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# *Isoëtes sabatina* (Isoëtaceae, Lycopodiopsida): Taxonomic distinctness and preliminary ecological insights

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**Abstract**

- Isoëtes sabatina* is the rarest aquatic quillwort in Europe. Although recently found (2013) in Lake Bracciano (central Italy), the species is just one step away from extinction with an estimated population not exceeding 400 individuals and a spatial range of a few hundred square metres.
- Lake Bracciano is a deep, oligo-mesotrophic Mediterranean volcanic lake that has been subjected to human activities. From January to October 2017, the lake experienced a dramatic water level decrease (up to  $-1.50$  m), which significantly affected the littoral zone and the habitat of *I. sabatina*.
- To improve the chances of survival of *I. sabatina*, the first eco-taxonomic investigation on this species was carried out to describe its genetic distinctness, physical and chemical requirements and companion species.
- The phylogenetic position of *I. sabatina* was investigated by applying standard DNA barcoding methods. Simultaneously, during summer 2019, the physical and chemical features of water and sediments of the *I. sabatina* population and five small Alpine lakes colonized by *Isoëtes echinospora* – a supposed close relative – were characterized. These data were then compared with the available data on the trophic requirements of the target obligate aquatic *Isoëtes*, together with *Isoëtes lacustris* and *Isoëtes malinverniana*.
- The present survey confirmed the taxonomic and ecological distinctness of *I. sabatina* – providing the first evidence of genetic differentiation from *I. echinospora*. *Isoëtes sabatina* grows in waters with temperature, conductivity and total alkalinity up to  $30^{\circ}\text{C}$ ,  $561\ \mu\text{S cm}^{-1}$  and  $3.45\ \text{meq L}^{-1}$ , respectively.
- The edaphic requirements of *I. sabatina* confirm its outstanding conservation value, and this study offers a basic understanding of how to prevent its extinction. Now, all possible actions must be taken immediately to save this species.

**KEYWORDS**

climate change, Isoetids, Mediterranean, submerged macrophytes, temperate, water abstraction

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## 1 | INTRODUCTION

Macrophytes are threatened globally (Zhang et al., 2017; Bolpagni et al., 2018) as a result of the high levels of exploitation and impairment of freshwater ecosystems by human activities (Cantonati et al., 2020). Pollution, water exploitation, changes in riparian and littoral land uses and alien species are widespread threats to the freshwater biota (Bolpagni, 2020; Hofstra et al., 2020), which represents about 10% of non-marine biodiversity, even though freshwater ecosystems cover only 2.3% of the Earth's surface area (Reid et al., 2019).

Among macrophytes, isoetids are particularly at risk (Smolders, Lucassen & Roelofs, 2002) because they are extremely sensitive to external perturbations and eutrophication of water (Free et al., 2009; Abeli et al., 2018) and sediment (Pulido et al., 2011). The lycophyte *Isoëtes* genus (quillworts) – embracing rooted plants characterized by a rosette shape and a preference for oligotrophic water bodies – is one of the most globally endangered freshwater taxa (Christenhusz et al., 2017). The *Isoëtes* species have a complex evolutionary history that has led to a morphological simplification of the genus by adaptive convergence (Taylor & Hickey, 1992). They can be strictly aquatic, amphibian or terrestrial (Troia & Greuter, 2015). In Europe, six out of the eight known obligate aquatic *Isoëtes* species are seriously endangered, occupying very restricted geographical areas (4–56 km<sup>2</sup>; Table 1). Among them, one, *Isoëtes heldreichii* Wettst., is considered Critically Endangered (Possibly Extinct) (García Criado et al., 2017) and two, including *Isoëtes sabatina* Troia & Azzella, fall into the Critically Endangered category (Troia & Azzella, 2013; Christenhusz et al., 2017; García Criado et al., 2017; Magrini et al., 2020).

Described as a new species in 2013 (Troia & Azzella, 2013), *I. sabatina* can be considered the rarest recently confirmed quillwort in Europe. Since its discovery, the presence of this lycophyte in Lake Bracciano, a deep oligo-mesotrophic lake near Rome (Italy), has raised a series of questions regarding both its taxonomic identity and edaphic conditions. Indeed, the biogeographical context and the chemical and physical characteristics of Lake Bracciano are very

dissimilar to the typical habitats of strictly aquatic *Isoëtes* species (Smolders, Lucassen & Roelofs, 2002; Brunton & Troia, 2018).

*Isoëtes sabatina* is point endemic to Lake Bracciano, which experienced a recent dramatic water level decrease compared with the reference level – of up to –1.50 m in October 2017 – as a result of a drop in precipitation caused by the local effects of climate change and the simultaneous increase in demand for drinking water (Azzella, 2014; Bolpagni, Laini & Azzella, 2016). Indeed, Lake Bracciano was used until a few months ago as one of the main water reserves for the nearby city of Rome. As a result, a substantial decline in the *I. sabatina* population, with the disappearance of about 60% of the known individuals, was recorded (Azzella M.M., pers. observ.). No more than 400 plants now survive in the wild. Changes in the water level are considered a critical threat to the conservation of *Isoëtes lacustris* L., as verified by Mjelde, Hellsten & Ecke (2013) for Nordic lakes. Furthermore, climate models indicate the accentuation of hydrological variability for Lake Bracciano and the Mediterranean region in the near future (Hoerling et al., 2012), reinforcing the risk of a sudden extinction of *I. sabatina*.

Consequently, it is essential to acquire genetic data on the taxonomic placement of *I. sabatina* and on the physical and chemical requirements of its unique population to give this species a chance of survival (Magrini et al., 2020). This was done using a multiple strategy consisting of: (i) molecular phylogenetic analysis (DNA barcoding) of *I. sabatina*; (ii) ecological characterization of the *locus classicus* of *I. sabatina*; (iii) comparison of *I. sabatina* habitat with growing sites of the supposed close relative *Isoëtes echinospora* Durieu in the Alpine chain (Trentino-Alto Adige region, northern Italy; Cavagna et al., 2019); and (iv) characterization of the floristic composition of the vegetation colonized by the two target isoetids. The present contribution offers the first results on the taxonomic position of *I. sabatina* within the *Isoëtes* genus and its edaphic range, with a preliminary comparison with the literature. Physical and chemical data on the supposed close relative *I. echinospora*, the ecologically close *I. lacustris* (erroneously reported for Italy in the past; Bolpagni et al., 2018) and the other aquatic isoetid endemic to Italy, *Isoëtes malinverniana* Ces. & De Not., are reported and discussed.

**TABLE 1** European aquatic *Isoëtes* species, with the indication of the IUCN Red List Category and the biogeographical region and range

Species	IUCN Red List Category	EU biogeographical region	AOO (km <sup>2</sup> )
<i>Isoëtes azorica</i> Durieu ex Milde	VU	Macaronesian	16
<i>Isoëtes boryana</i> Durieu	EN	Atlantic	56
<i>Isoëtes echinospora</i> Durieu	LC	Atlantic, Continental, Boreal, Alpine	n.a.
<i>Isoëtes fluitans</i> M.I. Romero	EN	Atlantic	12
<i>Isoëtes heldreichii</i> Wettst.	CR (PE)	Mediterranean	n.a.
<i>Isoëtes lacustris</i> L.	LC	Atlantic, Continental, Boreal, Alpine	n.a.
<i>Isoëtes malinverniana</i> Ces. & De Not.	CR	Continental	36
<i>Isoëtes sabatina</i> Troia & Azzella	CR	Mediterranean	4

Note: IUCN Red List categories: CR, Critically Endangered; CR (PE), Critically Endangered (Possibly Extinct); EN, Endangered; LC, Least Concern; VU, Vulnerable. AOO, Area of occupancy, when of interest (<100 km<sup>2</sup>), from Christenhusz et al., 2017; n.a., no information available.

## 2 | METHODS

### 2.1 | DNA extraction, amplification and data analysis

Genomic DNA was extracted from silica-gel-dried specimens (six per species) using the GeneElute Plant Genomic DNA Miniprep kit (Sigma Aldrich) following the manual's instructions. Amplification of the ITS1, 5.8 s and ITS2 regions of the nrDNA was done using the primers ITSu4 and ITS5 (Cheng et al., 2016). PCR reactions were carried out on a total volume of 25 µl containing 1× of Standard Taq Reaction buffer (New England Biolabs), 10 µM of each primer, 200 µM of each dNTP, 0.625 U of Taq DNA polymerase (New England Biolabs) and 10 ng of template DNA. The reactions were performed in a MiniAmp PCR system (Applied Biosystems, Thermo Fisher Scientific, USA). The PCR programme was 94°C for 4 min, followed by 25 cycles of 30 s at 94°C, 40 s at 55°C, and a final extension for 45 s at 72°C, with a final step of 10 min at 72°C. Automated DNA sequencing was performed from the purified PCR products using an AB3130xl sequencer (Applied Biosystems, Life Technologies Corporation).

The original sequences of *I. sabatina* and *I. echinospora* were multi-aligned with GenBank accessions of the most closely related taxa, as indicated by Larsén & Rydin (2016). Some names of species in GenBank were corrected according to Troia, Johnson & Taylor (2019). *Isoëtes setacea* (DQ285000) is here named *Isoëtes delilei* Rothm. and *Isoëtes hystrix* (DQ284994) is here named *Isoëtes phrygia* (Boiss.) Hausskn. The alignment was performed with MAFFT version 5, with Q-INS-I as the iterative refinement method for the multi-alignment (Katoh & Standley, 2013). Gaps were coded as separate characters (Simmons & Ochoterena, 2000) using FastGap version 1.0.8 (Borchsenius, 2009) and appended to the end of the dataset. The dataset was then analysed using Bayesian inference with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) following the manufacturer's indications. The tree was viewed and edited using TreeView (Page, 1996), with indications of the Bayesian posterior probabilities for the internal tree nodes. The number of site substitutions among *I. sabatina* and *I. echinospora* was also evaluated and appended as integration to the phylogenetic reconstruction.

### 2.2 | Physical and chemical features of *Isoëtes sabatina* and *I. echinospora* and companion species

The summer physical and chemical features of water and sediments of the unique population of *I. sabatina* (July 12, 2019) and five out of the 12 populations of *I. echinospora* only known in Italy (August 26–29, 2019) were characterized using a portable multiprobe (Hanna Instruments, HI 9828), for measuring temperature, pH, conductivity and dissolved oxygen, and by collecting water and sediment samples (intact cores) using a plastic bottle (1 L) and a manual corer, respectively. This enabled the ecological conditions of the two species during the growing peak to be preliminarily defined, focusing on the most critical period for

strictly aquatic *Isoëtes* taxa (i.e. maximum water temperatures and lower hydrological levels).

Water samples were collected just above the *I. sabatina* and *I. echinospora* stands (in the depth range 30–150 cm), and immediately processed and kept in cold storage at around 4°C for laboratory analysis (five replicates for *I. sabatina*, spaced at least 50 m apart, and one for each investigated population of *I. echinospora*). A water volume of 200 ml was filtered with GF/C glass-fibre filters (Whatman, Maidstone, UK) for NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup> and soluble reactive phosphorus (PO<sub>4</sub><sup>3-</sup>), and 100 ml of unfiltered water was collected for determination of total alkalinity, total phosphorus (TP) and total nitrogen. All analyses were performed using standard spectrophotometric methods (American Public Health Association, 2012), except for total alkalinity, which was determined according to Anderson et al. (1986). The sediment cores (five for each of *I. sabatina*, and *I. echinospora*) were collected using Plexiglass core tubes (20 × 4 i.d. cm). Subsequently, the first 5 cm of each core were extruded and homogenized to determine organic matter, determined as dry weight loss after ignition at 450°C for 2 h of 0.5 g of dry sediment. Comparative data were collected by exploring the literature on the aquatic *Isoëtes* species in Italy (Abeli et al., 2012; Barni et al., 2013 for *I. malinverniana* Ces. & De Not.) and on *I. echinospora* and the systematically and ecologically close *I. lacustris* in Norway and Scandinavia (Alahuhta et al., 2017; M. Mjelde, unpubl. data). Simultaneously with the physical and chemical characterization, data concerning the floristic composition of the two target *Isoëtes* species were also collected by visual inspection of standard plots of 1 m<sup>2</sup> (five for each species).

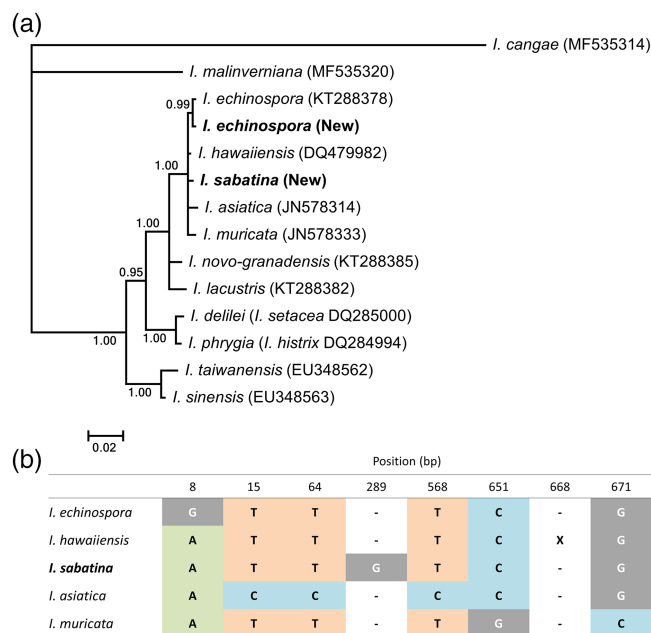
## 3 | RESULTS

### 3.1 | Genetic analyses

The alignment contains 14 taxa and 780 characters, including the position of the coded gaps (742–780), and 249 variable sites, of which 71 are informative. The phylogenetic tree using the Bayesian approach shows that the four taxa – *I. echinospora*, *Isoëtes hawaiiensis* W.C. Taylor & W.H. Wagner, *Isoëtes asiatica* Makino and *Isoëtes muricata* Durieu – are grouped in a terminal clade (posterior probability, 1.00) and separate from the other members of clade E: *Isoëtes novo-granadensis* H.P. Fuchs, *I. lacustris*, *I. phrygia* and *I. delilei* (Figure 1a). *Isoëtes sabatina* is included in the terminal clade consisting of *I. echinospora*, *I. hawaiiensis*, *I. asiatica* and *I. muricata*, a group of very close but distinct species that differ in a total of eight positions of alignment (Figure 1b).

### 3.2 | Water and sediment features of *I. sabatina* and *I. echinospora* and companion species

The water and sediment features of the only known population of *I. sabatina* are comparable with those of the littoral zones of meso-



**FIGURE 1** (a) Bayesian 50% majority-rule consensus tree from ITS1, 5.8 s and ITS2 sequences of *Isoetes*. Posterior probability values are shown when >50%. GenBank codes are shown in brackets; to solve the mismatch among GenBank repository and the updated taxonomy, the older taxonomic references of *Isoetes delilei* and *Isoetes phrygia* were also added. (b) Schematic representation of nucleotide polymorphisms among *Isoetes sabatina* and the phylogenetically closest species *Isoetes echinospora*, *Isoetes hawaiiensis*, *Isoetes asiatica* and *Isoetes muricata*

oligotrophic, large, carbonate-rich lakes, with pH, conductivity and total alkalinity values ranging from 7.3 to 8.5 pH units (7.8 on average), from 552 to 560  $\mu\text{S cm}^{-1}$  (557) and from 2.79 to 3.45  $\text{meq L}^{-1}$  (3.10), respectively (Table 2). Moreover, the sampled sites exhibit generally low concentrations of nutrients, with  $\text{NO}_3^-$  and soluble reactive phosphorus not exceeding 80  $\mu\text{g L}^{-1}$  and 5  $\mu\text{g L}^{-1}$  (method detection limit), respectively, whereas the TP in waters and the organic matter in surficial sediments are in the ranges 8.9–14.1  $\mu\text{g L}^{-1}$  (11.4) and 1.7–7.2% (3.8), respectively (Table 2). In contrast, the data related to the *I. echinospora* growing sites are typical of the mountains: very oligotrophic lakes, ponds and peatlands, with temperature, conductivity and TP not exceeding 18.2°C (late August; 14.8), 19  $\mu\text{S cm}^{-1}$  (16) and 10.5  $\mu\text{g L}^{-1}$  (8.1), respectively (Table 2). At the same time, the pH and the total alkalinity range from 5.92 to 6.48 pH units (6.05) and from 0.02 to 0.09  $\text{meq L}^{-1}$  (0.05) (Table 2).

In Lake Bracciano, *I. sabatina* grows in a mosaic environment dominated by several species, including perennial taxa [*Baldellia ranunculoides* (L.) Parl., *Eleocharis acicularis* (L.) Roem. & Schult., *Juncus articulatus* L., and *Schoenoplectus lacustris* (L.) Palla] and ephemeral taxa [*Chara aspera* Willd., *Chara vulgaris* L., and *Nitella hyalina* (DC.) C. Agardh], typical of the littoral zones of carbonate-rich lakes. Conversely, the studied populations of *I. echinospora* occur in small Alpine lakes, with only *Sparganium angustifolium* Michx. as a companion species.

**TABLE 2** Water chemical features of the aquatic *Isoetes* species obtained from a literature review and the present study (location and number of sites – in brackets – are also reported)

Species	Location and number of sites	Conductivity $\mu\text{S cm}^{-1}$	pH	Total phosphorus $\mu\text{g L}^{-1}$	Total alkalinity $\text{meq L}^{-1}$	Reference
<i>Isoetes echinospora</i>	Alps (Italy) (5)	16 (13–19)	6.1 (5.9–6.5)	8.1 (<5–10.5)	0.05 (0.02–0.09)	This article
	Norway (389)	n.a.	6.4	7.4	0.19	M. Mjelde (unpublished data)
	Scandinavia (47–341)	46 (3–265)	6.5 (4.4–8.8)	7.3 (<0.5–27.0)	0.18 (0.00–1.51)	Rørslett & Brettum (1989)
	Scandinavia (180)	48 (11–190)	6.9 (5.6–8.0)	15.5 (1.0–68.1)	0.20 (0.01–0.95)	Alahuhta et al. (2017)
<i>Isoetes lacustris</i>	Norway (424)	n.a.	6.3	7.2	0.17	M. Mjelde (unpublished data)
	Scandinavia (47–431)	46 (3–265)	6.6 (4.4–8.8)	7.3 (<0.5–27.0)	0.18 (0.00–1.51)	Rørslett & Brettum (1989)
	Europe (270)	n.a.	(5.3–8.4)	n.a.	(0.00–2.29)	Pietsch (1991)
<i>Isoetes malinverniana</i>	Scandinavia (213)	48	6.9 (5.6–8.0)	13.4 (1.0–64.0)	0.19 (0.01–1.75)	Alahuhta et al. (2017)
	Italy (12)	95 (101–93) <sup>a</sup>	7.5	n.a.	n.a.	Abeli et al. (2012)
<i>Isoetes sabatina</i>	Italy (3)	(81–105) <sup>b</sup>	(7.5–7.7)	n.a.	(0.40–0.43)	Barni et al. (2013)
	Lake Bracciano (Italy) (1)	557 (552–561)	7.8 (7.3–8.5)	11.4 (8.9–14.1)	3.10 (2.79–3.45)	This article

<sup>a</sup>Range of mean values.

Mean values when available and more than one site existing, and ranges in brackets.

n.a., No information available.

## 4 | DISCUSSION AND CONCLUSIONS

The data presented here confirm the taxonomic identity of *I. sabatina*, as proposed by Troia & Azzella (2013), placing the species in close relationship (based on currently available data) with *I. echinospora*. These two taxa have morphologically similar growth forms, but they differ in some diagnostic characteristics, such as the velum coverage and in the ornamentation of macrospores (Troia & Azzella, 2013). Furthermore, they differ in their ecological requirements, as clearly indicated by the differences in the physical and chemical features of the habitats colonized and in the floristic composition of colonized littoral areas. Indeed, along the littoral zone of Lake Bracciano *I. sabatina* is frequently found within the ephemeral *Chara* vegetation and in close association with submerged stands of perennial, amphibious (partially emergent) plants typical of lacustrine, riparian zones.

Specifically, *I. sabatina* is able to survive temperatures far higher than those measured for *I. echinospora* (with maximum values up to 30.0 vs 18.2°C, respectively), and higher mean pH values (7.8 vs. 6.3 pH units), although several authors have also reported values higher than 8.0 pH units both for *I. lacustris* (Rørslett & Brettum, 1989; Pietsch, 1991; Alahuhta et al., 2017) and for *I. echinospora* (Rørslett & Brettum, 1989; Alahuhta et al., 2017). The most relevant differences, however, emerge for conductivity and total alkalinity, which are generally higher in Lake Bracciano than the values measured for *I. echinospora* and *I. lacustris* (557 vs. 50  $\mu\text{S cm}^{-1}$ , and 3.10 vs. 0.30  $\text{meq L}^{-1}$ , on average, respectively). Indeed, these two latter species are typical of low- and very-low-alkalinity lakes and are frequently found in submerged stands co-dominated by *Lobelia dortmanna* L., *Littorella uniflora* (L.) Asch. and *Myriophyllum alterniflorum* DC., among other species. Similarly, *I. sabatina* clearly differs from the other Italian endemic isoetid species (*I. malinverniana*), which is typical of the *fontanili* systems (i.e. lowland springs) of northern Italy that are in turn characterized by low conductivity and alkalinity values (81–105  $\mu\text{S cm}^{-1}$  and 0.40–0.43  $\text{meq L}^{-1}$ , respectively; Abeli et al., 2012; Barni et al., 2013).

This is the first report of an *Isoëtes* species able to colonize water bodies with conductivity and total alkalinity higher than 265  $\mu\text{S cm}^{-1}$  and 2.29  $\text{meq L}^{-1}$ , respectively. The data discussed concerning the physical and chemical conditions of Lake Bracciano during summer 2019 are in line with those reported by Azzella, Bolpagni & Oggioni (2014) and Azzella et al. (2017) for the period 2009–2013.

These findings agree with the recent outputs of a preliminary *ex-situ* action for *I. sabatina*, which has shown a specific adaptation of *I. sabatina* to the Mediterranean climate. The megaspores of this species germinate at 20°C without any pre-treatment (Magrini et al., 2020), contrary to that observed for *I. echinospora* – the taxonomically closest species – which requires at least 3 months of cold stratification to break megaspore dormancy and germinate (Čtvrtlíková et al., 2012). Furthermore, *I. sabatina* lives in a deep lake dominated by charophyte species that shows a vegetation structure comparable with that of large calcareous lakes (Bolpagni et al., 2013;

Azzella et al., 2017). This evidence reinforces the existence of non-negligible ecological and edaphic differences between *I. sabatina* and aquatic congeners.

### 4.1 | Conservation insights

*Isoëtes sabatina* shows a set of typical features shared by most endemic species that give it a particular vulnerability to human threats or natural changes (Işık, 2011): restricted distribution, one small-sized declining population, specific habitat conditions and the need for a stable and constant environment. Owing to these characteristics, together with the drastic decline in its habitat since 2017 (the Lake Bracciano water level crisis), this species is highly threatened with extinction, although it occurs in a Regional Park (Bracciano-Martignano Regional Park; <https://www.parcobracciano.it/en/>). The endemic species should be carefully monitored and managed, and its conservation considered a global priority, to be managed at a national level (Işık, 2011; Orsenigo et al., 2018). Effective conservation efforts, including living plant collections in botanical gardens, *ex situ* spore conservation, habitat restoration, and translocations, are thus required to avoid further population decline or extinction. In this regard, the present results call for urgent actions to definitively halt the risk of losing *I. sabatina* as a valuable witness of past climate changes and, at the same time, call for deepening of the ecological determinants of aquatic isoetids to improve the management of effective global conservation strategies.

### 4.2 | Final remarks

A better understanding of the ecology of *I. sabatina* could enable predictions of the responses of the northern aquatic congeners to increases in water temperatures and changes in trophic as predicted by the leading climate forecast models. A further interesting result is that, despite the key roles played by *Isoëtes* ssp. and their conservation relevance, the available data concerning the water and sediment conditions are strikingly scarce and incomplete, preventing a robust ecological comparison among species. It is likely that a large amount of data is stored in 'grey' literature (i.e. national reports and papers). However, this kind of information is not available to a wider audience and, more specifically, to scientists and cross-national policy makers. Therefore, it seems crucial to stimulate a more intense collaboration between researchers to outline better ecological determinants of isoetids, including new systematic investigations, and develop strategies for adaptation to ensure the survival of this unique component of aquatic ecosystems.

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## CONFLICT OF INTEREST

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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