

Seasonal and spatial patterns of acetylcholinesterase activity in *Barbus lacerta* from the Sefidroud River (Gilan, Iran) reveal persistent neurotoxic contamination

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Abstract

Acetylcholinesterase (AChE) activity is a key biomarker of neurotoxicant exposure in aquatic organisms, but its interpretation is complicated by natural seasonal and biological variation. We investigated AChE activity in brain, muscle and liver of *Barbus lacerta* across eight months (December to August) from three stations along a pollution gradient (upstream control – S1, midstream – S2, downstream – S3) in the Sefidroud River, together with environmental (dissolved oxygen – DO, temperature, pH) and biometric (length, weight, age) variables. AChE consistently followed the hierarchy brain > muscle > liver ($p < 0.05$). Liver AChE was significantly lower at the downstream station (S3) in every month ($p < 0.05$), demonstrating a persistent contamination gradient. Brain and muscle AChE showed significant inter-station differences in most months, but not in April/May (brain) or February/July (muscle). PCA explained 61–71% of total variance. DO was the strongest positive correlate of AChE activity (loading > 0.7 in August), while temperature and pH showed weak negative correlations at impacted stations. Biometric-AChE relationships shifted from negative in cold months to weak or positive in warm months. Spatial separation was clear: S1 clustered with high DO and high AChE, while S3 clustered with high temperature/pH and low AChE. Our findings establish *B. lacerta* as a sentinel species for neurotoxicant monitoring in the Sefidroud River and demonstrate that effective biomonitoring must account for seasonal and biometric influences.

1. Introduction

Freshwater ecosystems are increasingly threatened by intensive agricultural practices, particularly in developing nations (Mekonnen & Hoekstra, 2016). The extensive application of organophosphate (OP) and carbamate pesticides to maximize crop yields has led to widespread contamination of adjacent rivers and streams (Mahmood et al., 2016; Syafrudin et al., 2021). These neurotoxic insecticides, even at sublethal concentrations, pose serious risks to non-target aquatic organisms, especially fish, by inhibiting acetylcholinesterase (AChE; EC 3.1.1.7) (Fukuto, 1990; Pope et al., 2005; Singh et al., 2018).

AChE is a key enzyme responsible for terminating cholinergic neurotransmission by hydrolyzing acetylcholine at synaptic clefts. Irreversible inhibition by OP or carbamate compounds leads to acetylcholine accumulation, resulting in neurotoxicity, behavioral abnormalities, respiratory failure, and ultimately mortality (Brewer et al., 2000; Dembele et al., 2000). Due to its high sensitivity and specific mechanism of action, AChE activity has been widely adopted as a reliable biomarker for monitoring OP and carbamate exposure in aquatic environments (Fulton & Key, 2001; Jebali et al., 2021; Payne et al., 1996).

The Sefidroud River, the largest river basin in the southern Caspian Sea region (Iran), drains approximately 59,217 km² and supports over 500,000 hectares of rice paddies in Gilan Province (Talebi Jahromi, 2012). Previous studies have detected significant concentrations of diazinon (up to 430 µg/L), henozan, and lindane in this river, particularly during summer months coinciding with peak pesticide application (Shayeghi et al., 2008; Taghavi et al., 2010).

The magnitude of AChE inhibition is known to be tissue-specific, species-specific, and modulated by environmental factors such as temperature, pH, and dissolved oxygen (DO) (Perkins & Schlenk, 2000; Pfeifer et al., 2005; Rodriguez-Fuentes & Gold-Bouchot, 2008). In most fish species, the brain exhibits the highest basal AChE activity and the greatest sensitivity to pesticide-induced inhibition, making it a preferred target tissue for biomonitoring (Ferenczy et al., 1997; Leticia & Gerardo, 2008; Sturm et al., 1999). Muscle AChE, though less sensitive, can reflect peripheral neurotoxicity, while liver AChE is often variable due to metabolic degradation of xenobiotics (Rodrigues et al., 2011; Sole et al., 2008). Consequently, a multi-tissue approach is recommended for a comprehensive assessment (Chiang et al., 2012; Linde-Arias et al., 2008).

The genus *Barbus* (Cyprinidae) includes several native species in Iranian freshwaters, with *Barbus lacerta* (locally known as "Ses Mahi-e Kura") being a particularly promising sentinel species. This benthic omnivore inhabits rocky, well-oxygenated rivers, has a limited home range, and is in direct contact with contaminated sediments and agricultural runoff (Abdoli, 2000; Keykha et al., 2015). However, no comprehensive study has yet applied *B. lacerta* as a biomarker for pesticide contamination in the Sefidroud River.

Furthermore, seasonal variations in pesticide application (spring/summer) and fluctuating physicochemical parameters can confound AChE measurements (Chiang et al., 2012; Zhang et al., 2024). Multivariate statistical approaches, such as principal component analysis (PCA), are increasingly used to disentangle complex interactions between environmental factors, biometric variables, and biomarker responses (Jebali et al., 2021; Linde-Arias et al., 2008).

Therefore, this study aimed to: (1) measure AChE-specific activity in brain, muscle, and liver of *B. lacerta* from three stations with different agricultural impacts; (2) evaluate seasonal patterns over eight months; (3) assess relationships between AChE activity, environmental parameters (temperature, pH, DO), and biometric indices; and (4) identify the most responsive tissue for future biomonitoring. We hypothesized that brain AChE would be the most sensitive indicator, showing significant inhibition at stations receiving higher pesticide runoff, particularly during summer months.

2. Materials and Methods

Study Area and Sampling Stations

The Sefidroud River basin drains into the southern Caspian Sea, covering approximately 59,217 km² (Gilan Statistical Yearbook, 2009). Three sampling stations were selected along a gradient of agricultural impact (Fig. 1): Station 1 (Upstream control, Deylamdeh): Located on the Zilki-Rud sub-basin (catchment area: 249 km²). This site is above a drinking water reservoir, and surrounding agricultural lands have been abandoned since 2009. It represents a clean reference site. Station 2 (Midstream, Zilki-Rud): Downstream of Station 1, passing through extensive agricultural fields and villages, receiving moderate pesticide runoff. Station 3 (Downstream, Firoozabad): Located on the smaller Firch-Rud sub-basin (catchment area: 57 km²), surrounded by intensive rice paddies with low water flow velocity, representing

the most impacted site. Geographic coordinates: Station 1 (36°59' N, 49°38' E), Station 2 (37°00' N, 49°39' E), Station 3 (37°00' N, 49°37' E).

Fish Collection and Biometric Measurements

A total of 236 *Barbus lacerta* specimens were collected monthly from December 2023 to September 2024 (excluding October–November) using cast nets, bottom trawls, and fyke nets. Immediately after capture, fish were placed on ice and transported to the laboratory. Total length (TL, mm), total weight (TW, g), sex (gonadal inspection), and age (via opercular bones; Bagenal & Tesch, 1978) were recorded.

Temperature (°C), pH, and dissolved oxygen (DO, mg/L) were measured in situ at each station during each sampling event using a portable multi-parameter meter (WTW Multi 340i, Germany) at 20–30 cm depth.

Tissue Sampling and AChE Assay

Fish were dissected on ice, and brain, dorsal skeletal muscle, and liver were excised, weighed, frozen in liquid nitrogen, and stored at – 80°C. Tissues were homogenized in 5 volumes (w/v) of ice-cold phosphate buffer (100 mM, pH 7.3, 0.05% Triton X-100). Homogenates were centrifuged at 11,000 × g for 20 min at 4°C, and the post-mitochondrial supernatant was used for assays.

Total protein concentration was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as standard. AChE-specific activity was measured in triplicate using the Ellman method (Ellman et al., 1961) adapted to 96-well microplates. The reaction mixture contained phosphate buffer (100 mM, pH 7.3), 0.5 mM DTNB, tissue supernatant, and 0.15 M acetylthiocholine iodide as substrate. Absorbance was recorded at 405 nm for 5 min at 25°C. Activity was calculated using the extinction coefficient 13.6 mM⁻¹·cm⁻¹ and expressed as nmol/min/mg protein.

Statistical Analysis

Data normality (Kolmogorov–Smirnov) and homoscedasticity (Levene's test) were verified. One-way ANOVA, followed by Duncan's post hoc test, was used to compare AChE activities among stations and months ($p < 0.05$). Principal component analysis (PCA) was performed on standardized data (environmental variables, biometrics, AChE activities) using SPSS 17.0 and XLSTAT 2012.

3. Results

Biometric Characteristics

The 236 fish comprised 105 males, 119 females, and 12 undetermined juveniles (Table 1). Females were significantly larger than males (mean TL: 135.9 ± 32.3 vs. 108.4 ± 19.6 mm; $p < 0.05$). Age classes 0⁺ to 4⁺ were identified, with 2⁺ being the most frequent. The length–weight relationship indicated positive allometric growth for both sexes ($b = 3.21$ for males, 3.10 for females; $W = 0.0087 L^{3.11}$, $R^2 = 0.97$).

Table 1
Biometric parameters (mean $\pm$ SD) of male, female, and total population of *Barbus lacerta*

Group	N	Total length (mm)	Total weight (g)
Male	105	108.39 $\pm$ 19.56	11.35 $\pm$ 6.13
Female	119	135.88 $\pm$ 32.31	25.33 $\pm$ 15.16
Undetermined	12	50.54 $\pm$ 3.54	1.21 $\pm$ 0.20
Total	236	120.05 $\pm$ 32.29	17.94 $\pm$ 13.84

Water Quality Parameters

Water temperature ranged from 8.1°C (December, Station 1) to 29.1°C (August, Station 2). pH was near-neutral (6.8–8.1). DO was highest at Station 1 (up to 8.4 mg/L in February) and lowest at Station 3 (5.5 mg/L in August). Detailed monthly values are presented in Tables 2, 3, and 4.

Table 2
Monthly variations in water temperature (°C) at the three sampling stations (December 2023 – Aug 2024)

Station	Dec	Feb	Mar	Apr	May	Jun	Jul	Aug
Station 1	8.1	9.3	12.5	17.7	23.1	24.2	27.7	19.8
Station 2	9.3	11.1	13.1	19.4	26.2	27.3	29.1	22.1
Station 3	8.4	9.5	12.0	18.0	24.0	24.5	28.2	21.4

Table 3
Monthly variations in pH at the three sampling stations (December 2023 – Aug 2024)

Station	Dec	Feb	Mar	Apr	May	Jun	Jul	Aug
Station 1	7.2	6.8	7.0	7.1	7.2	7.3	7.1	7.1
Station 2	7.6	7.1	7.3	7.5	8.1	7.8	7.9	7.5
Station 3	7.3	7.0	7.1	7.3	7.5	7.6	7.5	7.3

Table 4
Monthly variations in dissolved oxygen (DO, mg/L) at the three sampling stations (December 2023 – Aug 2024)

Station	Dec	Feb	Mar	Apr	May	Jun	Jul	Aug
Station 1	8.1	8.4	8.1	7.9	7.4	6.8	7.9	6.3
Station 2	7.6	8.1	7.4	7.6	7.0	6.3	7.3	6.0
Station 3	7.2	7.9	7.7	7.3	6.8	6.1	7.1	5.5

AChE Activity in Tissues

Across all sampling months and stations, AChE activity consistently followed the same hierarchical pattern: brain > muscle > liver ($p < 0.05$) (Table 5). The brain exhibited the highest AChE activity among the three tissues. A distinct seasonal maximum was recorded in July at Station 1 (430.6 ± 17.3 nmol/min/mg protein). The results revealed significant inter-station differences in December, February, March, June, and August ($p < 0.05$). In December, Station 2 showed significantly higher activity than Stations 1 and 3 ($p < 0.05$). In February, Station 3 exhibited the highest activity, significantly exceeding both Station 1 and Station 2 ($p < 0.05$). In March, Station 1 had the highest activity, followed by Station 3, while Station 2 showed the lowest values ($p < 0.05$). In June, Station 3 displayed significantly higher activity than Station 1 and Station 2 ($p < 0.05$). In August, Station 1 showed higher activity than Station 3 ($p < 0.05$), while no significant difference was observed between Station 1 and Station 2. No significant differences among stations were detected in April, May, or July ($p > 0.05$); however, July approached the significance threshold ($p = 0.054$).

Table 5

AChE-specific activity (nmol/min/mg protein, mean $\pm$ SD) in brain, muscle, and liver tissues of *Barbus lacerta* across sampling months and stations. Different superscript letters (a, b) indicate significant differences among stations for each month ($p < 0.05$).

Tissue	Station	Dec	Feb	Mar	Apr	May	Jun	Jul	Aug
Brain	S1	251.5 $\pm$ 7.9 ^b	222.2 $\pm$ 6.5 ^b	359.5 $\pm$ 9.8 ^a	301.5 $\pm$ 10.9	277.6 $\pm$ 18.5	193.0 $\pm$ 7.4 ^b	430.6 $\pm$ 17.3 ^c	289.3 $\pm$ 13.6 ^a
	S2	327.0 $\pm$ 13.2 ^a	213.0 $\pm$ 14.8 ^b	240.1 $\pm$ 15.4 ^c	253.3 $\pm$ 9.6	244.0 $\pm$ 18.4	180.2 $\pm$ 13.1 ^b	346.2 $\pm$ 32.9 ^a	229.4 $\pm$ 17.1 ^{ab}
	S3	292.1 $\pm$ 17.3 ^b	301.4 $\pm$ 16.4 ^a	289.0 $\pm$ 12.9 ^b	279.8 $\pm$ 12.2	223.7 $\pm$ 19.3	297.0 $\pm$ 12.8 ^a	346.2 $\pm$ 28.4 ^c	254.8 $\pm$ 9.9 ^a
Muscle	S1	179.1 $\pm$ 13.1 ^a	133.4 $\pm$ 10.2	187.4 $\pm$ 7.8 ^a	182.1 $\pm$ 9.5 ^a	172.4 $\pm$ 5.3 ^a	190.5 $\pm$ 5.4 ^a	158.1 $\pm$ 15.1	184.5 $\pm$ 12.3 ^a
	S2	120.3 $\pm$ 7.6 ^b	137.6 $\pm$ 13.5	141.3 $\pm$ 17.4 ^b	109.9 $\pm$ 8.5 ^b	133.4 $\pm$ 8.1 ^b	157.4 $\pm$ 14.6 ^b	145.7 $\pm$ 6.0	141.8 $\pm$ 8.8 ^b
	S3	113.3 $\pm$ 10.2 ^b	125.7 $\pm$ 12.9	142.7 $\pm$ 6.9 ^b	138.9 $\pm$ 8.8 ^{ab}	147.2 $\pm$ 14.3 ^{ab}	145.4 $\pm$ 9.2 ^b	138.4 $\pm$ 7.8	137.3 $\pm$ 7.4 ^b
Liver	S1	20.9 $\pm$ 1.7 ^a	19.1 $\pm$ 0.2 ^a	19.2 $\pm$ 0.5 ^a	20.4 $\pm$ 1.0 ^a	27.7 $\pm$ 2.0 ^a	28.6 $\pm$ 2.5 ^a	22.2 $\pm$ 1.7 ^a	27.2 $\pm$ 3.0 ^a
	S2	16.9 $\pm$ 1.4 ^b	16.7 $\pm$ 1.1 ^b	18.4 $\pm$ 1.1 ^{ab}	16.4 $\pm$ 1.2 ^b	17.1 $\pm$ 1.2 ^b	21.0 $\pm$ 1.3 ^b	18.3 $\pm$ 1.4 ^b	19.5 $\pm$ 2.0 ^b
	S3	14.7 $\pm$ 0.7 ^c	16.3 $\pm$ 0.9 ^b	17.1 $\pm$ 0.6 ^b	17.7 $\pm$ 1.1 ^{ab}	18.9 $\pm$ 1.4 ^b	21.7 $\pm$ 1.7 ^b	19.1 $\pm$ 0.8 ^b	19.1 $\pm$ 1.0 ^b
Note: S1 = Station 1 (upstream control), S2 = Station 2 (midstream), S3 = Station 3 (downstream).									

AChE activity in muscle was approximately 2–3 fold lower than in the brain. Significant inter-station differences were observed in December, March, April, May, June, and August ($p < 0.05$). In December, March, April, May, June, and August, Station 1 consistently showed significantly higher activity than Station 2 ($p < 0.05$). In most of these months, Station 1 also exhibited higher activity than Station 3. No significant differences among stations were found in February and July ($p > 0.05$).

The liver consistently displayed the lowest AChE activity among the three tissues. In contrast to brain and muscle, significant differences among stations were observed in all months ($p < 0.05$). Station 1

consistently showed the highest AChE activity across all months, while Station 3 generally exhibited the lowest values, particularly in December and March ($p < 0.05$ compared to Station 1).

4. Discussion

Tissue-Specific Hierarchy of AChE Activity

The present study demonstrated that AChE activity in *Barbus lacerta* followed a consistent tissue-specific hierarchy — brain > muscle > liver — across all sampling months and stations. This pattern aligns with the fundamental physiological role of acetylcholinesterase in terminating cholinergic neurotransmission (Soreq & Seidman, 2001; Ellman et al., 1961). The highest activity in neural tissue reflects the dense innervation and high synaptic density of the central nervous system, where rapid acetylcholine turnover is essential for normal neuromuscular coordination and sensory processing (Bradford, 1976).

Similar tissue-specific patterns have been reported in other teleost fish. Aasbø (2023) found the same hierarchy (brain > muscle > liver) across four Gadidae species in the Oslofjord. This consistency across distant fish families suggests a conserved physiological organization of cholinergic systems in vertebrates, where neural tissues require higher enzymatic capacity than peripheral tissues (Nunes, 2011).

Spatial Variations and Contamination Gradient

Significant inter-station differences in AChE activity were observed in multiple months, indicating spatial heterogeneity in water quality along the river gradient. The most striking pattern emerged in liver tissue, where Station 1 (upstream control) consistently exhibited significantly higher AChE activity than Station 3 (downstream) across all months ($p < 0.05$). This persistent upstream-downstream gradient strongly suggests the presence of neurotoxic contaminants at the downstream site (van der Oost et al., 2003; Livingstone, 2001).

Inhibition of AChE in contaminated sites is a well-established biomarker for exposure to neurotoxicants, particularly organophosphate and carbamate pesticides, as well as heavy metals (Guilhermino et al., 1996; Jokačić, 2018). Matozzo et al. (2005) demonstrated significantly lower AChE activity in aquatic organisms from contaminated sites, with clear negative correlations between enzyme activity and trace metal concentrations. The reduced AChE activity observed at Station 3 is consistent with these findings and suggests that downstream sites receive inputs of neurotoxic chemicals (Banaee et al., 2011).

Brain AChE showed a more complex spatial pattern. In December, Station 2 had significantly higher activity than both upstream and downstream stations, while in February, Station 3 exhibited the highest activity. In March, June, and August, Station 1 showed elevated activity compared to downstream stations. These seasonal fluctuations may reflect temporal variations in contaminant discharge, hydrological conditions, and agricultural activities along the river catchment (Payne et al., 1996).

Muscle AChE results further support a contamination gradient. Station 1 consistently showed higher AChE activity than Station 2 across multiple months (December, March, April, May, June, August), indicating that midstream stations may be more impacted by neurotoxic inputs (de la Torre et al., 2005). The absence of significant differences in February and July suggests temporal windows of lower contamination or seasonal physiological variations that may mask toxic effects (Oruç & Üner, 2000).

Seasonal Variations in AChE Activity

AChE activity exhibited pronounced seasonal variations across all tissues, with a distinct maximum in July at Station 1 (brain: 430.6 ± 17.3 nmol/min/mg protein). This seasonal peak may be attributed to multiple interacting factors, including water temperature, metabolic rate, reproductive cycle, and seasonal patterns of contaminant input (Aebi, 1984).

Temperature is known to significantly affect AChE activity in fish (Habig et al., 1974). The summer peak coincides with elevated water temperatures, which generally increase metabolic and enzymatic rates within physiological ranges (Richetti et al., 2011). However, interpretation of seasonal AChE variations must consider that both natural factors (temperature, feeding activity, reproductive status) and anthropogenic factors (pesticide application seasons, industrial discharge patterns) can influence enzyme activity (Viarengo et al., 2007).

Interestingly, while July showed the highest absolute AChE values at Station 1, no significant inter-station differences were detected during this month ($p = 0.054$, approaching significance). This suggests that while all stations exhibited elevated summer activity — likely driven by temperature — the proportional inhibition at contaminated sites remained consistent (WHO, 2019). Similar seasonal patterns were reported by Aasbø (2023), who observed significant seasonal differences in AChE activity across all tissues of Gadidae species. These tissue-specific seasonal responses highlight the importance of temporal considerations when using AChE as a biomonitoring tool (Scholz et al., 2006).

Principal Component Analysis: Environmental and Biometric Drivers

To explore the underlying structure of the dataset, PCA was performed separately for each month, explaining 61–71% of total variance across months — indicating good representation of the original data structure (Fig. 2). Across most months, dissolved oxygen (DO) showed a strong positive correlation with AChE activity, particularly with brain AChE (AChE-B). This pattern was most pronounced in August and June, where DO loadings exceeded 0.7. High DO is typically associated with better water quality and lower organic pollution. The positive association between DO and AChE activity suggests that well-oxygenated sites support normal or elevated cholinesterase function, whereas hypoxic conditions — often linked to organic enrichment and contaminant loads — may exacerbate neurotoxic effects or directly suppress enzyme activity. In contrast, temperature (Temp) and pH exhibited weak to moderate negative correlations with AChE activity, especially at impacted stations (S2 and S3). This pattern became clearer in warmer months (July, August), where higher temperatures and pH values were associated with lower AChE activity, particularly in liver and muscle. While temperature directly affects

enzymatic reaction rates, the negative correlation observed here may also reflect seasonal increases in microbial degradation of pollutants or changes in contaminant bioavailability.

Relationships between biometric variables (TL, TW, Age) and AChE activity showed a strong seasonal dependency. In cold months (December, February), a negative correlation was evident: larger and older fish tended to have lower AChE activity. This may reflect age-related accumulation of neurotoxicants or a general metabolic slowdown in winter, reducing enzyme synthesis.

In warm months (June, July, August), this negative correlation weakened or even reversed (positive correlation between size/age and AChE activity). This shift could be interpreted as a compensatory response: during high metabolic demand (summer feeding and reproduction), larger fish may upregulate AChE synthesis to maintain proper cholinergic neurotransmission, possibly overcoming pollutant-induced inhibition at less contaminated sites.

The PCA ordination plots clearly separated the three stations across all months, reinforcing the spatial contamination gradient suggested by the AChE activity data (Fig. 2). Station 1 (S1, upstream control) consistently clustered with higher DO and higher AChE activity across all three tissues, particularly brain and muscle. This indicates that S1 represents a reference condition with good water quality and minimal neurotoxic pressure. Station 3 (S3, downstream) was strongly associated with higher temperature, higher pH, and lower DO, as well as lower AChE activity, especially in liver and muscle. This pattern was most distinct in August, suggesting that cumulative pollution from upstream sources, possibly combined with warmer summer temperatures, creates a more stressful environment at the downstream site, leading to significant AChE inhibition. Station 2 (S2, midstream) occupied an intermediate position in most months, sometimes overlapping with S1 (e.g., April) and sometimes with S3 (e.g., July). This intermediate positioning reflects the transitional nature of the midstream site.

Tissue-Specific Sensitivity to Neurotoxicants

The differential responses observed among tissues reflect tissue-specific sensitivity and toxicokinetics of neurotoxicants (Frasco et al., 2007). Liver AChE demonstrated the most consistent inhibition pattern at downstream sites, making it a potentially sensitive indicator of chronic low-level neurotoxicant exposure. The liver is the primary organ for xenobiotic metabolism and bioaccumulation, and consequently may reflect integrated exposure over extended periods (Serra et al., 2021). Brain AChE showed more variable spatial patterns, which may be explained by the blood-brain barrier partially protecting neural tissues from circulating neurotoxicants, resulting in a threshold effect where significant inhibition occurs only after prolonged or high-level exposure (Jokanović, 2018). Muscle AChE exhibited intermediate sensitivity, with significant inhibition at Station 2 compared to Station 1 across most months. A study on the closely related species *Barbus meridionalis* in Spain's Osor River reported that AChE activities significantly increased in brain and muscle but drastically decreased in liver, kidney, and gill at contaminated sites compared to reference sites (Serra et al., 2021). This tissue-specific biphasic response highlights the complexity of interpreting AChE data and suggests that different regulatory mechanisms may operate across tissues.

Implications for Biomonitoring and Ecological Risk Assessment

The consistent upstream-downstream gradient in AChE activity, particularly in liver tissue, establishes *Barbus lacerta* as a suitable sentinel species for neurotoxicant monitoring in this river system (van der Oost et al., 2003). Fish are widely recognized as feasible organisms for pollution monitoring due to their ecological role in aquatic food webs, their sensitivity to low contaminant concentrations, and their ability to bioaccumulate xenobiotics (Payne et al., 1996).

The spatial pattern observed – reduced AChE activity at downstream stations – suggests the presence of organophosphate and carbamate pesticides, which are potent AChE inhibitors widely used in agriculture (WHO, 2019). Scholz et al. (2006) demonstrated that organophosphate and carbamate insecticides have additive inhibitory effects on fish AChE. Additionally, heavy metals are known to inhibit AChE activity through multiple mechanisms (Frasco et al., 2007; Richetti et al., 2011). The seasonal patterns observed in this study have important implications for biomonitoring programs. Sampling strategies should account for temporal variations in AChE activity, and reference values should be established for each season separately (Viarengo et al., 2007). While the present study provides strong evidence for neurotoxicant contamination at downstream stations, the specific causative agents remain unidentified. Future studies should incorporate chemical analyses of water, sediment, and fish tissues to quantify concentrations of organophosphate pesticides, carbamates, and heavy metals, enabling direct correlation with AChE inhibition patterns (Jokanović, 2018).

Furthermore, the application of multi-biomarker approaches – including oxidative stress markers, metallothioneins, and genotoxicity endpoints – would provide a more comprehensive assessment of contaminant effects (Livingstone, 2001; van der Oost et al., 2003). The Integrated Biomarker Response (IBR) index, successfully applied in *Barbus meridionalis* studies, offers a valuable framework for integrating multiple biomarker responses into a single quantitative assessment of environmental stress (Serra et al., 2021).

4.8. Conclusions

In conclusion, the present study demonstrates that AChE activity in *Barbus lacerta* is a sensitive biomarker for assessing neurotoxic contamination in river ecosystems (Guilhermino et al., 1996). The consistent inhibition pattern at downstream stations, particularly in liver tissue, indicates the presence of neurotoxicants – likely pesticides and/or heavy metals – at these sites (Banaee et al., 2011). The tissue-specific hierarchy (brain > muscle > liver) reflects the physiological distribution of cholinergic systems, and seasonal variations highlight the importance of temporal considerations in biomonitoring programs (Soreq & Seidman, 2001). The PCA results further revealed that dissolved oxygen is a key positive driver of AChE activity, while temperature and pH show negative correlations, and biometric relationships are seasonally dependent. These findings establish a baseline for future monitoring efforts and support the use of *Barbus lacerta* as a sentinel species for aquatic ecotoxicology studies in the region.

Declarations

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Competing interests: The authors have no relevant financial or non-financial interests to disclose.

Ethics approval: All animal handling and experimental procedures were approved by the Animal Ethics Committee of the University of Zabol (Approval No. UOZ-AEC-2023-045) and were performed in accordance with the ethical standards for animal welfare.

Data availability: The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Author contributions: All authors have read, understood, and have complied as applicable with the statement on "Ethical responsibilities of Authors" as found in the Instructions for Authors.

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Figures



Figure 1

Map of the Sefidroud River basin showing the three sampling stations. Station 1 (upstream control, Deylamdeh) is located in a forested area with no agricultural activity. Station 2 (midstream, Zilki-Rud) is situated downstream of agricultural lands and villages. Station 3 (downstream, Firch-Rud) is located in an intensively cultivated plain with low water flow velocity. The inset map shows the position of the Sefidroud River within Iran (dark rectangle) and its drainage into the Caspian Sea.

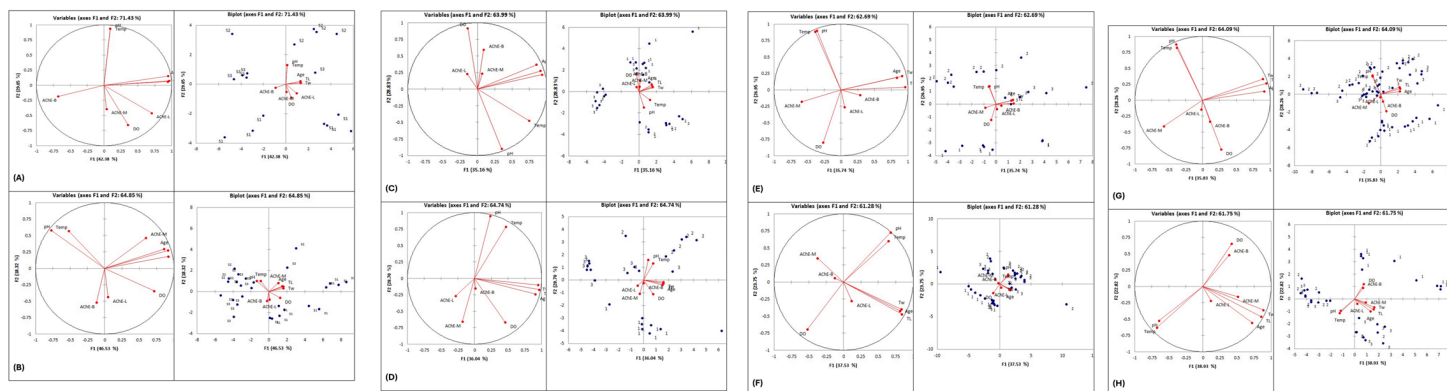


Figure 2

Principal Component Analysis (PCA) biplots (left panels) and ordination diagrams (right panels) showing the relationships among AChE activity in brain (AChE-B), muscle (AChE-M), liver (AChE-L), biometric variables (Age, total length – TL, total weight – TW), and environmental parameters (dissolved oxygen – DO, temperature – Temp, pH) in *Barbus lacerta* sampled from three stations (S1, S2, S3) along the Sefidroud River across eight months: (A) December, (B) February, (C) March, (D) April, (E) May, (F) June, (G) July, (H) August.

Vectors represent variable loadings. Ellipses or clusters indicate station grouping. The percentage of total variance explained by PC1 and PC2 is indicated on each axis. DO was consistently the strongest positive correlate of AChE activity (loading >0.7 in August), while temperature and pH showed weak negative correlations at impacted stations. Station separation was clear: S1 (upstream, green) clustered with high DO and high AChE; S3 (downstream, red) clustered with higher temperature/pH and lower AChE; S2 (midstream, yellow) occupied an intermediate position.