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REVIEW

Toward freshwater plant diversity surveys with eDNA barcoding and metabarcoding

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Abstract

Providing reliable, cost-effective data on species distribution is critical to ensuring biodiversity conservation. However, many species may go unrecorded by conventional surveys, especially in aquatic environments. Environmental DNA (eDNA) barcoding and metabarcoding are alternative approaches that could complete biodiversity estimates based on species observations. While eDNA surveys are being standardized for some animal groups (e.g., fish and amphibians), research on eDNA approaches for freshwater plant communities is just starting to bear fruit. Here, we synthesized the 22 studies that used eDNA barcoding and metabarcoding to survey plant biodiversity in freshwater systems. We present evidence that contemporary aquatic plants (macrophytes) and terrestrial plants can be detected in water and surficial sediment eDNA from lakes and rivers. The phenology (e.g., senescence) of the target taxa strongly influences species detection. The main application of eDNA barcoding targets the monitoring of invasive macrophytes, and barcoding assays are available for 14 taxa. The metabarcoding approach shows a range of applications: the detection of rare macrophytes, catchment-scale floristic inventories, and sediment fingerprinting. Barcodes on the plastid genome (cpDNA) are preferred for both approaches: *matK* and *trnH-psbA* for barcoding, *trnL*, and *rbcL* for metabarcoding. The intergenic transcribed spacer 1 (ITS1) from the nuclear ribosomal DNA (nrDNA) was used for designing eDNA barcoding assays on five invasive macrophytes. In contrast, only three metabarcoding studies used the ITS1 or ITS2 with newly designed primers. However, metabarcoding applications should routinely use a multi-locus approach, combining cpDNA and nrDNA barcodes. Barcode combinations and existing primers need further testing on eDNA samples. We recommend using local barcode reference databases (BRDs), ideally self-made, to circumvent taxonomic gaps and heterogeneous sequences in public BRDs. Finally, new technologies and developments like droplet digital PCR and hybridisation capture offer new perspectives for eDNA-based biodiversity monitoring approaches.

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KEYWORDS

aquatic ecosystems, aquatic plants, barcode reference databases, environmental DNA, monitoring, water

1 | INTRODUCTION

Monitoring biodiversity is an essential societal and scientific undertaking that demands both human and financial resources, time and ecological expertise. Traditionally, biodiversity monitoring relies on species observation, abundance estimation through visual surveys and the collection of individuals for later identification. These methods have inherent biases that are difficult to overcome. Species-level identification for some taxa cannot be done in the field and may depend on the developmental stage, the phenotypic plasticity or the researcher's experience. Field surveys may be limited to a short period when morphological characteristics are visible. In some environments, this approach does not allow for standardized surveys, and distortion may occur between observations and reality in the field. Traditional methods can also be invasive on the community in question, that is, dredging of benthic invertebrates or macrophyte sampling with rakes or grapnels.

In freshwater ecosystems, standard plant surveys could be limited by strong seasonal effects (floods, droughts) influencing plant biomass and the occurrence of diagnostic characters (e.g., flower structures). Macrophyte plant surveys are also known to overestimate floating-leaved taxa producing large biomass and underestimate more microscopic or bottom-dwelling taxa. The limited access to several aquatic ecosystems without heavy equipment also partly contributes to underestimating aquatic plant diversity. Aquatic life also impacts the trade-off between clonal and sexual reproduction, favoring plant clonality and limiting flowering (Silvertown, 2008). Many large genera of aquatic plants are also prone to hybridisation (e.g., *Potamogeton*, Kaplan & Fehrer, 2013); *Callitriche*, (Prančl et al., 2014); and *Ranunculus*, (Zalewska-Gałosz et al., 2015), a challenging bias for morphological determination.

Detection of species and communities with environmental DNA (eDNA) approaches is an option to overcome the drawbacks of traditional inventory methods. This was initially used in the early 2000s to study uncultured environmental bacteria communities. Almost a decade after the seminal eDNA-based analysis of ancient flora and vertebrates by Willerslev et al. (2003), the approach was applied to detect macroorganisms for an array of environmental substrates (Taberlet et al., 2018). Two different methodologies exist to study all levels of ecosystem organization: (i) eDNA barcoding is taxa-specific and uses PCR with specific primer pairs for species identification and (ii) eDNA metabarcoding identifies several species at once or entire communities relying on HTS. Early applications of eDNA approaches to the animal kingdom were favored by the identification of a main universal barcode: the COI region (Hebert et al., 2003). As such, applications of eDNA for animal monitoring are supported by a large corpus of literature and span numerous ecosystems.

The use of meta- and barcoding approaches is much less common for studying contemporary plants, and efforts are still dispersed. A reason for this could be the lack of a single universal plant barcode. Instead, combinations of several loci, dispersed mainly in the chloroplast genome (plastome) or nuclear ribosomal DNA (nrDNA), have been reported to enhance taxa discrimination, especially for metabarcoding. The recurrent search for convenient primers-barcodes increases laboratory work time and costs, unlike the use of a single universal barcode. Plant barcodes are also arguably harder to amplify than animal barcodes due to paralogy and hybridisation (Fazekas et al., 2009). Nevertheless, over the last decade, plant species detection from DNA fragments in environmental samples provided new perspectives for species occurrence and identification. Molecular identification has already been shown to be more effective for taxa with challenging morphological identification (Hollingsworth et al., 2016). It detects cryptic species across vast spatial scales, and it offers accessibility and standardization of samples for sites where visibility or accessibility are difficult (Beng & Corlett, 2020, and references therein). eDNA has been reported to complement traditional species survey methods, sometimes outperforming these on the number of detected species (e.g., Valentini et al., 2016). Moreover, the method is non-intrusive, offers fast presence/absence data, and does not necessarily require prior taxonomic knowledge making it accessible to many organizations.

Although efforts to consolidate eDNA surveys for plants are still ongoing, some consensus does appear in the literature. An extensive collection of reviews has treated the overall eDNA landscape, methodologies, and applications, for example, ancient plant eDNA (Gugerli et al., 2005; Parducci et al., 2017), eDNA in ecology and conservation (Beng & Corlett, 2020; Cordier et al., 2021; Deiner et al., 2021; Rees et al., 2014; Thomsen & Willerslev, 2015; Yoccoz, 2012), the metabarcoding approach (Deiner et al., 2017; Taberlet et al., 2012), ancient and modern eDNA (Pedersen et al., 2015), pollen DNA barcoding (Bell et al., 2016), and characteristics of aquatic eDNA (Mauvisseau et al., 2022). A detailed and comprehensive overview of eDNA is lacking for some taxonomic groups and ecosystems (Harper et al., 2019). Regarding plant eDNA, we welcomed the review detailing plant-animal interactions (Banerjee, Stewart, Antognazza, et al., 2022), and the broad overview outlining methods and applications (Banerjee, Stewart, Dey, et al., 2022). In this review, we examine plant biodiversity monitoring in freshwater ecosystems with eDNA barcoding and metabarcoding. Our goal is to highlight the potential utility of eDNA for monitoring freshwater plants by identifying and clarifying the successes and drawbacks of this method. We aim to encourage (molecular) ecologists and botanists to contribute to this field and accelerate the adoption of freshwater plant eDNA monitoring by practitioners. In this pursuit, our focus is on (i) providing background knowledge on the characteristics of freshwater

plant eDNA and presenting evidence that water eDNA from aquatic plants is recoverable from a variety of freshwater ecosystems.; (ii) discussing the advantages and limitations of current applications of eDNA surveys implementing barcoding and metabarcoding, as well as highlighting research priorities; (iii) presenting an approach-based (i.e., barcoding and metabarcoding) selection of the molecular tools (primers and barcodes) most adapted for a given study; and (iv) and discussing the current state of affairs and use of DNA BRDs, while encouraging fellow (molecular) ecologists and managers to create more regional and local flora barcode databases.

We reviewed the literature obtained from a term search in the Web of Science (WoS) Core database using the following search string: AB = (“eDNA” OR (“environmental-DNA”) OR barcoding OR metabarcoding NEAR/8) (“plant” OR “plants”) NOT (“ancient-DNA” OR “aDNA”). The list of articles was manually filtered to retain those exploring contemporary vegetation in freshwater biomes (rivers, lakes, wetlands, etc.) and was completed using cited references and a search on Google Scholar and PubMed with the same terms.

2 | IS THERE ANY PLANT EDNA IN FRESHWATERS?

2.1 | Studies have analyzed water plant eDNA

There is an overwhelming body of research on animal eDNA in freshwater, and it is widely accepted that DNA shed by animals can be used for eDNA barcoding and metabarcoding. The paucity of similar studies on plants raises the question of whether they shed DNA, and if so, how much, how long it remains in the environment, and whether it can be reliably used for eDNA meta- and barcoding. Scriver et al. (2015) were the first to test the barcoding of aquatic plant species from water eDNA. Under controlled conditions, they tested whether DNA from plants could be retrieved (extracted and amplified) in water samples from aquaria with the following experimental setup: 10 g of leaf material bathed in 1 L of water for 24 h before being removed prior to filtration of eDNA. Not only did they show that plant DNA quickly accumulates but also that barcode regions could be sequenced and assigned to target species using a combination of three plastid markers. It should be noted that their study design is similar to reference studies on animal eDNA (Dejean et al., 2011; Ficetola et al., 2008; Lodge et al., 2012; Minamoto et al., 2012; Thomsen et al., 2012).

These encouraging results in controlled mesocosms were then partly duplicated and tested in the field by Fujiwara et al. (2016) and Matsushashi et al. (2016). They conducted a series of experiments both in aquaria and in freshwater ponds to quantify eDNA concentration over time in lotic environments. For the first time, both studies detected aquatic plant eDNA in the field in variable concentrations: from 12 to over 30,000 copies/L, values being estimated with real-time polymerase chain reaction (qPCR). Fujiwara et al. (2016) and Matsushashi et al. (2016) found that eDNA could be detected as little as 24 h after living plants were introduced in

aquaria. Fujiwara et al. (2016) used 4 g of living plants in approximately 1 L, and Matsushashi et al. (2016) used 1-day-old 4 cm cuttings in 2 L of water. The concentration of eDNA reached its peak within one to 3 days but quickly faded after the removal of plant tissue. Aquaria with a single cutting of 1 cm had undetectable levels of eDNA in as little as 24 h (Matsushashi et al., 2016). On the flip side, both studies also detected eDNA traces in aquaria with qPCR for up to 1 week after plants were introduced. This detection boundary may even be a lower estimation since eDNA has already been consistently detected for up to 2 weeks (Gantz et al., 2018). In this study, the experimental setup consisted in taking water samples every second day for 2 weeks, the first week in planted aquaria and the second week after the removal of plants. In line with previous aquarium experiments, Gantz et al. (2018) proved that plant and animal eDNA degrade or accumulate similarly in water, anywhere from days to a week for degradation, and only 1–2 days for accumulation, supporting previous research (Fujiwara et al., 2016; Matsushashi et al., 2016; Thomsen & Willerslev, 2015). Interestingly, subjecting plants to herbivory showed a faster eDNA degradation, up to half the time. On the contrary, eDNA from whole plants could be detected at trace levels weeks after removal (Gantz et al., 2018). Confident from these early studies, an acceleration in the number of publications was observed and more notably in the number of laboratories investigating macrophyte eDNA since 2018. These results in controlled conditions must be carefully transposed to ecosystems, since numerous environmental factors are known to interfere with eDNA in polarized ways, accelerating degradation or enhancing its accumulation (Barnes et al., 2014; Dejean et al., 2011; Saito & Doi, 2021). Gantz et al. (2018) demonstrated that water plant eDNA could be detected in rivers and lakes in concentrations ranging from 0.7 copies/L to 60,000 copies/L. They further showed that qPCR on eDNA could detect the target species at concentrations above 0.7 copies/L. Although eDNA metabarcoding studies have tried to investigate the amount of captured eDNA in relation to species cover and dominance, establishing a direct correlation based on the number of reads has not always been conclusive. However, the fact that such studies have recovered eDNA from both dominant and scarce species in different waterbodies is congruent with eDNA barcoding results in that plant eDNA is indeed readily available.

In a nutshell, “Yes,” there is plant DNA in environmental matrices like river and pond waters. Environmental DNA from freshwater plants can serve species occurrence studies to accurately detect plant species and estimate abundances (barcoding) and describe communities (metabarcoding), as described in section 3.

2.2 | Capturing plant eDNA across different freshwater bodies

Our understanding of how eDNA behaves in lotic and lentic environments has started to settle and some consensus on sampling methods is emerging from the literature, mainly from animal eDNA studies. However, they are not fully applicable to plants, which

present significant structural and developmental differences (e.g., cell walls). In particular, untangling the interactions between eDNA concentration, seasonality and plant phenology is crucial for developing and standardizing eDNA sampling and capture protocols as these are known to affect DNA yields and the number of sequences (Deiner et al., 2015).

Detection of eDNA varies through the life cycle of the studied macrophytes. The work of Matsushashi et al. (2019) recommended sampling water eDNA in ponds during the growing season, but they did not sample during senescence. Kuehne et al. (2020) studied eDNA concentrations of *Egeria densa* in situ in lakes and ex-situ in a 10-week mesocosm experiment. For both setups, the authors reported a higher release of eDNA during the senescence season than during the growing season. Based on this result, Kuehne et al. (2020) recommended sampling during senescence periods to improve detection. These results are also true in lotic systems where Anglès d'Auriac et al. (2019) studied seasonal changes in the eDNA concentration of invasive aquatic plants in Norwegian rivers. In large river systems, Kodama et al. (2021) found that the release of eDNA progressively increases through the macrophytes' life cycle, reaching its maximum at senescence. Overall, phenology strongly influences macrophyte eDNA concentration, favoring protocols that sample during senescence periods.

Another contributing factor to eDNA capture is the distribution pattern of eDNA in different water bodies. Anglès d'Auriac et al. (2019) investigated the dispersal distance of eDNA in lotic and lentic water bodies (Figure 1). They found that the rate of

disappearance of eDNA was reduced by an order of magnitude over 230m in the lake, against over 1000m in the stream. They noted the limit of detection as close to 0.1 fg/mL at 1400m from the lake outlet, that is, the source of eDNA. In comparison, the eDNA concentration was two orders of magnitude higher (c. 10fg/mL) in the lake. Similarly, Doi et al. (2021) detected target eDNA up to 1600m downstream of the plant populations. However, the sampling design lacked points past 1600m away from the source of DNA. The authors also hypothesized that drifting stems and leaves may contribute to the homogenization of eDNA in rivers and mask the relation between eDNA concentration and macrophyte cover. The effect was most noted during the senescence season of the studied plant, that is, when most tissue is released. Miyazono et al. (2021) showed a correlation between the flux of eDNA and that of drifting plant tissue, thus supporting the observation of Doi et al. (2021, Figure 1). In addition, the relationship was stronger in December compared with August. Homogenization of eDNA not only occurs along rivers but has also been reported through their cross-section. Kodama et al. (2021) research on a large river system showed for the first time that the detection of water macrophyte eDNA did not vary with sampling location across the river section (left, middle and right bank, Figure 1). Similar results were shown on water fish eDNA by Sakata et al. (2020). However, the authors argue that further research on the spatiotemporal eDNA concentrations in lotic systems is needed, considering their vast geomorphologies and flow conditions. Additionally, rain events could modify the ratio of water eDNA and contribute to homogenization (Cannon et al., 2016). Based on

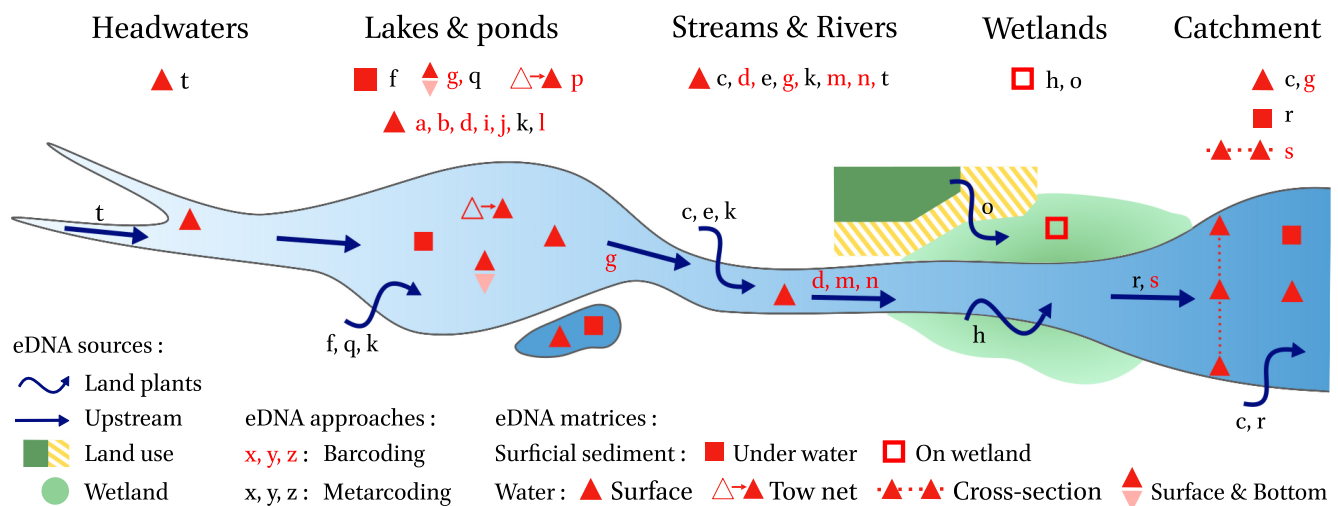


FIGURE 1 Freshwater systems studied with eDNA barcoding and metabarcoding show diverse eDNA sources and sampling methods. Sampling surface water (single solid triangle) is the preferred method. Derivatives like sampling surface and bottom water (up and down triangles) and transect sampling with a tow-net (empty to full triangle) have been tested in lakes. Sampling across the width of the river (triangles connected with a dotted line) has been tested in a large river system. Alternatively, surficial sediment samples, that is, the upper 2 cm layer of sediments, within the waterbody (solid square) or outside (empty square), on wetlands, have been used by metabarcoding studies only. Apart from autochthonous aquatic plant eDNA, two other sources have been reported: upstream eDNA (straight arrows) and terrestrial eDNA (curvy arrows). The supplementary Table S2 contains all the information in the figure and additional details on the sampling methods. Letters correspond to the following studies: a. Matsushashi et al. (2016); b. Fujiwara et al. (2016); c. Cannon et al. (2016); d. Gantz et al. (2018); e. Kuzmina et al. (2018); f. Alsos et al. (2018); g. Anglès d'Auriac et al. (2019); h. Shackleton et al. (2019); i. Matsushashi et al. (2019); j. Chase et al. (2020); k. Coghlan et al. (2021); l. Kuehne et al. (2020); m. Miyazono et al. (2021); n. Doi et al. (2021); o. Adame and Reef (2020); p. Schabacker et al. (2020); q. Drummond et al. (2021); r. Ji et al. (2021); s. Kodama et al. (2021); t. Tsukamoto et al. (2021).

these observations, the sampling protocol may be adapted to fit field requirements. For instance, the number of replicates taken at a site has a strong effect on the ability to detect a target species (Erickson et al., 2019). Sampling across the width of a river is recommended for sedimentary eDNA (Sakata et al., 2020), but for water eDNA, repeated samples from the middle of a river may be sufficient (Bedwell & Goldberg, 2020; Doi et al., 2021; Kodama et al., 2021). On a catchment scale, the ecology of the target taxa and the sampling efforts (number of samples) are important for defining the spatial distribution of samples (Carraro et al., 2021). In modeling the distribution of eDNA in a catchment, Carraro et al. (2021) recommended that sampling sites should preferably be located in downstream sections when taxa are clustered in hotspots and if sampling effort is limited. On the contrary, the authors recommend sampling upstream when taxa are more evenly distributed and if many sites can be sampled (large effort). Samples along a river are also important to highlight the distribution of target taxa. However, depending on the distance between sites, spatial autocorrelation can occur due to the downstream transport of plant debris, a source of eDNA observed by Doi et al. (2021) and Miyazono et al. (2021).

To maximize the chances of capturing species diversity with eDNA, sampling equipment can be designed that enhances the volume of water being filtered while reducing the undesired clogging of filters, specifically in lentic systems. Schabacker et al. (2020) have tested the use of a custom tow net (64 μm pore size) for sampling eDNA directly in the lake water column (Figure 1). They have shown increased capture of eDNA compared with standard filters (45 μm pore size) without affecting the success of PCR. In filtering a larger water volume (3–7.10³ L), this alternative method seems to capture more eDNA without increasing the concentration of inhibitors, a bias often shown on standard filters (Hata et al., 2011; McDevitt et al., 2007; Sepulveda et al., 2019). In lotic systems, no special equipment has been designed, aside from the different available pump systems, filters, and passive samplers (Bessey et al., 2021; Chen et al., 2022; Verdier et al., 2022). On the latter, sampling biofilm could be an alternative method, but such applications are currently in development (Rivera et al., 2021, Figure 2).

To capture contemporary macrophyte eDNA, the preferred sampling method consists of taking a single surface water sample, versus bottom or transect samples. The total volume of filtered water per sampling site was 2 L on average, ranging from 50 mL to 5 L (exceptionally 7100 L). Some studies successfully used the upper layer of sediments from lakes (Alsos et al., 2018), rivers (Ji et al., 2021) and floodplain wetlands (Adame & Reef, 2020; Shackleton et al., 2019, Figure 1). When replicates are taken, they may carry three distinct purposes: (i) spatial replicates, taken at different locations of a site to obtain a broader screening and avoid potential biases from the patchy distribution of eDNA (Chambert et al., 2018; Eichmiller et al., 2014); (ii) technical replicates, taken at the same sampling spot and treated independently in the lab to test the repeatability of the workflow; and (iii) repeated samples, taken at the same location and pooled together during lab work to increase the yield of eDNA and avoid false negative errors, that is, composite sample, but not strict

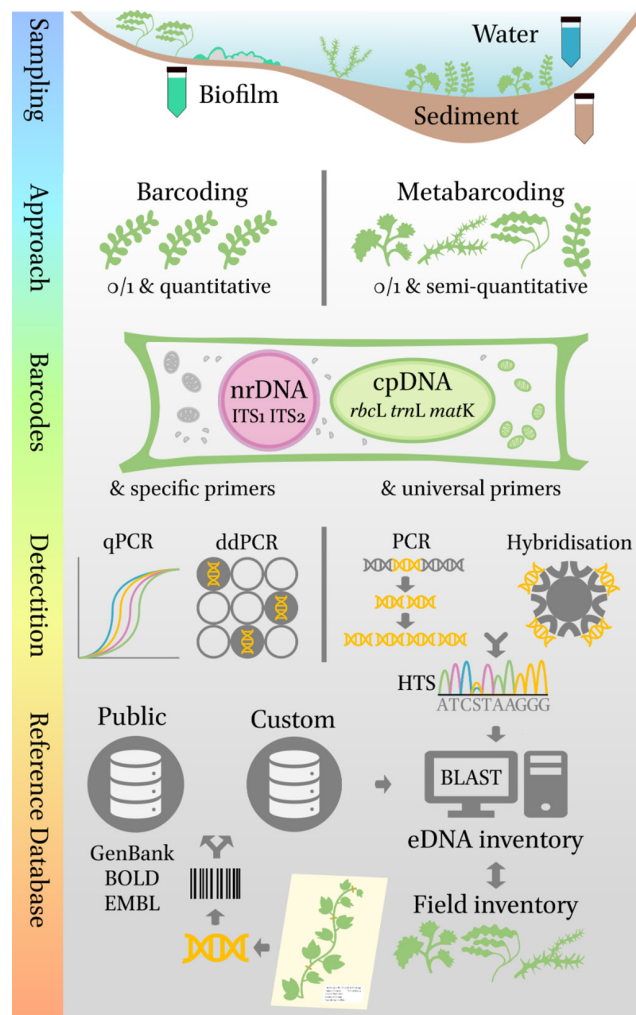


FIGURE 2 An overview of the environmental DNA barcoding and metabarcoding workflow shows the different choices associated with studying freshwater plant eDNA. The research question defines the sampling method (section 2 and Figure 1), and the eDNA approach (section 3). The selection of appropriate barcodes from the plastid or the nuclear DNA is common to both eDNA barcoding and metabarcoding approaches (section 4), but the primers used are approach-specific (Table 1 and Table 2). Similarly, the technologies used for species detection differ between approaches: quantitative PCR (qPCR) or droplet digital PCR (ddPCR) for barcoding, and conventional PCR or hybridisation capture followed by high-throughput sequencing (HTS) or shotgun sequencing for metabarcoding (section 3). Finally, for metabarcoding, taxonomic assignment of eDNA sequences could rely on public barcode reference databases (BRDs) or a much-preferred custom database from DNA extractions of plant specimens (section 5 and Table 3).

replicates (Alsos et al., 2018). However, if replicates are pooled together after eDNA extraction, replicability and semi-quantitative analysis cannot be done, that is, variation between replicates cannot be studied (Capo et al., 2021). In their study on eDNA detection of invasive macrophytes in lakes, Kuehne et al. (2020) demonstrated the significant variability of the amount of captured eDNA between technical replicates.

TABLE 1 Invasive macrophytes with an eDNA barcoding assay.

Family	Species	Barcode	Size (bp)	Study
Araceae	<i>Pistia stratiotes</i>	<i>matK</i>	374	Scriver et al. (2015)
		<i>trnH-psbA</i>	130	Scriver et al. (2015)
Cabombaceae	<i>Cabomba caroliniana</i>	<i>matK</i>	250	Scriver et al. (2015)
Haloragaceae	<i>Myriophyllum aquaticum</i>	<i>matK</i>	213	Scriver et al. (2015)
		<i>26S</i>	56	Schabacker et al. (2020)
		<i>ITS1</i>	93	Kuehne et al. (2020)
Hydrocharitaceae	<i>Elodea canadensis</i>	<i>matK</i>	150	Gantz et al. (2018)
		<i>trnL-trnF</i>	103	Anglès d'Auriac et al. (2019)
		<i>ITS1</i>	122	Gantz et al. (2018)
	<i>Egeria densa</i>	<i>matK</i>	232	Scriver et al. (2015)
		<i>trnL-trnF</i>	99	Fujiwara et al. (2016)
		<i>ITS1</i>	79	Chase et al. (2020)
	<i>Elodea nutalli</i>	<i>ITS1</i>	122	Gantz et al. (2018)
	<i>Hydrilla verticillata</i>	<i>matK</i>	110	Matsushashi et al. (2016)
		<i>matK</i>	186	Gantz et al. (2018)
		<i>ITS1</i>	154	Gantz et al. (2018)
	<i>Stratiotes aloides</i>	<i>matK</i>	359	Scriver et al. (2015)
		<i>matK</i>	316	Scriver et al. (2015)
		<i>rbcl</i>	437	Scriver et al. (2015)
Lythraceae	<i>Trapa natans</i>	<i>trnH-psbA</i>	116	Scriver et al. (2015)
Menyanthaceae	<i>Nymphoides peltata</i>	<i>matK</i>	472	Scriver et al. (2015)
		<i>trnH-psbA</i>	164	Scriver et al. (2015)
Pontederiaceae	<i>Eichhornia crassipes</i>	<i>matK</i>	344	Scriver et al. (2015)
		<i>trnH-psbA</i>	104	Scriver et al. (2015)
Salviniaceae	<i>Salvinia molesta</i>	<i>matK</i>	792	Scriver et al. (2015)
		<i>trnH-psbA</i>	285	Scriver et al. (2015)

Note: A detailed table with the original publication and primers can be found in [Table S1](#).

Overall, the most studied environmental factors influencing sampling design are seasonality and plant phenology. In light of recent findings, we suggest that eDNA projects compare samples taken during the same season (excluding phenological studies), preferentially during the peak season of plant senescence. Freshwater plant meta- and barcoding studies should focus on the following aspects to increase the chances of capturing all representatives of the community: (i) repeat both biological (spatial) and technical replicates; (ii) consider prior knowledge of the ecology of the target taxa. The detection of eDNA from a river segment—up to 1600 m from the source of eDNA—rather than a point location is not a limitation of the method, but rather a more field-efficient approach to traditional transect surveys (Deiner et al., 2016). Instead of starting an eDNA design from a blank canvas, we hope this overview will be helpful, in addition to other guidelines in Minamoto et al. (2021) manual for

eDNA research, Taberlet et al. (2018) book on environmental DNA, and Bruce et al. (2021).

3 | APPLICATIONS OF EDNA FOR MONITORING FRESHWATER PLANTS: BARCODING AND METABARCODING APPROACHES

3.1 | Tracking species through eDNA barcoding

The seminal research of Scriver et al. (2015) used water eDNA to test the detection of invasive macrophytes. They set the premises for good practice in this field, discussed in section 5, and gave undoubted proof that the benefits and challenges awaiting eDNA

TABLE 2 The markers used in eDNA meta- and barcoding studies of freshwater vegetation.

Studies	Plastid DNA						Nuclear ribosomal DNA			
	<i>rbcL</i>	<i>matK</i>	<i>trnL</i>	<i>trnL-trnF</i>	<i>trnH-psbA</i>	<i>atpB-rbcL</i>	ITS1	ITS2	18S	26S
Barcoding										
Scriver et al. (2015)										
Fujiwara et al. (2016)										
Matsushashi et al. (2016)										
Gantz et al. (2018)										
Anglès d'Auriac et al. (2019)										
Matsushashi et al. (2019)										
Chase et al. (2020)										
Doi et al. (2021)										
Kuehne et al. (2020)										
Miyazono et al. (2021)										
Schabacker et al. (2020)										
Kodama et al. (2021)										
Metabarcoding										
Cannon et al. (2016)			a		b					
Alsos et al. (2018)			a							
Kuzmina et al. (2018)										
Shackleton et al. (2019)			a							
Adame and Reef (2020) ^a										
Coghlan et al. (2021)										
Drummond et al. (2021)										
Ji et al. (2021)										
Jia et al. (2021)			a							
Tsukamoto et al. (2021)			a							

Note: A multimer approach is common in metabarcoding projects to enhance taxonomic assignment, contrary to eDNA barcoding where taxa-specific primers will define the detection of the target. Foster et al. (2021) do not appear in the table for clarity because the authors used 20 different barcodes with hybridisation capture. Letters a-d correspond to the primers used for amplifying eDNA (a) g-h Taberlet et al. (2007); (b) bryo P6 Epp et al. (2012); (c) All18S Hardy et al. (2010); and (d) *rbclF-R* Levin et al. (2003) and CBOL Plant Working Group et al. (2009).^aWe could not get the primers used in this study.

monitoring of invasive macrophytes and animals are much alike. A key concern for macrophyte monitoring with eDNA barcoding is validating the occurrence data with field surveys or records. Studies comparing eDNA and conventional approaches showed that eDNA can be complementary, if not better, for most freshwater bodies (Figure 1): ponds (Fujiwara et al., 2016; Matsuhashi et al., 2016; Matsuhashi et al., 2019), lakes (Anglès d'Auriac et al., 2019; Chase et al., 2020; Gantz et al., 2018; Kuehne et al., 2020), streams (Anglès d'Auriac et al., 2019; Doi et al., 2021; Gantz et al., 2018; Miyazono et al., 2021), and more recently, large rivers (Kodama et al., 2021). All studies consistently detected the target taxa where known populations existed. Some studies also detected previously unreported populations, either not observed at the time of sampling or lacking in records (Matsuhashi et al., 2016; Miyazono et al., 2021).

The detection of invasive alien species is the main subject of all 12 eDNA barcoding studies. The relevance of this application in rivers, lakes, and watersheds has been demonstrated by several groups in Canada, Japan and Europe (Anglès d'Auriac et al., 2019; Chase et al., 2020; Doi et al., 2021; Fujiwara et al., 2016; Gantz et al., 2018; Kuehne et al., 2020; Matsuhashi et al., 2016). For now, only hydrophytes (strictly aquatic taxa) have been surveyed with eDNA—14 worldwide invasive species have at least one eDNA barcoding assay (Table 1). For example, the assay for *E. densa* from Fujiwara et al. (2016) was adopted by fellow researchers in Japan (Doi et al., 2021; Matsuhashi et al., 2016; Miyazono et al., 2021), yet it remains to be tested outside of Japan where a different regional flora (with other related taxa) may modify its specificity. The proven applicability of eDNA assays for hydrophytes should encourage its development for invasive helophytes (semiaquatic or amphibious plants) like *Crassula helmsii* and *Hydrocotyle ranunculoides*, and terrestrial plants known to use riverbanks as a means of propagation (e.g., in Europe *Impatiens glandulifera* and *Reynoutria japonica*).

Plant eDNA barcoding not only gives presence data but has the potential to estimate biomass, and cover from eDNA concentration measurements (e.g., eDNA copies/L). This quantitative data have been correlated to plant density in aquarium experiments, but in situ, the breakdown of eDNA, dilution and adsorption, and range detection complicate eDNA-based abundance estimates (Anglès d'Auriac et al., 2019; Doi et al., 2017; Takahara et al., 2012). Matsuhashi et al. (2016) established a correlation between eDNA concentration (DNA copies/L) and macrophyte biomass in an aquarium experiment where different taxa were grown together (community setup) or alone and at different densities. The authors confirmed the ex-situ results by sampling eDNA from ponds with different plant biomass. Using the same sampling protocol for all ponds, those with higher plant biomass yielded higher eDNA concentrations. Additionally, the aquarium experiments showed that the correlation could be species-specific. The authors established a positive correlation for *E. densa* but no correlation for *Hydrilla verticillata*. Kuehne et al. (2020) reported that neither detection nor eDNA concentrations were good predictors of species abundance for *E. densa* and *Myriophyllum spicatum* in either the mesocosm experiments or field samples. In a mesocosm experiment, Gantz et al. (2018) found that, for the two

Elodea canadensis assays (ITS and *matK* barcode assays), density and the interaction variable time x density did not significantly explain eDNA copy numbers during the accumulation phase (first 6 days after the introduction of plants in aquaria). During the degradation phase, after the removal of plants, the authors found significant differences in the degradation rate of eDNA between high-density and low-density aquaria. This result cannot easily be transposed in situ, where numerous environmental factors are at play. Water temperature and pH were shown to influence eDNA concentration in ponds with *H. verticillata* (Matsuhashi et al., 2019). Phenology strongly influences eDNA concentration (section 2) and may locally cloud correlation with macrophyte cover and biomass during senescence in lotic systems (Doi et al., 2021; Kodama et al., 2021). Doi et al. (2021) suggested that “eDNA concentration could reflect the broader-scale biomass.” On the contrary, Miyazono et al. (2021) provided evidence of a positive correlation between the relative abundance and eDNA flux (eDNA copies/s) across seasons in the studied river. For managers to adopt quantitative eDNA barcoding assays for macrophyte monitoring, further research into its potential for estimating species abundance is required. To achieve accurate estimations of macrophyte biomass and cover, more quantitative eDNA data are required across different water bodies and seasons. We suggest that knowledge of plant–animal interactions (e.g., plant palatability) could be considered as it has been shown that predated plants release more eDNA (section 2 of this review and Gantz et al., 2018).

Only a few studies have used eDNA barcoding to detect rare or endangered macrophytes. The work from Matsuhashi et al. (2016) included an assay for *H. verticillata*, a native hydrophyte species in Japan, identified as threatened of extinction in some areas of the country. The authors detected eDNA from the target species in every pond where it was observed, and in a few ponds without sightings but with historic occurrence. Additionally, *H. verticillata* eDNA was detected in a pond where the species was never reported before. In the context of biodiversity erosion, eDNA barcoding assays are a promising monitoring tool for endangered species. Furthermore, adopting more sensitive technologies like droplet digital PCR (ddPCR) should increase the detection of rare taxa and species leaching small quantities of DNA. Alternatively, when considering more than one target species, and if quantification is not required, eDNA metabarcoding is a good solution (see subsection 3.2). Given that we cannot protect a species if we do not know where it is, eDNA assays could provide initial cost-effective distribution data crucial to enhance conservation plans.

To resume, eDNA barcoding is in the process of becoming a standard tool for surveying invasive macrophytes. The robustness of existing assays should be tested outside of the geographic region they were designed for. Expanding the application to more terrestrial plants should also be the focus of future work since these can be detected in water samples discussed in subsection 3.2. Finally, the conservation of threatened species would profit greatly from having species-specific assays which are surprisingly lacking. The sensitivity of qPCR used in all eDNA barcoding studies is undeniable, but we argue that adopting ddPCR could further increase the detection of

TABLE 3 Projects barcoding the flora of defined geographic regions. The barcodes generated by these projects come from amplifying and sequencing DNA extractions of identified plant specimens (field or herbarium).

Studies	Species	Vegetation	Study region	Markers
Lahaye et al. (2008) ^a	1100	Tropical and Temperate	Mesoamerica and S-A	<i>matK</i> , <i>trnH-psbA</i>
Kress et al. (2009)	296	Tropical	BCI, Panama ^b	<i>rbcL</i> , <i>matK</i> , <i>trnH-psbA</i>
Sønstebo et al. (2010)	856	Arctic	Oslo, Norway	<i>trnL</i>
Kuzmina et al. (2012)	312	Arctic	Churchill, Canada	<i>rbcL</i> , <i>matK</i> , ITS2
de Vere et al. (2012)	1143	Temperate	Wales (UK)	<i>rbcL</i> , <i>matK</i>
Saarela et al. (2013)	490	Arctic	Arctic Canada	<i>rbcL</i> , <i>matK</i>
Willerslev et al. (2014) ^c	842	Arctic	Oslo, Norway	ITS1
Kuzmina et al. (2017)	5076	Temperate to Arctic	Canada	<i>rbcL</i> , <i>matK</i> , ITS2
Li et al. (2018)	155	Tropical	Xisha Islands, China	<i>rbcL</i> , <i>matK</i> , ITS
Hernández-León et al. (2018)	25	Conifers	Hidalgo, Mexico	<i>rbcL</i> , <i>matK</i>
Bringloe et al. (2019)	134	Macroalgae	Breme, Norway	<i>rbcL</i> , COI-5P, <i>tuf A</i>
Gill et al. (2019)	460	Semi-arid savanna	MRC, Kenya ^d	<i>rbcL</i> , <i>matK</i> , <i>trnH-psbA</i> , <i>trnL-trnF</i> , ITS
Nitta et al. (2020)	176	Cloud forest Pteridophytes	Costa Rica	<i>rbcL</i>
Alsos et al. (2020)	5575	Arctic and Alpine	Circum-Arctic, Alps & Carpathians	cpDNA and nrDNA
Jones et al. (2021)	1437	Temperate	United Kingdom	<i>rbcL</i> , <i>matK</i> , ITS2
Garcés-Pastor et al. (2022) ^e	4437	Alpine	Alps & Carpathians	P6 loop <i>trnL</i> intron
Alsos et al. (2022) ^e	1899	Arctic	Norway	P6 loop <i>trnL</i> intron

Note: These barcode databases contain long barcode sequences (>900bp) aiming at providing reference data for different applications, including for eDNA studies.

^aThe proportion of sampled species versus those queried from public databases within this study is unclear and the number of species reported in the table comes from our analysis of appendix 10 Lahaye et al. (2008). We conclude that 232 species of orchids and 38 species of vascular plants were sampled in Mesoamerica and South Africa (S-A). The markers shown are the ones the authors recommend from a list of tested markers.

^bBarro Colorado Island (BCI), Smithsonian Research Center, Panama.

^cThe authors extended the database from Sønstebo et al. (2010) with ITS1 sequences.

^dMpala Research Center (MRC) and surrounding areas in Laikipia, Kenya. The number of barcoded species corresponds to 92% of all known species from the park.

^eThe authors extracted the P6 loop of the *trnL* intron from the previously created genome skimming database (Alsos et al., 2020).

early invasions or cryptic species, and potentially detect plants at different life stages (e.g., fern gametophytes).

3.2 | eDNA inventories of freshwater plant communities: The metabarcoding approach

Contrary to the abundant research using eDNA metabarcoding for surveying aquatic fauna, the method has seldom been used for contemporary freshwater plant communities, although it was already adopted in paleoecology as a complementary tool (Taberlet et al., 2018, Chapter 15; Thomsen & Willerslev, 2015, and references therein). Up to 2022, only 10 studies using eDNA metabarcoding to explore contemporary vegetation had been published, the first in 2016 (Cannon et al., 2016). These studies show how complementary the approach is to traditional monitoring methods.

Six studies use water eDNA to monitor macrophyte communities (Figure 1, Cannon et al., 2016; Coghlan et al., 2021; Drummond et al., 2021; Foster et al., 2021; Ji et al., 2021; Kuzmina et al., 2018).

Cannon et al. (2016) seminal research assessing the biodiversity of macroorganisms (fauna and flora) from the Cuyahoga River (USA) (137 km long river) was an unprecedented effort. The originality comes from using the same water samples (40 mL, $n = 91$) to create 12 libraries for the different taxonomic groups. Overall, their method showed that a preliminary evaluation of the global biodiversity in a river catchment is possible and cost-effective. In Europe, the only comparable large-scale water eDNA biomonitoring study was done by the Joint Danube Survey, an international monitoring effort grouping all countries through which the Danube flows. Their fourth monitoring campaign from 2019 to 2020 included eDNA monitoring of fish, macroinvertebrates, and diatoms, but not macrophytes, monitored with conventional methods. Arguably, there was insufficient evidence for macrophyte metabarcoding at the time considering that only two studies (Cannon et al., 2016; Kuzmina et al., 2018) had been published before. These studies demonstrated that eDNA was an appropriate strategy for monitoring river biodiversity, and notably underestimated hydrophytes, for example, rare pondweeds (Kuzmina et al., 2018) and early invasives (Coghlan et al., 2021).

Building on the promising results of previous research, Ji et al. (2021) conducted a contemporary plant surficial sediment eDNA metabarcoding study at the scale of the Chaobai River watershed in China sampling 17 sites from headwaters to floodplain waters. They showed complementary species occurrences between conventional methods and eDNA metabarcoding, with 30 species in common, but an additional 30 and 97 taxa, respectively, were also found by each method. Rare and cryptic species were better detected with eDNA, while certain taxa were only found visually (e.g., Rubiales, Helobiae, Liliales, Equisetales and Juncales). In the only study to use metabarcoding to survey threatened species, Tsukamoto et al. (2021) targeted the six species of Podostemaceae present in Japan and reported four species within their previously observed habitat range.

The detection of terrestrial plants (mesophytes) in freshwater eDNA was highlighted by several studies (Figure 1). A major contribution of Ji et al. (2021) work is the high detection of mesophyte in surficial sediment eDNA (48% of the total richness with eDNA). In terms of relative abundance, hydrophytes (strictly aquatic) and helophytes (amphibious) equally dominate in eDNA (45.89% and 41.39%, respectively) whereas helophytes were most reported (73.55%) in conventional surveys. Previous to this work, the fraction of eDNA from terrestrial plants in freshwater systems had received some attention: Cannon et al. (2016) detected helophytes and mesophytes in water eDNA from rivers; Coghlan et al. (2021) found eDNA of some invasive shoreline species in rivers and lakes; Shackleton et al. (2019) identified the riparian vegetation continuum along a river using wetland sediment eDNA; and Alsos et al. (2018) specifically studied eDNA representing the surrounding contemporary terrestrial vegetation in lake sediments. The authors showed a strong contribution of dominant taxa growing within 2 meters of the shore and some common species to the catchment area. However, several taxa, including dominants, were not recorded with eDNA, and in opposition, some overlooked taxa in field surveys were detected with eDNA. These partially mismatching results were also found by Ji et al. (2021) at a river catchment scale. What is more, Alsos et al. (2018) also showed that rare aquatic taxa were highly detected with eDNA, a result that contrasts with the detection of terrestrial plants which correlates to the species abundance in the vegetation. Not only can terrestrial plant eDNA be found in river and lake sediments but also within the water column, although it appears to be sporadic and does not seem to represent the entire surrounding terrestrial vegetation (Drummond et al., 2021).

Other recent applications include using flood sediment eDNA as a proxy for tracing the provenance of pollutants in floodplain wetlands (Figure 1), as an innovative approach to conventional fingerprints, for example, fallout radionuclides and organic matter properties (Adame & Reef, 2020; Evrard et al., 2019). The eDNA of the characteristic vegetation of dominant land uses can be found in the sediment of rivers as they drain the topsoil of their catchment. The spatial connectivity of the catchment and the coastal zone was demonstrated by Adame et al. (2012) and confirmed with eDNA metabarcoding by Adame and Reef (2020) (e.g., eDNA from sorghum, planted in grazing fields, was detected 15 km offshore). With the

sampling method of Evrard et al. (2019), plant sediment eDNA represented over 6 years of land use, thus acting as a memory of a catchment's transformations. An additional benefit of metabarcoding is to provide "by-catch" eDNA, referring to captured non-target eDNA (Macher et al., 2021). For example, Adame and Reef (2020) detected the occurrence of invasive plants with their metabarcoding protocol although not initially planned for.

Finally, eDNA metabarcoding can rely on two multilocus other molecular methods, shotgun sequencing and target DNA capture enrichment (hybridisation capture), to avoid the well-known qualitative and quantitative PCR biases (Nichols et al., 2018; Taberlet et al., 2018). Shotgun metabarcoding (as in Parducci et al., 2017) has the potential to retrieve all taxa within an environmental sample (ancient or contemporary eDNA) but requires high-sequencing depths and extensive reference databases for taxonomic assignment (Taberlet et al., 2012, 2018). Higher sequencing depth implies a higher cost for the experiment, which means that for the same budget, one can analyze fewer eDNA samples with shotgun sequencing than with PCR-based metabarcoding. Yet, the number of samples is not negligible and may be required to answer the ecological question (Taberlet et al., 2018). However, the cost advantage of metabarcoding degrades when considering multilocus approaches and shotgun genome skimming becomes more effective (Srivathsan et al., 2015, 2016). Differences in cost should also become less apparent in the future as sequencing cost decreases while sequencing depth increases. The main hurdle for shotgun sequencing is the gaps in reference databases that we discussed in section 4. Harbert (2018) also raised the lack of a standard bioinformatic pipeline for the taxonomic classification of short-read shotgun sequences for plants as a shortcoming of this method. Recommendations are given by the authors to use existing assembly and mapping approaches or classic local alignment search tools as we wait for more adapted bioinformatic tools. These tools should be tested for aquatic plant community reconstruction and compared with the standard HTS method using the same eDNA samples. We have not seen any study using shotgun sequencing on contemporary aquatic plant eDNA, but the method has already shown promising results for plant ancient DNA (Parducci et al., 2017, section 8 and references therein), and for other eDNA studies (e.g., Bell et al., 2021; Bovo et al., 2018; Parducci et al., 2019). Similarly, other sequencing technologies like nanopore should be competitive for eDNA studies in remote sites (Blanco et al., 2020).

The taxonomic resolution provided by the combination of different regions with shotgun sequencing could be achieved with hybridisation capture, although to a lesser extent so far. The aim is to enhance species-level detection, reliability and accuracy through the design of probes targeting barcodes of interest, but the method is still in its infancy (Foster et al., 2021; Murchie et al., 2021; Seeber et al., 2019; Taberlet et al., 2018). Similar to genome skimming, the drawback so far is the requirement of well-documented reference databases for all the regions of interest. Just as for shotgun sequencing, one can use hybridisation capture to build the required reference databases as described in section 4. Foster et al. (2021) proof of concept targeting 20 plastid regions on a 10-species mixture (mock

sample), showed that hybridisation capture detected six plants at the species level and four at the genus, or even all 10 at the species level using a restricted barcode database. The authors also applied the approach to sediment eDNA and captured 17 plastid regions, of which 11 indicated the presence of *Avicennaria marina*, also visually observed at the sampling sites. This approach could enable a more reliable and accurate eDNA vegetation monitoring, but more applications are needed to strengthen the interpretation of multigene-targeted capture results. To facilitate the use of this method, off-the-shelf sets of probes for eDNA studies are available and have been tested in eDNA studies (Cruz-Dávalos et al., 2017; Foster et al., 2021; Johnson, Pokorny, et al., 2019; Murchie et al., 2021; Peñalba et al., 2014). Probe-design software has also been developed to assist in the design of probes with either a specific targeted-enrichment approach (Anil et al., 2018; Faircloth, 2017; Johnson et al., 2016) or with an adaptable workflow (Campana, 2018; Chafin et al., 2018).

The potential of eDNA metabarcoding to study the biodiversity of plants in freshwater ecosystems is still poorly exploited. Plant metabarcoding and conventional surveys are congruent and complementary methods, and we recommend still using conventional surveys to test and improve plant eDNA metabarcoding. Floristic inventories obtained from water and sediment eDNA transcend the water boundary since they also provide occurrence data for terrestrial plants in a catchment. Applications have so far focused on biodiversity assessment, but other original research questions can be developed, such as surficial sediment fingerprinting.

4 | THE PRIMERS AND BARCODES FOR FRESHWATER PLANT EDNA

The selection of primers and DNA barcodes has become a core step in the workflow of any environmental DNA study or even a field of expertise alone (Freeland, 2017). Benefiting from the increasing abundance of sequence data, computer software and computational power, designing new primers has never been easier. Given that plants do not have a universal barcode (CBOL Plant Working Group et al., 2009; Chase et al., 2005; Coissac et al., 2016; Fazekas et al., 2008; Gostel & Kress, 2022; Hollingsworth et al., 2011; Hollingsworth et al., 2016; Kress et al., 2005; Kress et al., 2015), and sometimes weakly universal primer-binding sites (e.g., Kolter & Gemeinholzer, 2021a), the quest for primers is still ongoing and is not a straightforward path. Environmental DNA studies target short fragments of DNA (10–400 bp), thus requiring amplification of short barcodes within the predefined markers (e.g., *rbcLa* China Plant BOL Group et al., 2011 and Dong et al., 2014). For eDNA barcoding applications, primers can be designed in relatively variable regions (e.g., non-coding regions) to target a given species. For metabarcoding applications, primers must target conserved binding sites flanking interspecific variable regions to amplify a diversity of different taxa.

The design of new primers also depends on the availability of reference sequences in BRDs. The development of primers for eDNA

barcoding started earlier than for metabarcoding studies of freshwater plants. To date, 14 invasive macrophytes have tailor-made primers and eDNA assays (Table 1) tested on environmental samples. The first species-specific primer sets for macrophyte eDNA from Scriver et al. (2015) focused only on plastid barcodes. Macrophyte barcoding studies outside the scope of eDNA research are not included in this review because the long barcode regions do not directly suit eDNA studies. However, the regions of interest identified by these studies are an invaluable source for obtaining candidate primers and barcodes.

4.1 | Nuclear ribosomal primers and barcodes

Concerning the use of nuclear barcodes in eDNA (meta) barcoding, of the 22 eDNA studies, four used ITS1 and two used ITS2 (Table 2). Although recognized as a barcoding region for land plants (China Plant BOL Group et al., 2011; Hollingsworth et al., 2011; Kolter & Gemeinholzer, 2021b; Kress et al., 2005), and having well-documented primer sets, including all those cited in Kolter and Gemeinholzer (2021a), the ITS region is still seldom used in freshwater plant eDNA studies and a conservative preference for plastome DNA barcodes prevails. Conversely, other applications of eDNA for detecting plant species from different substrate types have welcomed the ITS barcode, for example, medicinal plants (Chen et al., 2010; Urumarudappa et al., 2020), illegal trading (de Boer et al., 2017), soil metabarcoding (Fahner et al., 2016; Timpano et al., 2020), plant–pollinator interactions (Bell et al., 2017; Richardson et al., 2015), honey composition (Khansaritoreh et al., 2020; Prosser & Hebert, 2017), and herbivory analysis (Moorhouse-Gann et al., 2018; Sobek & Walker, 2020). The ITS1 and ITS2 accumulate nucleotide substitutions more rapidly than coding genes (e.g., flanking nrDNA 18S), even between closely related species, hence offering better taxonomic resolution (China Plant BOL Group et al., 2011). Furthermore, ITS1 and ITS2 are considerably smaller compared with traditional plastid markers, about 241 bp and 221 bp, respectively, for angiosperms (Kolter & Gemeinholzer, 2021b). This small size (i) is more suitable for eDNA analyses since shorter DNA is more prevalent in the environment and (ii) the whole marker can be used as a barcode for taxonomic assignment (Kuzmina et al., 2018).

Only five species of invasive macrophytes have nrDNA barcoding assays on the ITS1 (Chase et al., 2020; Gantz et al., 2018; Kuehne et al., 2020), but there is currently no ITS2 species-specific primer, despite their recognized taxonomic resolution for angiosperms (Table 1). Overall, only a small set of primers are recurrently used in eDNA metabarcoding, owing to the difficulty of designing universal primers for nrDNA. Many universal ITS primers targeting land plants are modifications or complementary sequences to the most used primers from White et al. (1990) (Kolter & Gemeinholzer, 2021a). Additionally, through *in silico* and *in vitro* testing, Kolter and Gemeinholzer (2021a) propose new sets of improved primers for metabarcoding. Three of the 10 freshwater plant metabarcoding studies have designed primers for this region: Kuzmina et al. (2018)

designed Potamogetonaceae-specific ITS2 primers and Coghlan et al. (2021) and Drummond et al. (2021) designed de novo freshwater vascular plant primers for the ITS2 and the ITS1, respectively (Table 2). The authors reported better detection of target taxa with their new primers compared with the well-known primers ITS2F and ITS3R from Chen et al. (2010), or the former paired with the reverse primer ITS4 from White et al. (1990), or a modified version of this combination from Fahner et al. (2016). Drummond et al. (2021) ITS1 primer sets were designed to amplify a broad spectrum of 119 freshwater plant species from the Great Lakes (USA). The authors suggested their primers are “not intended to be widely used outside the area of [their] study,” but we found that their primers could be effectively used for a wide variety of freshwater European plants too (unpublished results).

4.2 | Plastid DNA primers and barcodes

Amplification of plastid barcodes also comes with its own set of challenges, and mixed results regarding barcode specificity are found in the literature. Commonly used markers for freshwater plant eDNA include the *rbcl* gene (mainly the *rbclA* region) and *atpB-rbcl* spacer, the *matK* gene, the *trnL* (UAA) intron and *trnL-trnF* spacer, and the *trnH-psbA* spacer (Table 2). Yet other overlooked but informative plastid markers could be considered for metabarcoding using hybridisation capture (Foster et al., 2021). This would use more of the captured eDNA and increase identification success. Besides, whole markers would be used for identification, thus avoiding any bias in barcode size and location within markers. These two biases can be observed in the *rbcl* marker where the primers from Prosser and Hebert (2017) and those used by Handy et al. (2021) and Khansaritoreh et al. (2020) amplify, respectively, 210 and 380bp in angiosperms and begin at the 5' end of the gene and 300bp away from it. Barcodes generated with the former yielded less taxonomic resolution (from our observation on European temperate plants). Regarding the *rbcl* in eDNA freshwater studies, the short barcodes on the 5' end of the gene are commonly used for metabarcoding, but only seldom for barcoding (Table 2). Coghlan et al. (2021) found that their new *rbcl* primers yielded more informative sequences, outperforming their new *matK* and ITS primers (see Table 1 for details on primers). Additionally, the taxonomic resolution was also better than for the metabarcodes amplified with the *rbcl* primers from Fahner et al. (2016). The latter used established primers, the *rbclA-F* from Levin et al. (2003) and the *rbclA-R* from Kress and Erickson (2007). This combination was also used by Ji et al. (2021) for metabarcoding freshwater vegetation. The authors detected an outstanding 127 aquatic plant species with the *rbcl* metabarcode alone, from a total of 17 surficial sediment eDNA samples along the 470km Chaobai River (China). That is double the number of species the authors detected with a traditional survey method in a paralleled field survey, of which 30 were detected by both methods. We argue that additional metabarcodes, especially the ITS nrDNA, could have resolved some genus to species level, especially for taxa lacking

resolution on the *rbcl* (e.g., *Rubia*). Hence, more species could have been detected with eDNA, including some or all of the 30 species only detected with conventional methods. On the contrary, the use of *rbcl* in eDNA barcoding projects is rare. Only Scriver et al. (2015) designed species-specific primers for 10 species but reported successful specific amplification of eDNA for only one target species, *Stratiotes aloides* (Table 1).

A different picture appears for *matK* barcodes which are seldom used for freshwater plant eDNA metabarcoding but readily used in eDNA barcoding. The best primers for standard specimen barcoding yield barcodes that discriminate most species but are too long for eDNA applications (see subsection 5.1 below; and Lahaye et al., 2008). The lack of satisfactory eDNA barcodes and universal primers makes it impractical for metabarcoding and difficult samples (Arulandhu et al., 2017; Fahner et al., 2016). In addition, Fahner et al. (2016) found inconsistent amplification and poor sequence quality. Instead, an alternative use of *matK* could consist in using family-specific primers to increase taxonomic resolution (Yang et al., 2016). Only Coghlan et al. (2021) used *matK* with their newly designed primer pairs for metabarcoding freshwater plants (Table 2). However, *matK* species-specific barcodes offer good discrimination against close relatives and the region is the most common marker for eDNA barcoding: 10 out of 14 invasive macrophytes have *matK* eDNA barcoding assays Table 1, eight of which were designed by Scriver et al. (2015), and two by Coghlan et al. (2021) (Table 1).

Similarly, the *trnH-psbA* intergenic spacer has not yet been used for metabarcoding, but eDNA barcoding assays have been developed for five invasive species (Scriver et al., 2015). The assays were shown to specifically amplify target species, but have not been reused since their publication. Kolter and Gemeinholzer (2021b) reported that reference databases are not yet well documented for this marker, which explains why it has not been considered for metabarcoding. However, conventional barcoding studies have shown that this marker contains considerable genetic divergence at intra- and interspecific levels, thus offering a competitive taxonomic resolution (Kress et al., 2009) including for Bryophytes (Liu et al., 2010). The *trnH-psbA* also shows complementary results to other markers for conventional barcoding (Fazekas et al., 2008; Kress et al., 2009; Parmentier et al., 2013). However, the greater intraspecific variability compared with other plastid markers may hinder species-level determination (Kress et al., 2009; Parmentier et al., 2013), which could be exacerbated by gaps in reference databases for metabarcoding. For complex taxonomic samples, more than one primer pair could be required to amplify all the diversity of taxa (García-Robledo et al., 2013; Meiklejohn et al., 2019). Overall, metabarcoding studies could consider using this marker, owing to its important interspecific divergence, but further testing of existing universal primers is necessary.

The small size of eDNA fragments also influences the choice of primers and barcodes, which becomes apparent for the *trnL* marker. On the one hand, eDNA barcoding is often based on the more

variable spacer *trnL-trnF*, keeping a small amplicon size (c. 100bp Table 1). The primers designed within this spacer by Fujiwara et al. (2016) for *E. densa* were used in three other eDNA barcoding applications (Doi et al., 2021; Matsuhashi et al., 2016; Miyazono et al., 2021) (Table 1). The *trnL-trnF* spacer was also described by Kolter and Gemeinholzer (2021b) as underestimated and with potential for barcoding compared with other plastid markers, based on species discrimination. Despite this, the authors found that databases contained the fewest sequences for this marker. On the other hand, all metabarcoding studies chose Taberlet et al. (2007) primers g and h amplifying the *trnL* (UAA) P6-loop (from 10 to 160bp amplicons). This primer pair is one of the most used for metabarcoding across all environmental substrates, particularly for ancient DNA (aDNA), and soil and sedimentary eDNA. However, this *trnL* metabarcode is known to provide minimal species resolution compared with other plastid and nuclear ribosomal metabarcodes. Shackleton et al. (2019) compared conventional with eDNA surveys and tested the complementarity of two metabarcodes, the P6 loop of the *trnL* intron and the 18S RNA gene. The authors offer a threefold explanation for the poor species correlation between the two methods: (i) when geographically restricted reference databases are not used, the short *trnL* metabarcode can at best yield genus and family taxonomic assignments which is sub-optimal for contemporary vegetation surveys (previously noticed by Alsos et al., 2018; Cannon et al., 2016; Taberlet et al., 2007); (ii) the 18S resolved algae better than vascular plants, such that the taxonomic overlap between the two metabarcodes was poor; and (iii) the BRD was not designed for the expected flora.

Due to its small size and amplification success of many plant families (Sønstebo et al., 2010), we recommend that metabarcoding studies continue using the P6 loop of the *trnL* intron with primers g and h (Taberlet et al., 2007) for samples where eDNA is expected to be highly degraded (e.g., ancient DNA and diet eDNA). Regarding the specificity of the P6 loop, Sønstebo et al. (2010) reported that from a total of 842 species, 77% and 100% of the genera and families were uniquely identified in silico, respectively, and 33% were identified to the species-level (including families represented by a single species), which is less than the reported specificity of ITS1 and ITS2 nrDNA metabarcodes (Gielly et al., 1996; Kolter & Gemeinholzer, 2021b; Yao et al., 2010). Thus, for contemporary eDNA studies requiring species detection, we suggest an alternative use of the P6 loop as a “keystone” metabarcode instead of a standalone use. Owing to the disposition of primer pair g-h to amplify a vast number of plant families (Sønstebo et al., 2010), we believe it is more suited to verify the amplification of plant eDNA and to consolidate genus and family identification by other barcodes. Additionally, to enhance the taxonomic resolution of *trnL* alone, we recommend using the primer pair c-h from Taberlet et al. (2007), amplifying on average an additional 80bp before the P6 loop (total amplicon size ca. 240bp). Sønstebo et al. (2010) already identified the first region of the *trnL* as a candidate for metabarcoding ancient DNA due to its short size, high coverage, and specificity. We suggest that the combined amplification of this region and the P6 loop with primers c-h lowers the taxonomic

resolution unpublished in silico observations, and Johnson, Cox, and Barnes (2019); Johnson et al. (2021, 2023) while still being compatible with contemporary aquatic plant eDNA studies (Shackleton et al., 2019; Yang et al., 2016).

Overall, a common feature of all meta- and barcoding projects of freshwater vegetation is the use of short universal barcodes widely documented in BRDs. The seeming lack of consensus for primers and barcodes across the reviewed studies is not discouraging, but evidence of a healthy, critical and developing field of research. Knowing that correct detection of taxa and diversity metrics are influenced by the primers and barcodes used, we can only encourage further testing of existing primer pairs (e.g., Coghlan et al., 2021), and the development of new ones (e.g., Kolter & Gemeinholzer, 2021a). In particular, future research should focus on developing primers for reasonable taxonomic groups (e.g., Angiosperms, Bryophytes) as opposed to universal ones, and targeting taxa of specific ecosystems (e.g., Drummond et al., 2021). Alongside this, appropriate primers and barcodes for certain overlooked taxa like Bryophytes and Characeae must be developed. In addition, a multilocus approach, the combined use of different metabarcodes including cpDNA and nrDNA, should alleviate the problem of identical sequences of closely related taxa and significantly increase taxonomic resolution.

5 | PLANT DNA BARCODES: PRIMERS, MARKERS, AND DATABASES

5.1 | Toward comprehensive plant barcode databases

The quest for universal plant genetic markers originated first from studies on plant phylogeny, gene evolution and genome structure (see Clegg & Zurawski, 1992, and references therein). In this context, Clegg and Zurawski (1992) (i) define genetic regions of interest, (ii) prospect the relationship between phylogeny and molecular evolution, and (iii) infer genetic species identification, known today as DNA barcoding sensu stricto (Valentini et al., 2009). The notion of the barcode was introduced by Hebert et al. (2003) to define a genetic marker used for species identification. Among the 15 plant barcodes outlined in Hollingsworth et al. (2011) reference work, only a handful are used today in eDNA meta- and barcoding studies. Plastid barcodes include the *trnL* (UAA) intron (Taberlet et al., 2007), the *rbcLa* region of the gene *rbcL* (Kress & Erickson, 2007), the *matK* gene (Chase et al., 2007; Ford et al., 2009; Lahaye et al., 2008), and the *trnH-psbA* intergenic spacer (Kress et al., 2005). Within the nuclear ribosomal DNA (nrDNA) we mainly find the Intergenic Transcribed Spacers (ITS1 and ITS2) (Chen et al., 2010).

The genesis of DNA BRDs and barcoding projects was in 2004, 1 year after the term barcode was defined. Today, barcode sequences are accessible from a few interconnected databases: (i) the Barcode Of Life Database (BOLD), the reference in the field (Ratnasingham & Hebert, 2007), (ii) GenBank from the National Center for Biotechnology Information (NCBI) (Benson et al., 2013), and (iii) the

European Molecular Laboratory Biology Sequence Database (EMBL) (Kanz et al., 2005). Two decades after the creation of BOLD, recent gap analyses and models have shown that the barcoding landscape of plants is patchy. For instance, BOLD contains over 500,000 published sequences, although these only represent an estimated 20% of all plant species (Gostel & Kress, 2022). Analyzing GenBank data, Kolter and Gemeinholzer (2021b) reported over 150,000 sequences of the ITS barcode for c. 75,000 Tracheophyte species (i.e., 25% of them). In comparison, the *rbcl* has over 102,000 sequences for over 42,000 species, followed by *matK* with 97,000 sequences for 46,000 species, and finally *trnL-trnF* (73,000 seq./44,500 sp.) and *trnF-psbA* (63,000 seq./30,000 sp.). Not all taxa are represented equally, with a stark bias toward taxa that are easy to identify and amplify with universal primers (Gostel & Kress, 2022). Gostel and Kress (2022) found 37% of green algae, 9% of Bryophytes, 37% of Ferns and Lycophytes, 52% of Gymnosperms and 18% of Angiosperms referenced in BOLD. While all groups are underrepresented in BRDs, the most challenging ones remain Bryophytes and Angiosperms. For example, GenBank contains 24,700 ITS, 22,400 *trnL* and 13,000 *rbcl* sequences of 5000 species of Bryophyta (as of 19 October 2022), while there are an estimated 20,000 species (Stech & Quandt, 2014). This is primarily because some unsequenced taxa have challenging morphological identification, requiring a high level of botanical expertise. Second, the current universal primers and markers are unfit for these taxa, and searching for new ones is a vast project (e.g., for Bryophytes Hassel et al., 2013). A few studies on Bryophytes tried to standardize barcodes (Liu et al., 2010) and proposed primers (Epp et al., 2012; Taberlet et al., 2018).

Investigating how European vascular freshwater plants are represented in BOLD, Weigand et al. (2019) found that from a total of 683 species, an encouraging 83% have at least one sequence, and 69% have at least five sequences. However, 66 and 46% of macrophytes have, respectively, one barcode sequence of *rbcl* or *matK* publicly available, while only 46% have sequences for both barcodes. We have further observed that regarding the ITS barcodes (ITS1 and ITS2), both loci are not always available or are just partially present in BRDs. Important taxa for aquatic biomonitoring such as Characeae are just beginning to have quality sequences. As of 19 October 2022, GenBank contains 200 ITS and 817 *rbcl* sequences of 80 and 116 species of Characeae, respectively, but there are over 700 described species (Guiry & Guiry, 2022). The development of eDNA-suited primers for Characeae is also slow due to morphological uncertainty (Schneider et al., 2015) and poor genetic differentiation (Nowak et al., 2016). Another family with challenging morphological criteria is Potamogetonaceae, considered among the most phenotypically reduced and plastic Angiosperms (Lindqvist et al., 2006). This family also represents one of the largest biomasses in aquatic ecosystems, often dominant in plant communities. Du et al. (2011) reported ITS as being the most informative marker for this family based on barcoding 17 Potamogetonaceae species. Kuzmina et al. (2018) successfully used ITS2 and *atpB-rbcl* for detecting *Potamogeton* species in environmental samples. The authors further reported incomplete reference databases for certain taxa and markers in GenBank.

These figures not only are encouraging but also show that further barcoding projects are needed to complete BRDs and enhance taxonomic resolution, even for previously sequenced species. Kolter and Gemeinholzer (2021b) found that identification success rapidly increases with the number of reference sequences per species for the five established plant barcodes (*rbcl*, *matK*, *trnL-trnF*, *psbA-trnH*, ITS). The authors also observed a reduction in species misidentification with increasing sequencing depth. From these results, it seems that we can rapidly and significantly increase correct identifications by ensuring at least two reference sequences per barcode for every species. However, adding more sequences per species does not necessarily increase identification success when different species share the same barcode due to a lack of genetic divergence (Wilkinson et al., 2017).

The taxonomic assignment is affected not only by the size of BRDs but also by the quality of available sequences. Sequence length is known to diverge greatly interspecifically due to different evolutionary paths. Size and quality also differ depending on how species were sequenced—for example, trimming Sanger sequences for quality assurance is a common barcoding practice. Two studies (Kolter & Gemeinholzer, 2021b; Wilkinson et al., 2017) reported that the quality and size of reference sequences are positively correlated with the taxonomic assignment. As a result, smaller sequences from BRDs cannot be used for species identification, except for barcodes smaller than 300bp Wilkinson et al. (2017). The authors noted that *matK* suffered the most from this variability in the size of reference sequences compared with *rbcl*.

What is more, the source of DNA used for barcoding is crucial for correct taxonomic assignment. The best practice in this field is to source reference barcode sequences from recent herbarium specimens, ideally collected for this purpose, and deposited in collection institutions (Cf. Index Herbariorum) Barcode sequences and herbarium specimens should be linked with common metadata to allow for continuous referencing and corrections. In this regard, stark differences appear between public BRDs, for example, BOLD exclusively references curated barcode sequences for *rbcl* and *matK*, while GenBank contains all DNA sequences but with variable sizes and quality. However, assessing the reliability and accuracy of BOLD and GenBank, Meiklejohn et al. (2019) found no significant differences in terms of correct species assignment when using *rbcl* and *matK*. Even so, GenBank contains more markers, thus allowing multiloci metabarcoding. This approach increases correct taxonomic assignment compared with a two-loci approach with BOLD, according to Meiklejohn et al. (2019). Most notably, the transition from Sanger to HTS platforms allows multiplexing large libraries, thus reducing sequencing time while increasing sequencing depth and base call accuracy (Liu et al., 2021; Wilkinson et al., 2017). To keep up with current and future needs, alternative barcoding methods coined super- and ultrabarcoding are being implemented, which consist of whole genome or plastome sequencing, that is, genome skimming (Coissac et al., 2016; Gostel & Kress, 2022 and references in Li et al., 2015). For one sample, this sequencing strategy gets the whole plastome and ribosomal DNA cluster (including nrDNA ITS1 and ITS2) for a

constantly falling price, making it competitive for enriching BRDs. In addition, genome skimming provides a significant sequencing depth useful to detect heterozygosity and heteroplasmy, especially in recombinant markers such as the nrDNA. This is crucial, especially for plant taxa where hybrid genesis often occurs.

In light of the state of BRDs (number of barcodes, sequence length, number of species) and how it influences the results of eDNA studies, we should not neglect (i) how we use and get sequences from public BRDs, (ii) which BRDs we choose, and (iii) how we curate the obtained sequences for our plant eDNA studies. Hereafter, we detailed the standards of reference sequences in freshwater plant eDNA studies.

5.2 | How to create barcode reference databases for freshwater plant eDNA studies

Most eDNA studies rely on some form of database of reference sequences for species identification, but these could be a source of errors if not properly managed. The cost of generating DNA barcode references for a consequent number of species is high enough that most eDNA studies rely on previously published barcode works. However, several studies recommend self-developed, research-specific, and local reference databases to enhance taxonomic resolution (e.g., Alsos et al., 2018; Fahner et al., 2016; García-Robledo et al., 2013; Jurado-Rivera et al., 2009; Moorhouse-Gann et al., 2018).

Outside of the eDNA context, plant DNA barcoding projects began in the 2000s with a major contribution being Lahaye et al. (2008) research. Other barcoding projects can be resumed to Table 3 and two additional reviews that meta-analyze barcoding projects in the Arabian Peninsula and China (Mosa et al., 2019; Yang et al., 2020). Barcoding projects have been using the *rbcl*, *trnL* and *matK* markers since the beginning, and quickly but not unanimously adopted the nrDNA ITS following international consortia (CBOL Plant Working Group et al., 2009; China Plant BOL Group et al., 2011; Hollingsworth et al., 2011). The common workflow for self-developed barcode references is as follows (Figure 2, and Elliott & Davies, 2014): (i) plant samples are collected from the field or herbarium collections, (ii) DNA is extracted from young leaves using the CTAB method or commercial DNA extraction kits, (iii) long barcodes (>900bp) or whole markers (e.g., ITS) are amplified with universal or group-specific primers, (iv) amplicons are Sanger sequenced and sequences are manually curated, and (v) barcodes are compared with public BRDs (e.g., BLAST), and reference plant samples are re-identified in case of taxonomic mismatch. A more common alternative to Sanger sequencing (fourth step) is the use of next-generation sequencing, a cost-effective method, which significantly improves the quality and quantity of sequences and accelerates the creation of barcode data (own observations and Liu et al., 2021; Wilkinson et al., 2017). Shotgun sequencing has also been used as it provides more sequencing depth, albeit at a higher cost per sequence, which may not be cost-effective for barcoding entire floras. Coissac et al. (2016) suggested a workflow for building extended barcode

reference libraries based on shotgun sequencing. Chua et al. (2021) used genome skimming to generate an extended BRD for 184 plant species from Denmark. Alsos et al. (2020) used genome skimming to build a reference database for the full chloroplast genome and the nrDNA cluster. The cpDNA and nrDNA regions were successfully assembled for 67% and 86% from a total of 6655 plant samples (2051 herbarium specimens and 4604 freshly collected, silica gel dried specimens). The authors showed that large-scale genome skimming is an efficient method for enriching reference databases from herbarium and fresh plant samples. This approach yields qualitative data because of its higher sequencing depth compared with other sequencing methods. The extended regions (i.e., extended barcodes), including all high-copy fractions of the genome, can be used in different applications compared with a barcode-only database that is more eDNA-oriented.

The targeted PCR step of the standard workflow for creating barcode databases could also be replaced with target DNA capture enrichment (hybridisation capture), yielding even more barcode references and whole markers (Dodsworth et al., 2019; Foster et al., 2022). Capture probes are available for the plastid genome of vascular plants (Nicholls et al., 2015; Peñalba et al., 2014) and more recently a set of probes targeting 353 nuclear genes has been developed (Johnson, Pokorny, et al., 2019). These tools could be used as a cost-effective approach for complete or targeted enrichment of plant DNA extractions for creating reference databases (Bethune et al., 2019; Foster et al., 2022). Some limitations intrinsic to the quality of the plant sample still affect the success of hybridisation capture. Brewer et al. (2019) identified that efficiency decreases with the age of herbarium specimens since genomic DNA decays with time. The authors also identified biases related to the geographic origin of the taxa (e.g., herbarium specimens from tropical taxa yielded less target DNA than silica-gel-dried specimens). This method requires further calibration but is a promising tool for building reference databases (Brewer et al., 2019). Along with shotgun sequencing, alternative workflows for generating reference databases should help alleviate the gaps in current public databases and provide quality sequence data for different uses from phylogenetics to eDNA studies (Baker et al., 2022; Coissac et al., 2016).

Despite recommendations for self-developed barcode references, this practice is uncommon in aquatic plant metabarcoding probably due to the investment that is required (Elliott & Davies, 2014). For barcoding, only reference sequences of target and relative (non-target) species are needed for the design of specific primers. All the reviewed eDNA barcoding studies designed assays using sequences from public BRDs, except (Scriver et al., 2015) who completed their database for some target species following the procedure described above (Figure 2). Three out of 10 metabarcoding studies created their reference database from plant specimens (Foster et al., 2021; Kuzmina et al., 2018; Tsukamoto et al., 2021). Alsos et al. (2018) gathered a local reference database from previous eDNA meta- and barcoding studies—with only 3% of the local flora missing—and showed that the taxonomic coverage benefited from such a complete reference database. Five others directly used the

NCBI GenBank nucleotide (nt) database for the taxonomic assignment of eDNA sequences. The simplest method consists in blasting cleaned unique eDNA sequences directly to the entire NCBI nt database using Blastn (Figure 2, Cannon et al., 2016; Coghlan et al., 2021; Drummond et al., 2021; Ji et al., 2021). With this method, sequences can match multiple taxa, even those from different geographic areas, and additional analyses after species assignment are needed to check for false and true positives (e.g., read count and match quality). Alternatively, a more refined database can be obtained by limiting the NCBI database to the regional pool of species (Adame & Reef, 2020). On the other hand, Shackleton et al. (2019) skipped creating reference databases and taxonomic assignments altogether by interpreting ecological results solely from Operational Taxonomic Units. The downside is poor species correlation with conventional surveys, although the authors established good correlations at the OTU level, that is, before the taxonomic assignment of eDNA sequences.

We identified a potential reproducibility issue in that studies did not provide database release information, only Alsos et al. (2018) reported the EMBL release they used. Another way to address reproducibility is to filter downloaded sequences with an in silico PCR generating a smaller barcode database (Bellemain et al., 2010; Ficetola et al., 2010; Shehzad et al., 2012). Alternatively, adopting new software for creating reference databases, such as the MetaCurator software (Richardson et al., 2020) and BCdatabaser command-line and web interface (Keller et al., 2020). Users without coding experience can compile standardized large meta- and barcoding databases directly from BCdatabaser online interface (<https://bcdatabaser.molecular.eco/>). This circumvents the powerful but cumbersome queries with Entrez Direct from NCBI (Kans, 2013). Most interestingly, the generated databases, authored by the user, can be instantly published and shared with the community. The latest methodological advances should simplify many key steps of eDNA studies and push toward standardization and community-driven research.

We have shown that BRDs already offer quality sequences and that alternative analyses (e.g., OTUs) and methods (e.g., hybridisation capture) can complete or circumvent database gaps. These should be resolved with the increasing number of available reference sequences, and past data sets can be reanalyzed at will. Barcoding projects should focus on (i) quality: complete sequences including priming sites, (ii) redundancy: reducing sequencing error, and increasing infraspecific diversity with the number of sequences per barcode per species, (iii) de novo taxa: sequencing more species, (iv) sequencing method: go a step further in quality and quantity of barcodes with HTS, (v) herbaria: voucher specimens allow overall traceability of the genetic information (e.g., reproducibility, consultation, taxonomic reassignment), and (vi) standardization: adopt strong guidelines established by reference databases like BOLD.

6 | CONCLUSION

It should be now accepted that all plant types, from aquatic to terrestrial, can be detected with eDNA barcoding and metabarcoding.

Both approaches could be used in complement with traditional freshwater vegetation surveys as is the case for animals. The use of eDNA for monitoring the vegetation of freshwater ecosystems is supported by 22 studies published from 2015 to 2022, 12 with a barcoding approach and 10 with metabarcoding. This highlights the growing interest in this method and supports continued research in this area. We provided a contrasted review, addressing the current limitations and successes of both approaches, to give a comprehensive picture and offer research perspectives. Four cornerstones of eDNA studies were the focus of this review. We first focused on understanding the characteristics of freshwater plant eDNA that are necessary to make informed methodological choices (e.g., sample volume). Specific aspects of plant eDNA should be accounted for when planning sampling campaigns, such as eDNA spatiotemporal distribution and the life cycle of the studied taxa. In this regard, seasonal leaf senescence is often designated as the most suitable period for presence-absence eDNA surveys. It is, however, less suited for estimating abundances in lotic systems due to homogenization of the eDNA along the river. This observation has not been verified in lentic systems, which may benefit from this homogenization for estimating abundances. The growing season seems most suited for estimating abundances in lotic systems as the correlation between eDNA concentration and biomass and cover is stronger and persistent across seasons. More data are needed to consolidate these early observations. The choice of sampling matrix (e.g., water, sediments, biofilm) also seems to influence occurrence results. We should further investigate if these matrices record distinct patterns of eDNA release and accumulation (e.g., origin, retention time). Second, through our investigation of the current applications, we demonstrated the broad applicability across diverse freshwater systems and highlighted where there is room for improvement and exploration of other uses (e.g., sediment tracing). Conservation efforts should start benefitting from barcoding assays which can detect and quantify early invasions and rare species, but for the latter assays remain to be developed. Existing assays should be tested outside of the geographic region they were designed for before they can be used by practitioners. Barcoding assays for semi-aquatic and terrestrial plants that can be detected in water samples should also interest end users of this method and need development and testing (e.g., *Reynoutria japonica* an exotic invasive species in Europe). Metabarcoding has been used for biodiversity assessments across many freshwater ecosystems, showing congruent results with traditional survey methods. Alternative uses of this eDNA approach have already started and should receive further attention (e.g., sediment fingerprinting). Semi-quantitative eDNA metabarcoding assays should further be explored and calibrated to exploit all the potential of metabarcoding. These examples should inspire further research projects and encourage end users to include plant eDNA approaches in their ecological monitoring toolbox. Third, we concentrated on the choice of appropriate primers for plant eDNA, which should be determined through a research-specific and approach-based selection of primers and barcodes. This is supported by the ever-growing number of available primers which are proof tested in silico and in vitro. We have identified that

marker preferences differ between eDNA barcoding and metabarcoding. Metabarcoding studies by far use the short *trnL* (UAA) P6 loop metabarcode with primers g-h (Taberlet et al., 2007), despite its poor taxonomic resolution. In contrast, eDNA barcoding studies rely mainly on *matK*, *trnH-psbA* and ITS1. The best answer to improve taxonomic resolution would be the combined use of several markers from the plastid and the nuclear DNA. Particularly for eDNA metabarcoding, we encourage the adoption of the ITS2 nuclear barcode, which has so far been underused in freshwater applications despite its recognition as a core barcode for plants. New technologies and applications such as ddPCR, genome skimming, and hybridisation capture could be adopted to overcome some methodological biases like the limits of detection and PCR pitfalls. Finally, we discussed the importance of reference databases, which are continuously being populated with more sequences. We encourage fellow (molecular) ecologists and botanists to collaborate in the creation of regional and local barcode databases. Several recent reviews of plant eDNA applications (e.g., Banerjee, Stewart, Dey, et al., 2022; Johnson et al., 2023) and analyses of reference databases and markers (e.g., Kolter & Gemeinholzer, 2021b; Weigand et al., 2019) provide critical guidelines. To support research in this field and improve species identification success, the construction of regional or local DNA BRDs is of utmost importance as they overcome taxonomic gaps in public BRDs. Alternatively, when using public BRDs to build a reference database, standard reporting of how it was obtained and the use of new software for building databases is strongly recommended to allow repeatability. We hope that the studies presented will encourage more eDNA surveys of freshwater vegetation, which constitute the basis of biodiversity management projects.

AUTHOR CONTRIBUTIONS

The conception or design of the study: AEP, LH, and JNB. The acquisition, analysis, or interpretation of the data: AEP, Writing of the manuscript: AEP, LH, and PV

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CONFLICT OF INTEREST STATEMENT

We confirm that all authors have seen and approved the submitted version of the manuscript and that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere. We have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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