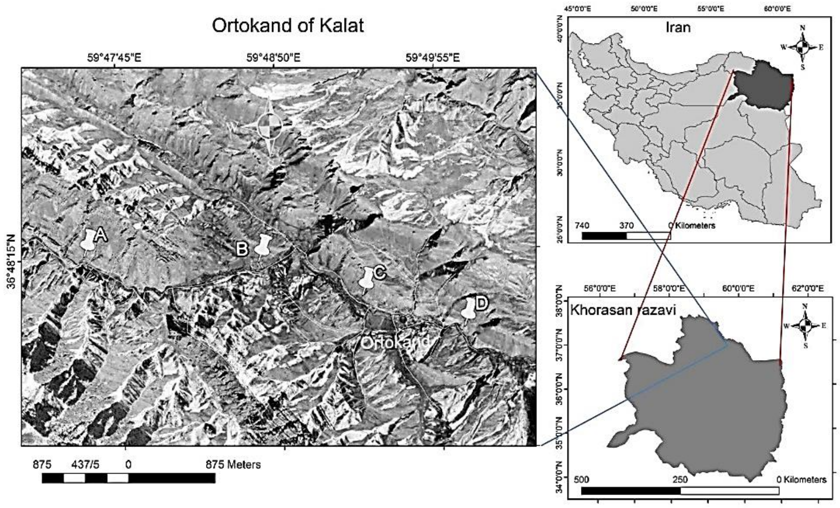

Figure 1. Location of the sampling stations along the Ortkand River, Khorasan Razavi province, Iran.

and distribution of macrobenthos groups in the environment. If species are distributed equally among all ecosystem groups, the Shannon index will be higher (Yazdian et al., 2014). This index was calculated using the formula of $H` = -\sum PilnPi$ to characterize species diversity at the stations (Shannon and Weaver, 1964).

To calculate the EPT richness index, the number of all individuals belonging to Ephemeroptera, Plecoptera, and Trichoptera was counted and using the formula of EPT% = (NE+P+T/Ns)×100 was calculated. in this formula, Ns is the sum of all collected macroinvertebrates, and N_{E+P+T} is the number of single-day families with hairy wings. Increased levels of this index indicate higher water quality (Fries and Bowles, 2002; Kenney et al., 2009). The Hilsenhoff Family Biotic Index (HFBI) (Hilsenhoff, 1988), was used to assess the water quality at each station using the equation of HFBI = $(X_i \times T_i)/N$, where, X_i: The number of individuals for each species, T_i: Tolerance value, and N: The density of macroinvertebrates. According to HFBI, the water quality is classified into five levels: high (0.00-3.75), very good (3.76-4.25), good (4.26-5.00), fair (5.01-5.75), and poor (6.51-7.25).

Physical and chemical parameters: The concentration of dissolved oxygen, electrical conductivity, temperature, and pH were measured with a portable apparatus (Hanna Instrument, Model: HI98193, Romania). BOD5 (5-Day Biochemical Oxygen Demand) and COD (Chemical Oxygen Demand) of the water samples from each sampling station were measured at the laboratory for each sampling station. The mean depth of the river, speed, and discharge were also measured at each station. Also, the Reynolds number (Re) was calculated according to the following formulas: Re = UDv-1, where U: velocity proximal to the substrate, D: depth of water column and V: kinematic viscosity. The following formula was used to calculate discharge: Q = S×V, where S: Surface area (m^2), V: Average flow velocity (m/s) and Q: Water discharge (m^3/s).

Morphological analyses: To study the morphological differences between specimens from stations A to C (away from the point source of pollution and just after entry of wastewater from the fish farm), the photographs were taken with a Nikon D70 digital reflex camera (using a Sigma 105 mm macro lens at five megapixels) using a standardized protocol. The camera was placed on a tripod). Left-right symmetry on the ventral and dorsal sides was the criteria used to allow a standardized positioning of the specimens. The homolog points of the species' body parts were digitized, with landmark points using TpsDig2 software. A total of 18 landmark points were digitized on the dorsal side (Fig. 2), and 16 landmark points on their ventral side (Fig. 3).

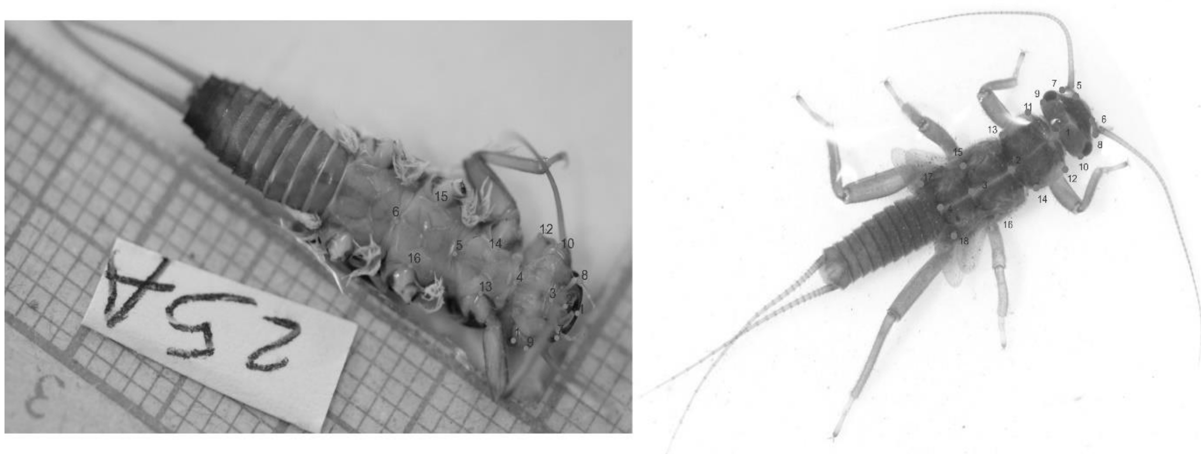

Figure 2. Landmarks on the ventral and dorsal side of *Nemoura cinerea.*

Table 1. Landmark points definitions on the ventral side of specimens.

Anatomical definition	Landmark's number
Anterior point of mouthpart	1
Groove length of inferior lip	2-3
Prosternum length	4-5
Mesosternum length	5-6
Head width at mouthpart	7-8
Postgena width just behind the compound eye	9-10
Post occiput width	11-12
Distance between the coxa of forelegs	13-14
Distance between the coxa of middle legs	15-16

Table 2. Landmark points definitions on the dorsal side of specimens.

Anatomical definition	Landmark's numbers
Pronotum length	1-2
Mesonotum length	2-3
Metanotum length	3-4
length of the upper lip	5-6
Scape distance at pedicle base	7-8
Head width behind the compound eyes	9-10
Width of anterior Pronotum	11-12
Width of posterior Pronotum	13-14
Width of posterior metanotum	15-16
Posterior tips of metanotum	17-18

Nine numerical attributes were determined for the ventral side (Table 1), and 11 numerical attributes for the dorsal side (Table 2).

A Generalized Procrustes Analysis (GPA) was performed using PAST (Paleontological Statistics) ver. 3.22 (Hammer et al., 2001) to eliminate the effect of non-shape data, including size, rotation, and displacement (Rohlf and Marcus, 1993). One-way ANOVA was used for comparing the mean sizes (centroid size) between the groups, using STATISTICA (StatSoft, version 12.0, www.statsoft.com). To investigate morphological variation and determine those morphological variables that discriminate the studied groups, Principal Component Analysis (PCA) was done using PAST, and Discriminant Function Analysis (DFA) in MorphoJ ver. 1.02c (Klingenberg, 2011), on the shape weight matrix of each data set separately for the dorsal and ventral sides. The scatter plots that illustrate the results of PCA analysis on the dorsal side (a more informative plot compared to the ventral side) were generated to visualize how

Table 3. The mean (±standard deviation) Hilsenhoff and Shannon index of Macrobenthos in the Ortkand river at the studied stations (n=2).

Station	Spring		Summer		Autumn		Winter	
	HFBI	Shannon-Wiener	HFBI	Shannon-Wiener	HFBI	Shannon-Wiener	HFBI	Shannon-Wiener
Upstream (A)	2.33±0.02^{b,B}	3.12±0.02b^{,C}	1.38±0.06^{a,A}	3.14±0.09^{b,C}	3.03±0.05^{a,A}	2.04±0.07^{a,A}	3.50±0.05^{a,A}	1.99±0.09^{a,A}
The entry point (B)	8.01±0.02^{a,C}	1.38±0.06^{a,A}	8.99±0.03^{a,C}	1.40±0.04^{a,A}	5.16±0.20^{b,B}	1.61±0.05^{c,C}	6.630.05^{c,B}	1.33±0.01^{c,C}
Downstream1 (C)	7.63±0.01^{c,C}	1.86±0.05^{a,A}	7.75±0.1^{c,C}	1.97±0.08^{a,A}	5.14±0.02^{b,A}	1.48±0.09^{c,B}	5.51±0.01^{c,A}	1.38±0.07^{c,B}
Downstream2 (D)	2.45±0.02^{b,B}	2.58±0.05^{bc,C}	2.74±0.03^{b,B}	3.01±0.05^{b,C}	4.10±0.08^{a,A}	2.01±0.04^{a,B}	4.26±0.02^{a,A}	1.82±0.06^{a,B}

Table 4. The mean (±standard deviation) EPT richness index of Macrobenthos in the Ortkand river at the studied stations for every season (n=2).

Stations	Spring	Summer	Autumn	Winter
Upstream (A)	0.78±0.015^{c,A}	0.90±0.011^{b,A}	0.86±0.085^{c,A}	0.81±0.016^{b,A}
The entry point (B)	0.04±0.00 $^{a, A}$	0.03±0.0^{a,A}	0.16±0.00^{a,B}	0.15±0.035^{a,B}
Downstream 1(C)	0.22±0.046^{ab,A}	0.22±0.04^{a,A}	0.32±0.04$^{a, B}$	0.24 ±0.099^{a,B}
Downstream 2 (D)	0.77±0.05$^{c, B}$	0.85±0.07^{b,B}	0.67±0.028$^{c, A}$	0.35±0.017^{ab,A}

the specimens are distributed in the morphospace. Thin-plate spline deformation grids were used to visualize shape variation expressed by the first two PCs (RWs axes). Illustrating the shape difference between the groups was made using MorphoJ ver. 1.02c. Cluster analysis (UPGMA) was done in PAST by combining the partial warp scores of specimens' dorsal and ventral sides.

Statistical analyses: Except for geometric morphometric analyses, the statistical analyses were performed using R ver. 3.3.0.

Results

The highest Shannon index was observed at stations A and D during summer at 3.14, and 3.01, respectively. Station B showed the lowest Shannon index. In the summer, the Shannon-Weiner index increased at station A, compared to winter, autumn, and spring. The difference in this index between summer and winter was significant (P<0.05). The highest value of the Hilsenhoff index was observed in summer at stations B (8.99, at the entry point of farm wastewater to the river) and station C (7.75). The lowest Hilsenhoff index was recorded at station A in spring. The average Hilsenhoff index was 2.51

The highest EPT index at the 95 percent confidence level (0.90) was observed in summer at station A, and the lowest value of this indicator was observed at the entry point of farm wastewater in summer (0.03) (Table 3). The EPT index increases in summer compared to winter, autumn, and spring (Table 4). Calculating Pearson correlation between biological indices and physicochemical variables showed a significant correlation between Shannon-Wiener and EPT richness indices with DO and BOD (Table 5). The Hilsenhoff index showed a positive correlation with TDS, BOD, and COD and a negative correlation with DO (Table 5).

Geometric morphometric analysis: The correlation between Procrustes distances and their corresponding Euclidean distances after projection in shape space was tested using TpsSmall (Viscosi and Cardini, 2011). The correlation value of 0.999 between the Euclidean and tangent distances for the dorsal side indicates that the shape data matrix can be used for further analyses.

An ANOVA on the mean sizes on ventral and dorsal sides showed a significant difference (P<0.01) between the two studied groups (A vs. C). PCA showed considerable variation in body shape i.e. the populations occupy different spaces in the scatter plot. However, there was some overlap between the two populations, and PCA was unable to distinguish the two populations of *N. cinere* i.e. The populations of station A from the upstream station and the population of the first downstream station (station C). The first two axes (PCs) could explain a total of 76.02% of variations (48.05 and 27.97% for PC1 and PC2, respectively) (Fig. 3).

The results of the PCA analysis for the dorsal side

Table 5. Pearson correlation between biological indices and physicochemical variables**.**

Index	Q ($m^3.s^{-1}$)	Re	EC (μmohs.cm^{-1}.s)	COD ($mg.l^{-1}$)	BOD ($mg.l^{-1}$)	TDS ($mg.l^{-1}$)	DO ($mg.l^{-1}$)
H′	-0.3	-0.9	0.6^{**}	-0.6^{*}	-0.7^{**}	-0.5^{**}	0.5^{**}
EPT	-0.4	-0.8	0.6^{**}	-0.5^{*}	-0.7^{**}	-0.4	0.7^{**}
HFBI	0.5^{**}	0.6	0.5^{**}	0.8^{**}	0.8^{**}	0.5^{**}	-0.6^{**}

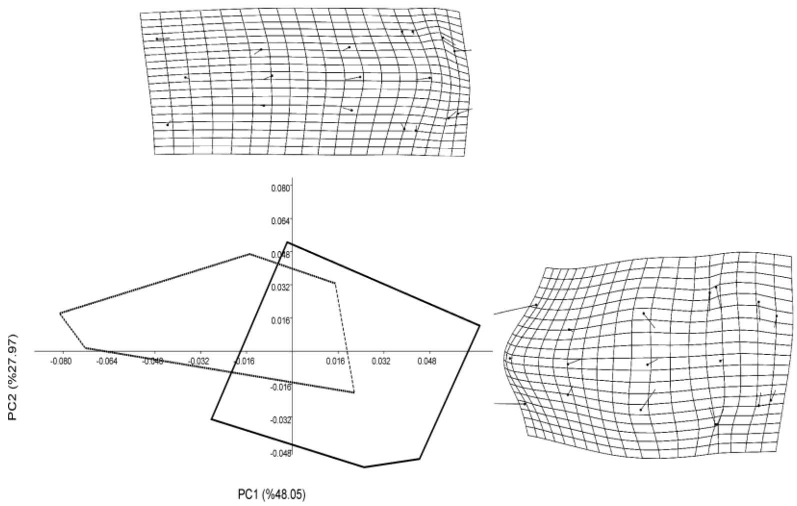

Figure 3. Scatter plot of PC1 versus PC2 of the dorsal side. Deformation grids represent shape differences along each axis and correspond to PCs' highest values. The specimens from station A are represented by the black solid polygon, and those from station B are represented by a grey dashed polygon. For the numbering of the landmarks, see Figure 2.

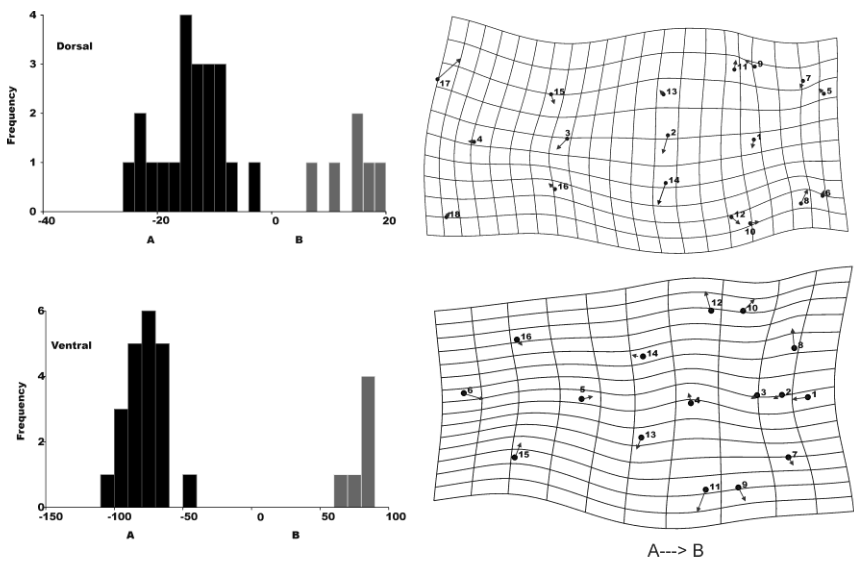

Figure 4. Histogram of DFA analysis and the deformation grids, including the arrows (3X magnified), which show shape differences between the groups, on the dorsal (above) and ventral (below) sides.

showed that considerable morphological variations along PC1 occur across the head and chest (mainly at the pronotum), but the highest variations along PC2 were observed along with the components of the mouth and body length (Fig. 3). The specimens captured away from the point source of pollution (station A) have higher values on the PC1 axis and are characterizes by broader chests.

DFA analysis of the dorsal side of the specimens (with 10,000 replicas) showed no overlap between

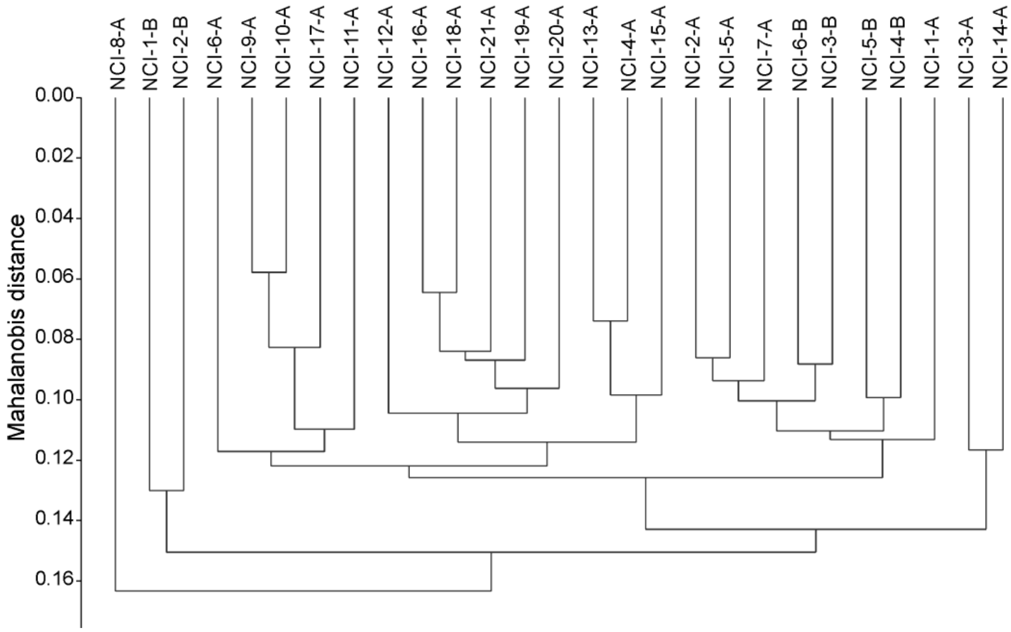

Figure 5. A UPGMA cluster analysis on the combined ventral and dorsal data.

the two populations (Fig. 4). This analysis of the ventral side's data (with 10,000 replicas) shows a clear separation of the two groups (Fig. 4). The differences between groups are mainly in the metanotum, head width (Fig. 4, the distance of landmarks 16-18 on the dorsal side), and chest (Fig. 2. distances between 13-14 and 15-16 landmarks). A UPGMA cluster analysis based on Euclidean distance showed that specimens belonging to different groups (sampled from stations A and C) are relatively well-clustered (Fig. 5).

Discussion

The environmental problems of the Ortkand River will increase with the expansion of anthropological activities such as trout farms and direct release of their effluents into the river. The results of the diversity index showed that the number of macrobenthos decreased in stations B and C because of the wastewater entry from fish farms, which led to the elimination of macrobenthos such as Ephemeroptera, followed by an increase in the number of resistant species such as Chironomidae. Yokoyama et al. (2007), in a study of macrobenthos diversity at the point of wastewater entry, pointed out that the highest self-purification occurs in summer. Based on the results, wastewater entry could not affect the richness of macrobenthos at the second downstream station since pollutants were decomposed before reaching the station. In this station, the Shannon diversity index was 1.4-3.14 in summer, showing poor to moderate water quality.

The results also showed that with increased production in fish farm and the reduction of dissolved oxygen, especially in the summer, species diversity is decreased at station B, which consequently lead to an increase in the Hilsenhoff index (classifying as "poor" level), and a considerable decrease in the number of pollutant-sensitive groups such as Ephemeroptera. The Hilsenhoff index increased in stations downstream from the fish farm, indicating the accumulation of organic matter from fish farms and reduced riverbed quality at these stations. Station D had considerably lower levels of HFBI due to its distance from the fish farms. This fact indicates that the water quality at the upstream and the second downstream station (about four kilometers from the last fish farm) is considerably different and demonstrates the river's ability to self-purify. Additionally, improvement of the environmental conditions at the second downstream station shows that the pollution load could be noticeably lower after passing through an almost short distance. These findings are consistent with the results of Young et al. (2014), which showed that water quality is improved by increasing the distance from the pollution sources.

The results showed a reduction in the EPT richness index at the stations affected by aquaculture. These results agree with Kani and Murugesan (2014), who pointed out that pollution reduced the diversity of sensitive species while the density of resistant species is increased. Releasing treated wastewater did not have much effect on TDS (Noroozrajabi et al., 2013). Environmental conditions worsen as the dissolved solids increase, and consequently, sensitive organisms were significantly reduced (Sarker, 2016).

Our results showed that the Shannon-Wiener index has a positive correlation with DO, but a negative correlation with TDS, which agrees with the previous studies (Yazdian et al., 2014). In the present study, physicochemical parameters (TDS and EC) and discharge showed a positive correlation with the Hilsenhoff index and a negative correlation with DO. The turbulence increased at the first and second downstream stations, which could reflect the substrate conditions. During the self-purification process, the dissolves organic matter, e.g. ammonia and phosphorus, convert to soluble salts, which cause an increase in EC. Water quality gradually decreases, and electrical conductivity gradually increases down the river at the first and second downstream stations due to entry. Since EC increased down the river, it can be concluded that changes in EC are mainly influenced by geological factors.

Regarding the morphological differences, the shape differences between the studied groups could be in response to environmental conditions and adaptation to different habitats (Haas et al., 2010). As shown by the deformation grids, the most differences were in the width of the head, which was observed in the group affected by pollutants. The reason for this can be a morphological adaptation of Macrobenthos to prevent being washed away. Also, the chest width was smaller at the wastewater entry point, which can be a morphological adaptation and biological advantage for living in a polluted environment. This fact was previously reported in the Perlidae and Nemouridae families (Briers, 2009). The differences in morphology are affected by various factors, such as environmental stressors, pollutants (Taylor and Kennedy, 2006), or even by access to food (Johnson, 1987). Using benthic organisms, especially macrobenthos indicators greatly enhances states' ability to identify and subsequently improve water quality.

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