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Article

Nitrogen Pathways and Bioindicators in River Systems: A Pilot Study

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Abstract

The evaluation of nitrogen pathways in river systems and the identification of suitable aquatic ecosystem response indicators are essential for maintaining river health. Nitrogen, in its various forms, is a key physico-chemical quality element that underpins the biological communities determining the ecological status of surface waters. Elevated nitrogen loads in freshwater ecosystems can trigger adverse processes such as eutrophication and acidification, both of which are linked to biodiversity loss. We hypothesize that specific biotic indicators can reflect nitrogen-related stress in aquatic ecosystems, acting simultaneously as biodiversity components and as bioaccumulating indicators. To test this hypothesis, we examined four study sites within the Koiliaris River watershed (Crete, Greece). Our approach aims to enhance understanding of nitrogen dynamics at the river-basin scale by providing complementary insights into how nitrogen is processed, stored, and transferred within the aquatic ecosystem. To this end, total nitrogen content was quantified in four matrices: water, sediments, aquatic mosses, and macroinvertebrates. This study represents a pilot, exploratory assessment indicating that water may act as the primary pathway of nitrogen availability for aquatic biota in the studied streams.

Keywords: total nitrogen (TN); karstic rivers; aquatic bryophytes and macroinvertebrates; mediterranean

1. Introduction

Nitrogen plays a central role in regulating ecological processes in freshwater ecosystems, and its various dissolved and particulate forms directly influence primary production, community structure, and overall ecosystem functioning. Recent studies have systematically investigated sediment–water nitrogen dynamics, emphasizing the importance of identifying pathways through which nitrogen is transported, stored, and transformed within river networks [1-2]. Understanding these pathways is critical for assessing ecological status, as nitrogen enrichment can trigger eutrophication, alter biogeochemical fluxes, and modify biological communities, particularly in sensitive or nutrient-limited systems.

Karstic river basins represent especially complex environments for studying nitrogen dynamics. Their hydrological regimes are shaped by rapid infiltration, heterogeneous subsurface flow, and strong groundwater–surface water interactions, all of which affect nutrient availability and transport. The Koiliaris Critical Zone Observatory (CZO), located in the Mediterranean region, is a representative karst watershed characterized by highly permeable carbonate geology and long-term human influence, including agriculture, grazing, and localized groundwater abstraction [3]. These combined natural and anthropogenic drivers create spatial variability in nitrogen concentrations and

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offer an opportunity to investigate nitrogen pathways under contrasting hydrological and ecological conditions.

Although nitrogen concentrations in water have been frequently studied in Mediterranean karst systems, much less is known about nitrogen accumulation in sediments and biotic components such as aquatic mosses and benthic macroinvertebrates. These biological compartments can act as integrative indicators of nitrogen availability because they accumulate nitrogen over longer time periods than water and therefore reflect both chronic exposure and trophic transfer. Aquatic bryophytes, in particular, have been widely used as biomonitors due to their ability to incorporate dissolved nutrients directly from the water column, while benthic macroinvertebrates integrate nitrogen through feeding pathways and thus reflect conditions across multiple trophic levels [1, 4].

Despite their relevance, comparative assessments of nitrogen content across abiotic (water, sediments) and biotic (mosses, macroinvertebrates) matrices remain scarce, especially in Mediterranean karst rivers. Filling this knowledge gap is essential for developing reliable ecosystem response indicators and improving our understanding of nitrogen cycling at the river-basin scale. The Koiliaris River Basin CZO provides an ideal natural laboratory for such investigations due to its well-studied hydrology and the presence of distinct source areas, including karstic springs, agriculturally influenced tributaries, and reaches affected by seawater intrusion. The focus of the present study is therefore the characterization and comparison of total nitrogen (TN) concentrations in four environmental matrices—water, sediments, aquatic mosses, and macroinvertebrates—across four sites within the Koiliaris River Basin CZO. Bryophytes and macroinvertebrates were selected because they are biological quality elements and integrate nitrogen exposure over longer time scales than algae, while reflecting both dissolved and trophic nitrogen pathways. By integrating abiotic and biotic measurements, the study aims to (i) identify spatial patterns of nitrogen distribution, (ii) explore the potential of bryophytes and macroinvertebrates as bioindicators of nitrogen exposure, and (iii) provide insights into nitrogen pathways and trophic transfer in a Mediterranean karstic watershed. This pilot comparative approach contributes to a more comprehensive understanding of nitrogen cycling and offers a basis for future ecological assessments within similar karst river systems.

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2. Materials and Methods

2.1. Study area and river sites

The Koiliaris Critical Zone Observatory (CZO) lies in northwestern Crete near Chania, covering watershed of 132 km² under a Mediterranean semi-arid climate [3]. Land use is dominated by grazed shrubland and pasture, with agriculture (olive and citrus groves, vineyards, vegetables) and minor forest patches. Its 44.8 km drainage network includes the Keramianos tributary, ephemeral streams feeding the Anavreti tributary, and the karstic springs of Stylos and Anavreti, which join to form the main Koiliaris River.

The sampling campaign carried out in 2025 includes 4 locations in the Koiliaris River Basin CZO, Crete, Greece (Figure 1). The karst spring of Stilos, located at 17 m a.s.l., provides a permanent flow throughout the year [5]. Agios Georgios is a hydrological and water-quality monitoring station situated at the junction of the Stylos and Keramianos streams. Armeni Spring, also a water-quality station, is located near the village of Armeni. Zourpos Spring, another water-quality monitoring site, is situated in the village of Kalives.

2.2. Field Survey and Additional Data Acquisition

River water, sediment, aquatic plants and macroinvertebrates were sampled in July 2025. Basic physicochemical parameters (water temperature, pH, electrical conductivity and dissolved oxygen, Table 1) were measured in situ with a WTW meter. The nomenclature followed Hill et al. [6] for mosses.

2.3. Analysis of Total Nitrogen

Total nitrogen was selected as an integrative metric capturing both dissolved and particulate nitrogen pools relevant for biotic uptake. Water samples were collected in Nalgene bottles, stored at 4 0C, transferred to laboratory and filtered through Whatman® qualitative grade 4 papers to remove coarse particles and gelatinous precipitates. Sediment samples (approximately 300–500 g per site) were collected and transported in clean polyethylene bags for subsequent nitrogen analysis. Representative plant samples consisting of 5 to 10 subsamples were collected depending on the stream width and the "patchiness" of the selected biomonitor assemblages [7]. Plants were washed briefly in the stream to remove admixtures. In laboratory conditions, they were cleaned of mineral and organic particles. Benthic macroinvertebrates were collected using a hand net (250 μm mesh size) by kick sampling over an area of approximately 1 m² at each site, following the multi-habitat approach [8-9] and according to the ISO 5667-3:2024 [10]. Sampling effort was proportional to the relative abundance of available microhabitats (e.g., cobbles, gravel, macrophyte patches). Samples were rinsed in situ to remove sediment and coarse debris and preserved in 70% ethanol for laboratory analysis. In the laboratory, individuals were sorted under a stereomicroscope and identified to family or genus level.

Total nitrogen was analyzed in water, sediment and biota (aquatic mosses and macroinvertebrates) with a multi N/C 2100S Reactor (N/C elemental analyzer, Analytik Jena, Jena, Germany). In water samples the analyzer converted nitrogen content to nitrogen monoxide (NO) using combustion (800 0C) in the presence of platinum (Pt) catalyst and oxygen (O2). Quantification of nitrogen was achieved with a chemiluminescence detector (CLD) and a calibration curve created with analysis of potassium nitrate (KNO_3), ammonium sulfate ($(NH_4)2SO_4$) and και ethylenediaminetetraacetic acid (EDTA). Sediments, aquatic mosses and macroinvertebrates were dried at 750° C and micronized prior to the analysis in order to remove humidity and increase reaction surface. Combustion was carried out at 650° C. From each sample 8-15 mg (sediments) or 0.5-2 mg (biota) were weighed inside quartz cups, using a five-figure KERN ABT 120-5DNM balance and loaded into the furnace. Each sample was analyzed as triplicate while every 5 samples (15 runs) a

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blank was run in order to avoid instrumental drifts. The concentrations are presented in g/kg dry weight.

2.4. Statistical Analysis

Multiple regression analysis was performed to assess the relationship between total nitrogen (TN) concentrations in abiotic and biotic matrices, using Statistica 12 software [11].

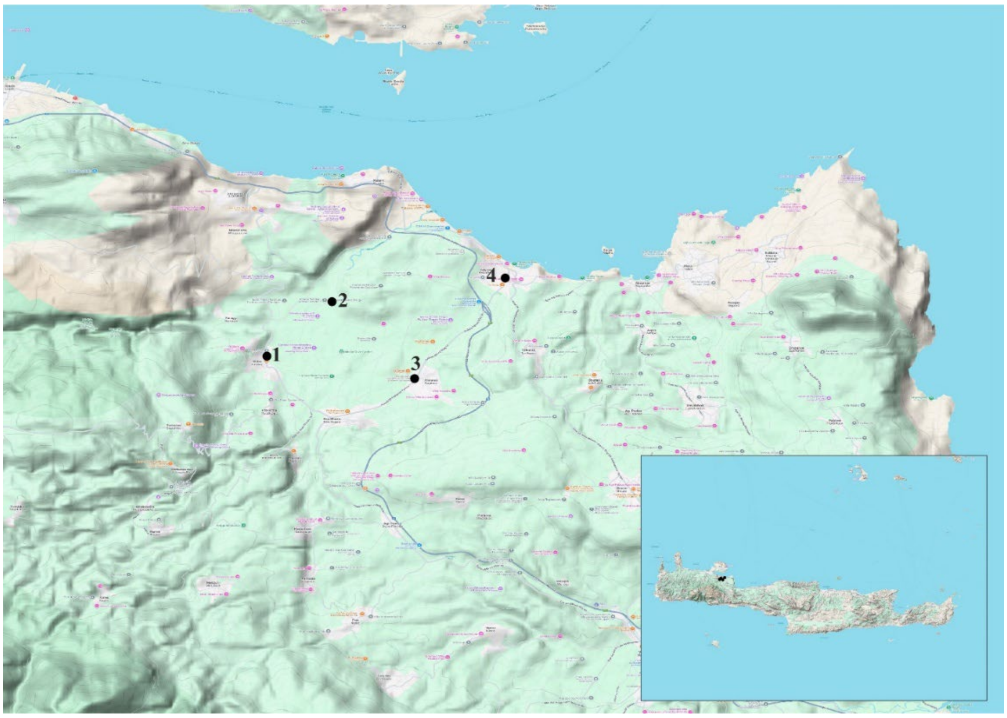

Figure 1. Location of the studied sites: 1- Stylos Spring, Water Quality Station at the center of Stylos village; 2- Agios Georgios, Hydrological/Water Quality Station at the junction of Stylos Karst Springs and Keramianos; 3- Armenoi Spring, Water Quality Station at Armeni village next to the church of the Assumption, 4- Zourpos Spring, Water Quality Station at Kalives village.

3. Results

3.1. Water Characteristics

The studied running waters were slightly alkaline (pH 7.8–8.2) and characterized by medium electrical conductivity, except at Zourpos, where conductivity was markedly higher (1526 µS/cm). Dissolved oxygen concentrations were high at all sites (9.0–11.1 mg/L) (Table 1).

Table 1. Coordinates of the sampling site and basic physico-chemical parameters.

	Spring/Stream	**Coordinates**		**T, °C**	**pH**	**EC, µS/cm**	**DO, mg/L**
		N	E				
1	Stylos	35.43423042	24.12621967	13.7	7.91	237	11.05
2	Agios Georgios	35.4450669	24.1391838	17.4	8.08	248	9.03
3	Armenoi	35.42976973	24.15562563	15.0	8.18	236	9.34
4	Zourpos	35.44977297	24.17366747	15.4	7.82	1526	9.32

3.2. Biota Indicators

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Hygroamblystegium tenax (Hedw.) Jenn. dominated aquatic macrophyte assemblages at Stylos, Agios Georgios and Armenoi, while *Cinclidotus fontinaloides* (Hedw.) P.Beauv. as dominant species was sampled at Zourpos spring. At all sampled sites, a high abundance of aquatic mosses, typical of cold-water aquatic macrophyte assemblages, was recorded. Nitrogen concentrations in moss tissues ranged from 16.9 to 20.4 g/kg across the studied sites. Sites dominated by H. tenax (Stylos, Agios Georgios, and Armenoi) showed comparable moss nitrogen levels (16.9–20.4 g/kg), while a similar concentration (20.0 g/kg) was recorded at Zourpos spring, where *C. fontinaloides* was the dominant species. Overall, total nitrogen levels in mosses were relatively consistent among sites despite differences in water and sediment nitrogen concentrations.

Macroinvertebrate assemblages showed site-specific differences rather than a clear longitudinal gradient along the sites from Koiliaris River Basin. Sensitive EPT taxa (Ephemeroptera, Plecoptera, Trichoptera) occurred mainly at Agios Georgios and Armenoi, where *Protonemura* sp., *Leuctra* sp., and Glossosomatidae sp. were recorded together with low tissue nitrogen content (29.8–30.5 g/kg). These sites represent cold, well-oxygenated and relatively undisturbed habitats with low nutrient availability. Gastropods, especially *Theodoxus* sp. (Neritidae) and fam. Hydrobiidae, together with *Gammarus* sp. (Amphipoda), were abundant across all sampling sites, forming the structural core of the benthic community. At Stylos, macroinvertebrate diversity remained high, including representatives of several Trichoptera families (Glossosomatidae, Limnephilidae, Sericostomatidae/Goeridae), in addition to abundant *Gammarus* sp., fam. Hydrobiidae, and *Theodoxus* sp. but nitrogen content in biota was elevated (44.5 g/kg). Gammarus sp., fam. Hydrobiidae and *Theodoxus* sp. dominated, indicating a stable karstic substrate with moderate nutrient enrichment. In contrast, Zourpos displayed a poorer community dominated by *Gammarus* sp., fam. Hydrobiidae, and fam. Neritidae, with the absence of sensitive EPT taxa. The highest nitrogen concentration in biota (47.1 g/kg) was registered (Table 2).

Table 2. TN content in matrices.

	Spring/Stream	water, mg/L	sediment g/kg	moss g/kg	macroinvertebrates, g/kg
1	Stylos	1.1	0.3	20.4	44.5
2	Agios Georgios	0.9	1.1	17.5	29.8
3	Armenoi	0.9	0.3	16.9	30.5
4	Zourpos	1.4	0.2	20.0	47.1

3.3. Comparison Between Matrices

Total nitrogen (TN) concentrations varied among sampling sites and four sampled matrices (Table 2). In water, TN levels were relatively low, ranging from 0.85 mg/L at Agios Georgios to 1.36 mg/L at Zourpos. In sediments, concentrations ranged from 0.23 to 1.14 g/kg, with the highest value recorded at Agios Georgios. TN content in moss was relatively similar across sites (16.85–20.39 g/kg), but levels were markedly higher than in water and sediments - ranging from 15- to 85-fold greater. While macroinvertebrates exhibited notably higher concentrations, ranging from 29.8 to 47.1 g/kg, with the maximum observed at Zourpos. Nitrogen concentrations in water, moss, and macroinvertebrates were highest at Zourpos, with Stylos showing the second-highest values.

A relatively strong positive relationship was found between water and moss ($b^* = 0.78$, $p = 0.54$) and between water and macroinvertebrates ($b^* = 0.83$, $p = 0.40$), whereas the relationships between moss and sediment ($b^* = 0.05$, $p = 0.96$) and between sediment and macroinvertebrates ($b^* = -0.09$, $p = 0.91$) were weak or negligible. Although the coefficients suggest possible positive trends between water and biotic components (moss and macroinvertebrates), none of the relationships were statistically significant.

4. Discussion

Similar average values of pH, EC, and DO for these streams during 2010–2019 were reported by Lilli et al. [3], indicating stable hydrochemical conditions over time. Overall, the study sites formed a coherent group with comparable water characteristics and generally low nutrient concentrations, with the exception of Zourpos Spring, which is influenced by seawater intrusion.

Our results indicated spatial variability in nitrogen accumulation among sites and differences in TN retention across environmental matrices. Nitrogen is a major nutrient in sediments, and reported TN values from the Wuding River Basin, China (0.36 g/kg upstream and 0.48 g/kg downstream) [12] are consistent with our measurements (0.23–0.34 g/kg), except at Agios Georgios (Site 2), where TN reached 1.14 g/kg. Similarly, sediments in the Taipu River—an area influenced by aquaculture and croplands—contained lower TN concentrations (0.555 g/kg) [13], about half of the value measured at Agios Georgios. This elevated TN level at Site 2 may be linked to localized nitrogen inputs, such as agricultural runoff, livestock activity, or groundwater inflow enriched in dissolved nitrogen.

Hygroamblystegium tenax has been documented from Crete [e.g., 14-15], whereas *C. fontinaloides* is known from mainland Greece [16], with no published evidence confirming its presence on Crete. *Hygroamblystegium tenax* typically occurs in calcareous rivers under low agricultural pressure and forest-dominated catchments [17]. In contrast, *C. fontinaloides* exhibits wide ecological tolerance [18], which corresponds to its occurrence at Zourpos, where nitrogen reached its maximum values in three of the sampled matrices.

Nitrogen, as an essential macronutrient, is required for amino acid, chlorophyll, and hormone synthesis in bryophytes and can stimulate growth; however, excessive nitrogen may lead to growth reduction [19]. Across all sites, nitrogen concentrations in moss tissues (up to 2.04%) were below the 3.1% reported for *Fontinalis antipyretica* at an undisturbed site [4]. To our knowledge, no published data exist on total nitrogen content in the tissues of *H. tenax* or *C. fontinaloides*. Previous studies have primarily considered these species as indicators along nitrogen or trophic gradients [18, 20], focusing on water chemistry rather than on tissue nitrogen concentrations. Comparison with the average nitrogen concentration of 19.224 g/kg, determined in 38 moss samples from upland and lowland sites along the Maritsa River, Bulgaria [21], showed similar values to those obtained in the present study (average 18.7 g/kg). This confirms the authors' finding that nitrogen is an element exhibiting the narrowest range of variation among bryophyte species.

Macroinvertebrate community composition was consistent with known biodiversity patterns in karstic rivers of Crete. Fam. Gammaridae and fam. Hydrobiidae are widely distributed throughout the Mediterranean and often dominate karstic stream assemblages [22-23]. Small water snails of genus *Theodoxus* are typical of hardwater springs and limestone streams, and recent molecular evidence indicates that Cretan populations belong to *T. fluviatilis* [23]. The occurrence of *Protonemura* and *Leuctra* species corresponds to the limited distribution of cold-stenothermic Plecoptera taxa in southern Greece [24-25]. The Trichoptera families recorded here (Glossosomatidae, Limnephilidae, Sericostomatidae/Goeridae) have also been documented in western Crete [26] and indicate well-oxygenated, pristine karstic headwaters.

Nitrogen is essential for protein and chitin synthesis in aquatic invertebrates and supports metabolic processes [27]. Excessive nitrogen availability, however, can alter community composition by favoring tolerant taxa. In our study, macroinvertebrate nitrogen levels ranged from 29.8 to 47.1 g/kg. The highest values were observed at Zourpos where poorest benthic community without Insecta species was registered. Beck et al. [28] emphasized on very high and variable N content of insects compared to other taxonomic groups. Insecta could thus be more prone to N-limitation and stoichiometrically-induced taxonomic shifts in communities than other taxa even at intermediate levels of nitrogen [29]. At Zourpos, more tolerant taxa belonging to only three families were identified – Gammaridae (Amphipoda), Neritidae and Hydrobiidae (Gastropoda). This suggested nitrogen accumulation within the food web and possible influence of nutrient-rich groundwater or seawater intrusion near the coastal zone. Increasing of the nitrogen content indicates moderate level of

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enrichment, reduced habitat heterogeneity and shift towards tolerant taxa compared to upstream sites. Similar patterns were observed in the karstic Loue River (France), where Frossard et al. [2] reported enhanced nitrogen accumulation in water and biota linked to nutrient-rich groundwater and altered trophic transfer. Although nitrogen dynamics in karst rivers have been studied previously [2, 22, 30-31], most research has focused on water chemistry rather than on nitrogen content in benthic organisms. Our findings therefore contribute valuable evidence on nitrogen incorporation and accumulation within benthic macroinvertebrates in karstic Mediterranean catchments, emphasizing the strong link between trophic status and community composition.

The observed patterns suggest that water may represent the primary pathway of nitrogen availability for aquatic biota in the studied streams. The relatively high standardized coefficients between water and both moss ($b^* = 0.78$) and macroinvertebrates ($b^* = 0.83$) indicate a potential link between dissolved nitrogen and its accumulation in primary producers and consumers. In contrast, the very low or negative coefficients involving sediment ($b^* = 0.05$ for moss–sediment and –0.09 for sediment–macroinvertebrates) imply that sedimentary nitrogen contributes little to the overall nitrogen pool accessible to these organisms. However, none of the relationships were statistically significant ($p > 0.05$), which is likely a result of the small sample size ($n = 4$). Despite this limitation, the data point to a possible dominance of waterborne nitrogen inputs over sedimentary sources in shaping the nitrogen content of the aquatic food web. These findings are consistent with previous observations on *Rhynchostegium riparioides*, where the elemental composition of the species was shown to depend strongly on the chemical characteristics of the surrounding water and to follow linear relationships for both intracellular and exchangeable elements [32].

The resulting ecological conditions correspond to a high ecological status based on macrophytes as a biological quality element. Similarly, lower nitrogen concentrations at Agios Georgios and Armenoi coincided with the presence of sensitive EPT benthic taxa, indicating limited nutrient availability and a high ecological status. The observed patterns are consistent with a high ecological status as defined under the Water Framework Directive, where low nutrient concentrations and the presence of sensitive EPT taxa are indicative of minimal anthropogenic pressure.

Understanding how nitrogen influences water quality is essential for informed policy decisions and improved environmental outcomes [33]. Although statistical significance was not achieved due to the limited sample size ($n = 4$), the magnitude and direction of the standardized coefficients suggest ecologically meaningful trends and support the proposed hypotheses. This study is preliminary, and further research is needed to evaluate the bioindicator potential of aquatic communities for nitrogen exposure and to better characterize nitrogen pathways and trophic transfer in Mediterranean karstic watersheds. Given the predicted sensitivity of nitrogen levels to hydroclimatic variables, improving our understanding of these processes is particularly important in the context of global climate change [20].

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Abbreviations

The following abbreviations are used in this manuscript:

CLD – Chemiluminescence detector
CZO – Critical Zone Observatory
DO – Dissolved oxygen
EC – Electrical conductivity
EDTA – Ethylenediaminetetraacetic acid
EPT – Ephemeroptera, Plecoptera, Trichoptera
ISO – International Organization for Standardization
TN – Total nitrogen

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