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FRESHWATER MOLLUSCS

Environmental DNA metabarcoding for freshwater bivalves biodiversity assessment: methods and results for the Western Palearctic (European sub-region)

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Abstract Freshwater ecosystems are the most vulnerable worldwide and freshwater bivalves rank amongst the most threatened animals in the world. Surveying and monitoring freshwater bivalves are difficult tasks: they are difficult to find, hard to identify (taxonomic expertise is needed), and working underwater is technically challenging. It is therefore crucial to find more efficient methods to survey and monitor these species. Here, we present the first metabarcoding approach for freshwater bivalves and compare environmental DNA (eDNA) and traditional surveys. We describe two sets of primers (for Unionida and Venerida) developed for freshwater bivalves eDNA metabarcoding. These primers have been tested in the field, with about 300 studied sites. Results were compared to freshwater bivalves' surveys using traditional methods, with eDNA always detecting more species than traditional surveys, especially when Sphaerids were taken into account. While our study initially focused on Western Palearctic freshwater bivalve species, our primers were confronted in silico with available sequences and have proven to be effective at a global scale. The results show that eDNA metabarcoding, with our developed primers, is a remarkable tool allowing for non-invasive surveys, detection of rare and inconspicuous species, absence

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data and overall freshwater bivalves routine monitoring.

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Introduction

Freshwater ecosystems are the most vulnerable ecosystems worldwide (Dudgeon et al., 2006; Dudgeon 2019; Albert et al. 2020) and freshwater bivalves rank amongst the most threatened animals in the world (Lydeard et al., 2004; Lopes-Lima et al., 2016). The surveying of freshwater bivalves is a challenging task, mainly by two reasons. First, because freshwater ecosystems are difficult to access for the biologist, because of turbidity, current, sometimes navigation (see Prié et al., 2018). Second, because freshwater bivalves are difficult to sample. Some live buried in the sediment or are covered with algae or mud, making them hard to distinguish in the wild. Some species are rare and scarce, others are minute, with shells measuring about a millimeter. For all these reasons, we lack efficient methods to sample all freshwater bivalve species. Moreover, freshwater bivalve species are difficult to identify in the field, leading to a need for taxonomic expertise. Indeed, species identifications rely on few diagnostic characteristics, with a high variability. Shell plasticity in Unionids is documented by plethoric classical and modern literature (e.g., Ortmann, 1920; Agrell, 1948; Nagel, 1992; Zieritz & Aldridge, 2009; Zieritz et al., 2010; Prié et al. 2012; Prié & Puillandre, 2014). Furthermore, these bivalves have shown to express high levels of hidden cryptic diversity or species that cannot be differentiated by morphology but only by molecular tools (eg. Prié & Puillandre 2014; Froufe et al. 2017). Consequently, and despite their worrying conservation status, freshwater bivalves are often overlooked by conservation policies and environmental impact studies.

For some rare or hard to survey freshwater organisms, several authors have shown that the use of environmental DNA (eDNA, Taberlet et al., 2012) is a potentially valuable survey technique for amphibians (Ficeola et al., 2008; Dejean et al., 2011; Dejean et al., 2012), fishes (Darling & Mahon, 2012; Kelly et al., 2014; Evans et al., 2015; Klymus et al., 2015) and other aquatic organisms (see Thomsen & Willerslev, 2015 for a review). Although invertebrates in general and freshwater molluscs in particular are noticeably underrepresented in eDNA studies (Belle et al. 2019), some experiments with gastropods (Goldberg et al., 2013) or bivalves (Deiner & Altermatt, 2014; Stoeckle et al., 2016; Gasparini et al. 2020) have proven to be successful. However, these were aimed at only one species or at most, one genus (de Ventura et al., 2017). Alternatively, a multi-specific approach, such as eDNA metabarcoding, can be applied to describe the community of a given site. This approach employs the use of group-specific primers, high-throughput sequencing technology and simultaneously identifies several taxa from an environmental sample without a priori knowledge of the species likely to be present in the sampled ecosystem (Taberlet et al., 2012).

First results with eDNA metabarcoding of freshwaters organisms were obtained by Thomsen et al. (2012); Miya et al. (2015) for fishes and Valentini et al. (2016) for fishes and amphibians. Recently, Klymus et al. (2017) have used the eDNA metabarcoding approach to detect invertebrate fauna in the Laurentian Great Lakes and surrounding waterways, with a special focus on invasive bivalve (Sphaeriidae) and gastropod species monitoring.

Here, we present the first metabarcoding approach for all freshwater bivalves of the Western Palearctic, and compare eDNA surveys to traditional surveys and general knowledge about species distribution and ecology. For the first time, an eDNA metabarcoding survey of bivalves covers a very large area and over 300 sampling sites with a standardized method.

False negatives (species not detected while present) and false positives (species detected as a result of contamination or inappropriate field or lab protocols,

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but not actually present in the sampling place) are a crucial issue to test, whereas or not eDNA analysis with our primers and our field protocol is a trustful method. Here for the first time, eDNA analysis could be challenged by (i) the comparison with traditional one-off surveys performed by a team of skilled professional malacologists using every available field sampling method in various contexts for testing false negatives, (ii) different environmental conditions and therefore available knowledge on species ecological requirements, (iii) species distribution knowledge at a very large scale and (iv) a wide array of taxa in each sampled site, allowing testing false positive with (ii) and (iii).

Our results show that eDNA metabarcoding, with the primers presented here, is a reliable and revolutionary tool, for surveying and monitoring rare and threatened species, and for the early detection of introduced ones.

Materials and methods

Systematics

The taxonomic classification used is based on the molecular phylogenies of Huff et al. (2004) and Lopes-Lima et al. (2018) for margaritiferids; Khalloufi et al. (2011), Prié & Puillandre (2014), Froufe et al. (2014), Froufe et al. (2016a, b) Froufe et al. (2017) and Lopes-Lima et al. (2017) for Unionids. We follow Lee and Foighil (2003) for the Sphaeriidae, with subsequent results of Bespalaya et al. (2015) for *Odhneripisidium conventus* (Clessin, 1877) and Mouthon & Forcellini (2017) for *Euglesa compressa* (Prime, 1852). Nomenclature for Sphaerids follows Gargominy et al. (2011). *Dreissena rostriformis* (Deshayes, 1838) and *D. bugensis* (Andrusov, 1897) are considered synonyms following the conclusions of the molecular phylogenies established by Therriault et al. (2005) and Stepien et al. (2014). However, because *D. rostriformis rostriformis* is a marine deep-water mussel presumably endemic to the Caspian Sea (Prié & Fruget 2017), we here refer to the European quagga mussel as *Dreissena rostriformis bugensis* (Andrusov, 1897). The genus *Corbicula* is taxonomically complex (see Pigneur et al., 2011 for a review in France based on molecular markers) and the gene fragment we amplify here does not allow distinguishing the different taxa of *Corbicula*. Therefore, we only refer to them as *Corbicula* spp.

Primers and reference database

The targeted gene was the 16S because (i) it is a mitochondrial gene, more abundant than nuclear ones in the cells, and hence in the environment, (ii) it has been traditionally used in the major molecular phylogenies of freshwater bivalves and there is a large amount of data available, (iii) it is reliable to distinguish freshwater bivalve taxa. COI could have been chosen, but was too variable and does not contain suitable conserved regions, leading to difficulties in universal primers' design (Deagle et al., 2014; Klymus et al., 2017). Group-specific primers were designed on a collection of all Eukaryote mitochondrial DNA sequences present in GenBank for Unionida and Venerida using the *ecoprimers* software (Riaz et al. 2011). The designed primers were then tested in silico using the ecoPCR program on all available DNA sequences in release 138 (standard sequences) of the EMBL database. For each primer, a sequence logo was generated (Crooks et al., 2004) based on the in silico PCR results for the target group and a mismatch analysis was performed, both for the target taxonomic group and for the non-target group (maximum of three mismatches allowed, excluding the last two nucleotides on the 3′end) to assess the specificity of the primer pairs using ggplot2 (Wickham, 2016), ggseqlogo (Wagih, 2017) and scatterpie packages under R (version 3.5.2, R Core Team, 2018). Finally, the length distribution of the amplified sequences (excluding primers) was analyzed.

A reference database of all species of freshwater bivalves of the Western Palearctic (Table 1) was first established for the target genes including female and male mitochondrial sequences for species using doubly uniparental inheritance (DUI, Zouros et al., 1994; Curole & Kocher 2005; Breton et al., 2007) mining all available sequences from GenBank and including our own unpublished sequences for missing 16S haplotypes to complete the database. Eventually, wrongly identified sequences from GenBank—such as EU518636 identified as *Pyganodon grandis* (Say, 1829) but actually a male sequence of *Sinanodonta woodiana* (Lea, 1834)—were withdrawn from the reference database.

Table 1 Western Palearctic bivalve species, 16S gene fragments available data and eDNA analyses results

European freshwater bivalve species' reference list	Nb of known 16S female haplotypes	Nb of known 16S male haplotypes	Discriminable in silico	Identified in situ
Order Unionida Stoliczka, 1871				
Family Margaritiferidae Haas, 1940				
Pseudunio auricularius (Spengler, 1793)	1	0	1	1
Pseudunio marocanus Pallary, 1918	2	1	1	1
Margaritifera margaritifera (Linnaeus, 1758)	1	1	1	1
Family Unionidae Rafinesque, 1820				
Anodonta anatina (Linnaeus, 1758)	12	4	1	1
Anodonta cygnea (Linnaeus, 1758)	2	0	1	1
Anodonta exulcerata Porro, 1838	2	0	1	1
Pseudanodonta complanata (Rossmässler, 1835)	3	0	1	1
Sinanodonta woodiana (Lea, 1834)	9	2	1	1
Microcondylaea bonellii (Férussac, 1827)	2	1	1	1
Potomida littoralis (Cuvier, 1798)	23	2	1	1
Potomida semirugata (Lamarck, 1819)	8	0	1	0
Potomida acarnanica (Kobelt, 1879)	2	0	1	0
Leguminaia wheatleyi (Lea, 1862)	1	0	1	0
Unio c. crassus Philipsson, 1788	14	2	1	1
Unio c. courtillieri Hattemann, 1859	6		1	1
Unio bruguierianus Bourguignat, 1853	2	0	1	0
Unio delphinus Spengler, 1793	9	1	1	0
Unio durieui (Deshayes, 1847)	1	0	1	0
Unio elongatulus C. Pfeiffer, 1825	8	1	1	1
Unio foucauldianus Pallary, 1936	2	1	1	1
Unio gibbus Spengler, 1793	2	0	1	0
Unio ionicus Drouët, 1879	1	0	1	0
Unio mancus Lamarck, 1819	5	1	1	1
Unio pictorum (Linnaeus, 1758)	10	1	1	1
Unio ravoisieri (Deshayes, 1848)	3	0	1	0
Unio tigridis Bourguignat, 1852	2	0	1	0
Unio tumidiformis da Silva e Castro, 1885	4	0	1	0
Unio tumidus Philipsson, 1788	3	1	1	1
Unio terminalis Bourguignat, 1852	1	1	1	0
Order Venerida J.E. Gray, 1854				
Family Cyrenidae J.E. Gray, 1840				
Corbicula fluminalis (O.F. Müller, 1774)	7		(1)	1
Corbicula fluminea (O.F. Müller, 1774)	2		1	1
Corbicula leana Prime, 1867	1			
Corbicula largillierti (Philippi, 1844)	0		0	0
Family Dreissenidae J.E. Gray, 1840				
Congeria jalzici Morton & Bilandzija, 2013	3		1	0
Congeria kusceri Bole, 1962	2		1	0
Congeria mulaomerovici Morton & Bilandzija, 2013	1		1	0
Dreissena blanci Westerlund, 1890	1		0	0
Dreissena presbensis Kobelt, 1915	1		0	0

Table 1 continued

European freshwater bivalve species' reference list	Nb of known 16S female haplotypes	Nb of known 16S male haplotypes	Discriminable in silico	Identified in situ
Dreissena stankovici Lvova & Strabogatov, 1982	6		(1)	0
Dreissena polymorpha (Pallas, 1771)	1		1	1
Dreissena rostriformis bugensis (Andrusov, 1897)	6		1	1
Mytilopsis leucophaeata (Conrad, 1831)	4		1	0
Family Sphaeriidae Deshayes, 1855 (1820)				
Euglesa casertana (Poli, 1791)	36		1	1
Euglesa compressa (Prime, 1852)	3		1	1
Euglesa edlaueri Kuiper, 1960	2		0	0
Euglesa globularis (Clessin, 1873)	2		1	1
Euglesa henslowana (Sheppard, 1823)	1		(1)	1
Euglesa hibernica (Westerlund, 1894)	1		1	1
Euglesa hinzi (Kuiper, 1975)	0		0	0
Euglesa lilljeborgii (Clessin, 1886)	2		1	1
Euglesa maasseni Kuiper, 1987	0		0	0
Euglesa milium (Held, 1836)	3		1	1
Euglesa nitida (Jenyns, 1832)	4		(1)	1
Euglesa obtusalis (Lamarck, 1818)	1		1	1
Euglesa personata (Malm, 1855)	7		1	1
Euglesa pseudosphaerium (J. Favre, 1927)	1		1	1
Euglesa pulchella (Jenyns, 1832)	1		1	1
Euglesa subtruncata (Malm, 1855)	7		(1)	1
Euglesa supina (A. Schmidt, 1851)	1		(1)	1
Euglesa waldeni (Kuiper, 1975)	1		1	0
Odhneripisidium annandalei Prashad, 1925	3		1	0
Odhneripisidium conventus (Clessin, 1877)	1		1	0
Odhneripisidium moitessierianum (Paladilhe, 1866)	2		1	1
Odhneripisidium tenuilineatum (Stelfox, 1918)	1		1	1
Pisidium amnicum (O.F. Müller, 1774)	1		1	1
Sphaerium asiaticum (Martens, 1864)	0		0	0
Sphaerium corneum (Linnaeus, 1758)	5		(1)	1
Sphaerium lacustre (O.F. Müller, 1774)	3		1	1
Sphaerium nitidum Clessin, 1876	0		0	0
Sphaerium nucleus (S. Studer, 1820)	4		(1)	1
Sphaerium ovale (A. Férussac, 1807)	1		1	1
Sphaerium rivicola (Lamarck, 1818)	1		1	1
Sphaerium solidum (Normand, 1844)	2		1	0
Sphaerium transversum (Say, 1829)	1		1	0

The number of haplotypes here refers to our short fragment. (): taxa for which some of the haplotypes are also found in another species

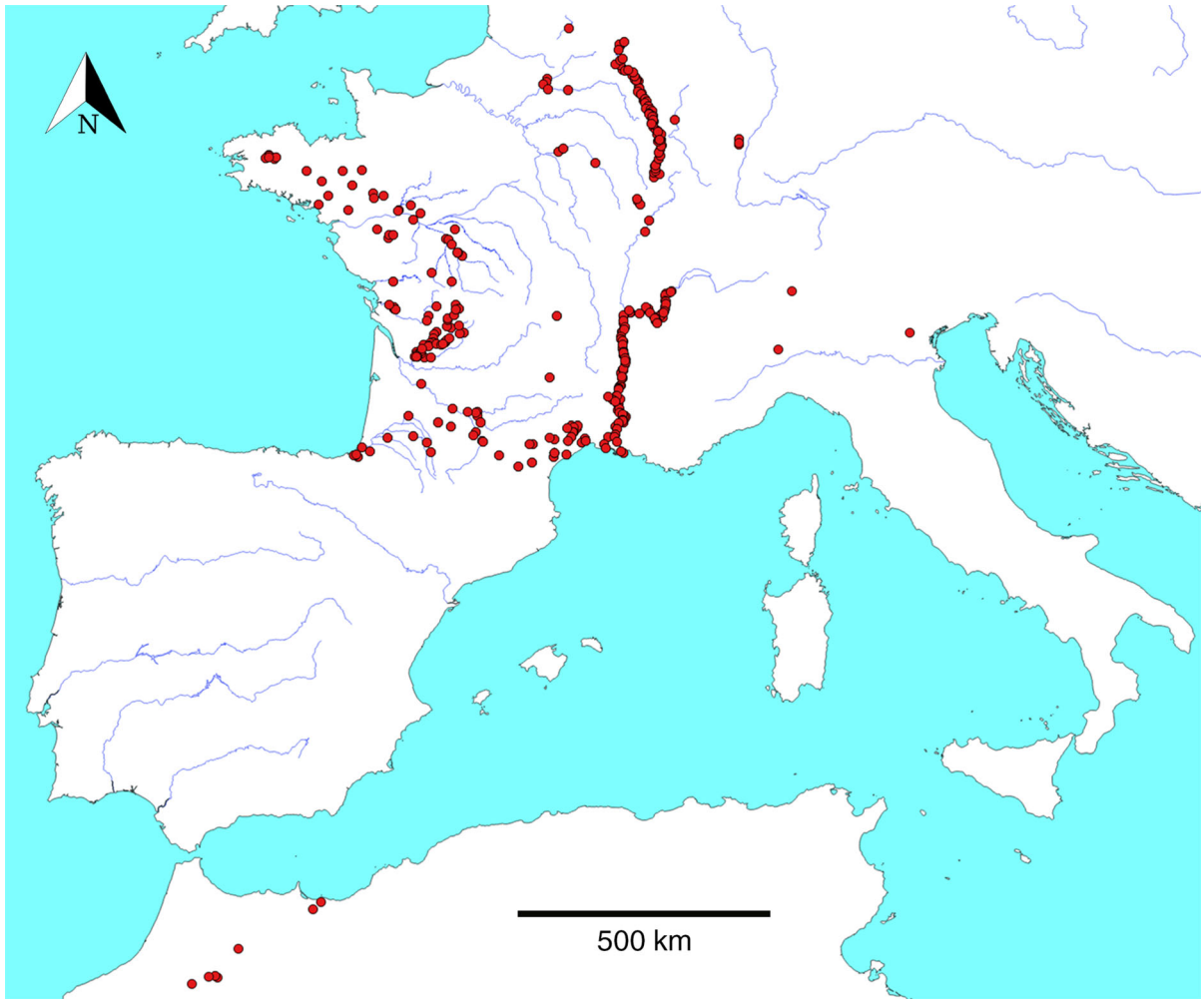

Fig. 1 Freshwater bivalves eDNA sampling sites, from years 2015 to 2019

The final reference database is available as supplementary material (Online Resource 1).

Taxon and ecological conditions sampling

Sampling was performed between 2015 and 2019, mainly in summer months, between May and October. Our sampling plan aimed at testing (i) the potential of eDNA to detect in the field all freshwater bivalve taxa of France, Italy and Morocco, (ii) the reliability of the method in different environmental conditions, and (iii) the comparison between eDNA and traditional surveys.

Sampling was performed mostly in France, where most Western Palearctic taxa can be found, with additional seven sites in Morocco, two in Italy and one in Switzerland (Fig. 1).

The sampling plan covered very different environmental conditions such as standing waters vs. running waters, springs and small streams vs. large rivers, and different pH conditions. However, most of the sampling was done in large rivers, in lowland and with limestone substrate. We lack sampling sites in alpine lakes (only one site, Lake Maggiore in Switzerland) and brackish waters (only one site, the Adour estuary in South-west France). For the Rhône River, the DNA sampled by Pont et al. (2018) was re-used for freshwater bivalve's analysis.

Sampling protocols and environmental DNA metabarcoding analysis

The water sampling protocol was performed using a Vampire Sampler (Bürkle GmbH), a VigiDNA® 0.45 µM filtration capsule (SPYGEN), and disposable sterile tubing for each sample. The water was filtered for about 30 min with the Vampire Sampler on speed 1 or until the full saturation of the capsule. Volumes of water pumped from each sample site varied according to water turbidity but are estimated to be about 30 l in good conditions. Then the filtration capsule was drained by filtrating air, and the filter was filled with 80 ml of CL1 buffer (SPYGEN) and stored at room temperature until the extraction. Two water filtrations were performed per site, as two replicates were enough to detect 96.9% of fish species in the Rhône River (Pont et al., 2018; and similar results obtained by Cantera et al., 2019 in French Guyana). For the Meuse River, the sampling protocol was the one described in Pont et al. (2018), using a small sampling boat.

For DNA extraction, the filtration capsules were emptied into one 50 ml tube each. The tube was then centrifuged for 15 min at 15,000×*g*. The supernatant was removed with a sterile pipette, leaving 15 ml of liquid at the bottom of the tube. Subsequently, 33 ml of ethanol and 1.5 ml of acetate 3 M Sodium were added to each 50 mL tube. After a quick manual shaking, samples were stored for 24 h at − 20 °C. The tubes were then centrifuged for 15 min at 15,000×*g* and 6 °C and the supernatant was discarded. After this step, 360 µl of ATL Buffer of the DNeasy Blood & Tissue Extraction Kit (Qiagen) were added in the tube, the tube was vortexed and the supernatant was transferred in a 2 ml tube and the DNA extraction was performed using NucleoSpin soil kit (MN) from step 6 following the manufacturer's instructions. The DNA was eluted with 100 µL of SE buffer twice.

The amplification mixture contained 1 U of AmpliTaq Gold Polymerase (Applied Biosystems), 1 × PCR Gold buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.5 µM of each tagged forward and reverse primers and 0.2 mg/mL of bovine serum albumin (BSA, Roche Diagnostics). The final volume was 25 µl including 3 µl of eDNA extraction. Each sample was amplified in twelve replicates per each group-specific primer pair. The amplifications started with an initial denaturation for 3 min at 95 °C, followed by 50 cycles of 30 s at 95 °C, 30 s at 50 °C and 30 s at 72 °C, with a final elongation at 72 °C for 5 min for both group-specific primer couple. For each sample, forward and reverse primers were 5' labeled with identical eight nucleotides tags in order to enable the subsequent assignment of sequences to their respective sample. Tags were designed with the oligoTag program included in the OBITools package (https://metabarcoding.org/obitools; Boyer et al., 2016), with at least four differences between tags to provide the assignment of reads to samples. Sixty-five negative extraction controls and 35 PCR negatives controls (ultrapure water) were also performed. Two different extraction controls were performed for each extraction session. One extraction control was a 50 ml tube filled by CL1 buffer that was centrifuged for 15 min at 15,000×*g* in parallel with the samples. The supernatant was removed with a sterile pipette, leaving 15 ml of liquid at the bottom of the tube. Subsequently, 33 ml of ethanol and 1.5 ml of acetate 3 M Sodium were added to each 50 ml tube and then the extraction was performed as described. The second one was done by adding 360 µl of ATL Buffer of the DNeasy Blood & Tissue Extraction Kit (Qiagen) in an empty tube and extracted in parallel to normal samples. Extraction controls were amplified and sequenced in parallel to the samples to monitor possible contaminations. After the filtering pipeline, the extraction and PCR negative controls were completely clean, and no sequence reads remained in those samples.

The products of the twelve replicates per primer pair were pooled after the PCR and visualized using capillary electrophoresis (QIAxcel; Qiagen GmbH) and purified using a MinElute PCR purification kit (Qiagen GmbH) with a final elution in 15 µl buffer. Before sequencing, purified DNA was titrated again using capillary electrophoresis. Several purified PCR products were pooled in equal volumes, to achieve an expected sequencing depth of 300,000 reads per DNA sample per group-specific primer pair (i.e., 600,000 per sample). Library preparation was performed at Fasteris facilities (Geneva, Switzerland) using Metafast protocol (www.fasteris.com/metafast), in total 43 libraries were analyzed. Six libraries were sequenced using an Illumina HiSeq 2500 (2 × 125 bp) (Illumina, San Diego, CA, USA) and the HiSeq SBS Kit v4 (Illumina, San Diego, CA, USA), six using an Illumina HiSeq 2500 (2 × 125 bp) (Illumina, San Diego, CA, USA), HiSeq Rapid flow cell v2 (Illumina, San Diego,

CA, USA) and the HiSeq Rapid SBS Kit v2 (Illumina, San Diego, CA, USA); 28 using a MiSeq (2 × 125 bp, Illumina, San Diego, CA, USA) and the MiSeq Flow Cell Kit Version3 (Illumina, San Diego, CA, USA) and three using a NextSeq (2 × 150 bp + 8, Illumina, San Diego, CA, USA) and the NextSeq Mid kit (Illumina, San Diego, CA, USA). The libraries ran on the NextSeq were equally distributed in four lanes. Sequencing were performed using following the manufacturer's instructions at Fasteris facilities (Geneva, Switzerland).

The sequence analysis of the metabarcodes obtained after the NGS was done as described in Valentini et al. (2016), using the OBITools package (Boyer et al., 2016). Briefly, forward and reverse reads were assembled using *illuminapairedend* program. Subsequently, the *ngsfilter* program was used to assign the sequences to each sample. A separate dataset was created for each sample by splitting the original dataset in several files using *obisplit.* Sequences shorter than 20 bp, or occurring less than 10 times per sample or labeled "internal" by the *obiclean* program, corresponding most likely to PCR errors, were discarded. The program *ecotag* was used for the taxonomic assignment of MOTUs with both the curated reference database and the sequences extracted from the release 138 (standard sequences), and only sequences with a similarity of higher than 98% were kept. We discarded all MOTUs with a frequency of occurrence below 0.001 per library in each sample, considered as tag-jumps (Schnell et al. 2015). For the samples sequenced with the NextSeq, only species presents in at least two lanes were retrieved.

Traditional vs eDNA surveys

The successful application of eDNA monitoring in the field relies on the absence of false positives and false negatives. We here checked for false positives and false negatives at three scales: (i) at the scale of France, comparing our c.a. 300 sampling sites' results to the species known distribution, (ii) at the scale of intensively sampled rivers, to check for false negatives (our sampling design is dense enough to presumably detect every species known from these two rivers) and (iii) at the scale of one-off surveys performed in a few hundreds of meters of river, to compare traditional survey's results to eDNA.

(i) For the whole of France, we compared the results of our sampling with the national database of the INPN (www.inpn.mnhn.fr, see Gargominy et al. 2016 for a detailed presentation), which gather almost all freshwater bivalves data available for France, with no limit of time, i.e., all the available data were included, from 1758 to 2019, so that the recent decrease of freshwater bivalves was not taken into account in the a priori species distribution). About 32 900 freshwater bivalve data are entered on the INPN database, which made it possible to compare the results obtained by eDNA with known ranges for all species (see Prié, 2017 for species distribution maps based on this database).

(ii) At the scale of large rivers, we use two eDNA sampling campaigns carried out (i) on the Rhône River, with 65 sampling points over 575 km of linear distance, and (ii) on the Meuse River, with 52 sampling points over 480 km of linear distance (Fig. 2). The data obtained were compared with the INPN database.

(iii) Finally, one-off surveys covering a few hundreds of meters of watercourses, carried out using traditional methods (direct observation, dredging, and screening of sediment, exploitation of floodline debris) were compared with the results of eDNA analysis in the immediate vicinity (less than 10 km downstream). These traditional inventories generally combined data from shells and individuals observed alive. We selected only inventories for which (i) we had the details of the observation (living individual, shell or subfossil shell); (ii) the inventory had been carried out intensively, involving either a team of several malacologists or repeated field surveys over several years, and always at least one hyperbaric diving survey; (iii) the eDNA analyses were carried out on site or less than 10 km downstream. Fifteen sites were thus studied for the comparison between eDNA and traditional surveys.

Ten of these sites are in the Meuse River, where inventories targeting molluscs have been carried out by a private consultancy (Biotope, 2009) as part of an

impact study on the renovation of dams. These inventories involved four malacologists, including two professional scuba divers. Surveys were carried out wading on the banks and shallow areas and hyperbaric diving (bank-to-bank transects carried out by the two malacologist divers). Two to three hours were spent on each site by the team. These surveys aimed primarily at protected species (*Unio crassus* and *Pseudunio auricularius*) and were mainly limited to the search for Unionida species. However, at two of these sites, a more complete inventory including gastropods and Sphaeriidae was also carried out, with sediment sampling for minute species. Sediment samples were sieved in the field or in the lab using a set of sieves, the latter one with a 1 mm mesh, and sorted out under a stereomicroscope.

Two other sites were located on the Saône River, which has been surveyed by a team of malacologists including four scuba divers and two people wading in shallower areas, in search of *Pseudunio auricularius* and *Unio crassus*. These surveys were mainly aimed at large species and sphaerids were not searched for. On each site, the time spent in prospecting was about one hour. Finally, the Lez (Montpellier), Ardèche and Charente Rivers have been the subject of extensive surveys over several years. For the Lez River, our data

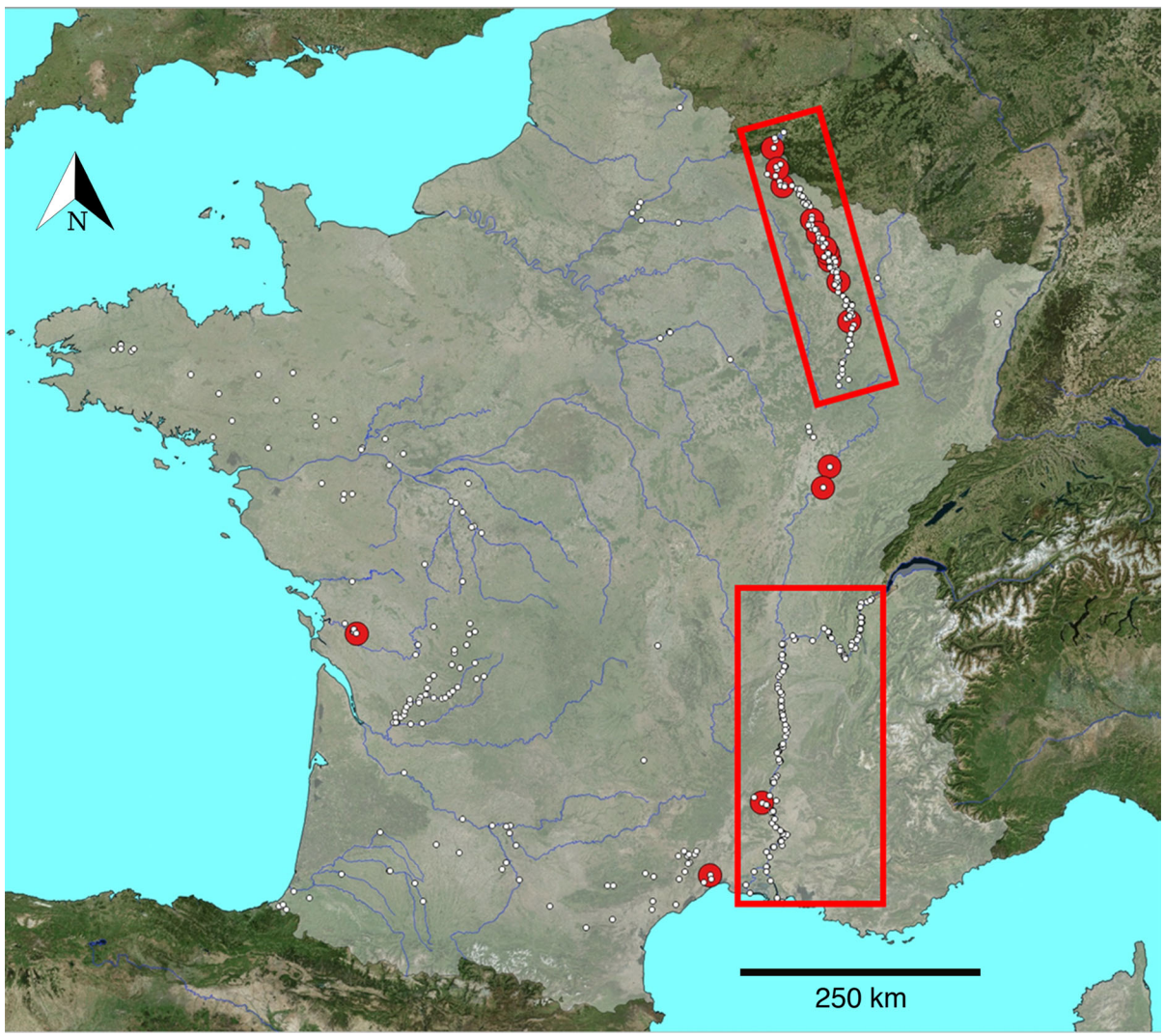

Fig. 2 Chosen sites for the comparison of traditional surveys and eDNA analysis. White dots: eDNA sampling sites in France; red squares: regional eDNA surveys at a river basin scale for comparison eDNA data *vs* available data (Meuse River in the north and Rhône River in the south); red dots: one-off surveys for comparison eDNA data vs traditional methods

include micro-molluscs search for in sediment and floodline debris samples (Prié 2003 and unpublished data) and scuba diving from large species. Data were acquired between 2003 and 2016. The data, therefore, included all bivalve species but suffer from a lack of expertise concerning Sphaerids. On the Ardèche River, two surveys aiming at Unionida were performed in 2010 and 2016 using mainly scuba diving, with additional observations carried out by scuba diving in between. On the Charente River various surveys were carried out between 2007 and 2016 (Prié et al., 2018), by scuba diving, focusing on *Pseudunio auricularius* but including all species. The data therefore only concern large species and exclude Sphaeriidae, for both the Ardèche and Charente Rivers.

For most of these inventories carried out using traditional methods, only Unionida species were targeted and unfortunately, we do not have such extensive comparisons for the Sphaeriidae, for which only three inventory sites could be compared with DNA analyses (Le Lez upstream of the city of Montpellier and two sites near the villages of Dieue-sur-Meuse and Saint-Mihiel on the Meuse). On the other hand, on the basis of the available distribution maps and general knowledge on the ecology of this group, we were able to compare our eDNA sampling results with the knowledge available at both national and regional levels (Rhone and Meuse catchments, and spot inventory stations).

Results

Primers and reference database

Two sets of primers were defined, one for the Venerida and one for the Unionida (Fig. 3).

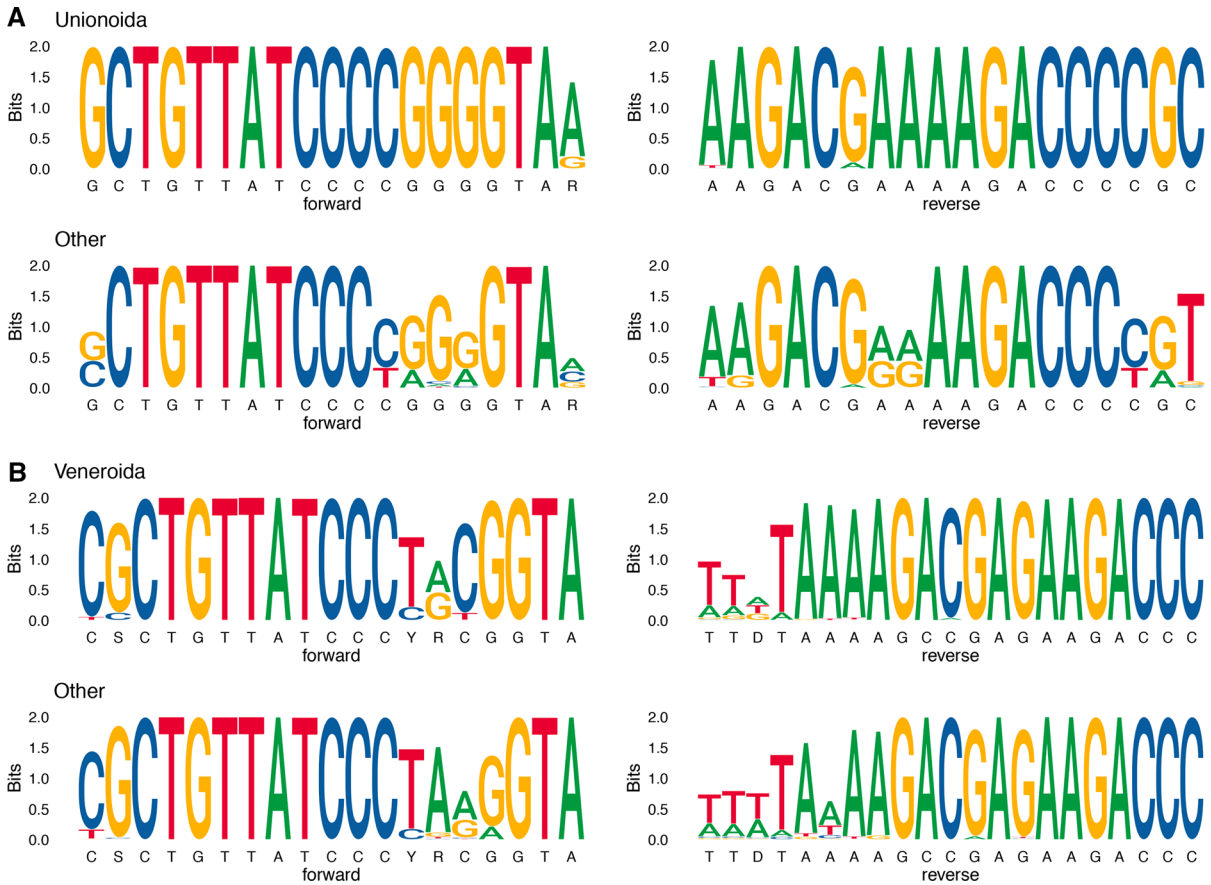

Fig. 3 Results of the in silico validation of the 'Unionoida' (**a**) and 'Veneroida' (**b**) primer pairs. Sequence logo of the primer illustrates the quality of the match between the primer and its target sequences

Our primers target a small fragment of the 16S gene (137 base pairs on average for the Unionida; 128 on average for the Venerida). This small fragment is effective in distinguishing most of the Western Palearctic freshwater bivalve species in silico (Table 1). The exceptions are the following: regarding the Sphaerids, *S. corneum* and *S. nucleus* shared one haplotype; *E. edlaueri* and *E. maasseni*, two species endemic from the Ohrid Lake cannot be distinguished from two of the haplotypes of *E. nitida*; two haplotypes of *E. henslowana* are shared respectively with *E. subtruncata* and *E. supina*. The subspecies of *Unio mancus* (Prié et al., 2012) cannot be distinguished with our small fragment, whereas the two subspecies of *U. crassus* (*U. c. crassus* and *U. c. courtillieri sensus* Prié & Puillandre, 2014) have different haplotypes and can be distinguished. We also distinguish the two lineages of male mtDNA, corresponding to those identified by Mioduchowska et al. (2016) but do not know which male fragment belongs to which female lineage, nor even if male and female lineages are the same or not.

The *Corbicula* species cannot be sorted out as some haplotypes are shared by the different taxa. Only one haplotype of *C. fluminalis* (O.F. Müller, 1774) differs from the other species, making it possible to reveal this species in some cases. *Dreissena blanci* and *D. presbensis* have the same 16S haplotype and therefore cannot be distinguished. This haplotype is also shared with some specimens of *D. stankovici* in our short fragment (provided sequences mined from GenBank come from properly identified specimens). Hence these three species cannot be distinguished with our primers, except for some haplotypes of *D. stankovici*.

The primers described here also amplify male mtDNA in silico for most Unionid species having DUI. While male 16S sequences are not described for all species, our primers could be tested in silico at least for *Pseudunio marocanus*, *Margaritifera margaritifera*, *Anodonta anatina* (four haplotypes), *Sinanodonta woodiana*, *Microcondylea bonelli*, *Potomida littoralis*, *Unio crassus crassus*, *U. crassus courtillieri*, *U. delphinus*, *U. elongatulus, U. foucauldianus* (two haplotypes), *U. mancus, U. pictorum* and *U. terminalis.*

Our reference database includes 270 haplotypes (244 female mitochondrial haplotypes, plus 26 male haplotypes for the species having DUI) and covers all the Western Palearctic taxa except *Euglesa hinzi* (Kuiper, 1975), *Sphaerium asiaticum* (Martens, 1864) and *Sphaerium nitidum* Clessin, 1876 for which no molecular data are available. For *Sphaerium ovale* (A. Ferussac, 1807) we used as a reference sequence the one produced by Prié & Lecaplain (in prep.) based on specimens collected in Normandie and in the Seine drainage. The identification of these specimens is based on the shape of the nephridium and the pore spacing on the inner shell. Indeed, these specimens have a distinct haplotype from *S. corneum* and *S. nucleus*. The taxonomic status of this taxon is not a matter of consensus within the malacological community and our results concerning it must be considered with caution. For *Euglesa pulchella* (Jenyns, 1832), the only reference sequence is the one available on GenBank, accession number KY202894 published under the reference Mouthon & Forcellini (2017), although this sequence of the 16S gene is not mentioned in this paper, which bases its conclusions only on the analysis of the ITS-1 gene fragment.

In silico PCR (Ficetola et al. 2010) ran on a collection of all mitochondrial DNA sequences, and all available DNA sequences in release 138 (standard sequences) of the EMBL database lead to the amplification of 596 haplotypes belonging to 267 species of Unionida; and 741 haplotypes belonging to 490 taxa of freshwater Veneroida. If marine species are included, the Venerida primers amplify 1057 haplotypes belonging to 683 taxa (Online Resource 2).

Field sampling

Our database compiles 302 sampling sites at the end of 2019. Most of the sampling was performed in France, with only a few sites in Switzerland, Italy and Morocco. Regarding the French checklist, 38 species, i.e., 90% of the species currently accepted in France could be successfully detected by the eDNA analysis. Additionally, *Pseudunio marocanus* and *Unio foucauldianus* were identified in Moroccan rivers; *Anodonta exulcerata* was identified in Lake Maggiore in Italy; *Unio elongatulus* and *Microcondylaea bonellii* were detected in Italian rivers. Hence 42 species, i.e., about 60% of the Western Palearctic fauna was successfully identified in the field.

Male mtDNA was collected in the field for *Margaritifera margaritifera, Anodonta anatina, Potomida littoralis, Sinanodonta woodiana, Unio c. crassus, Unio c. courtillieri, Unio elongatulus, Unio foucauldianus, Unio tumidus.* In some of the sampled

sites, especially for *Potomida littoralis*, male mtDNA yielded much more reads than females. The explanation of this unexpected result remains unknown.

Out of a sample of 162 sites for which we have two reliable replicates (i.e., replicates taken exactly in the same site, not one in each bank of the river for example), 60% of the two replicates yielded identical results in terms of species richness. Quantities of reads between replicates often differed up to a tenfold.

Traditional vs eDNA surveys

Our eDNA sampling is not evenly distributed in the studied area, and available traditional data may include ancient data, shell-only data and misidentified species. However, species distribution at a large scale is pretty well known in France and we could compare our results to the available knowledge, species by species.

At the scale of France, our eDNA results were overall congruent with the known distribution of all species and with their known ecology. Nevertheless, in some cases, we rediscovered species out of their known range, but still in their historical range according to ancient literature. Not surprisingly, this happened with introduced species which are still under settlement in France (ex. *Euglesa compressa*, *Dreissena rostriformis bugensis* and *Sinanodonta woodiana*) but also with *Unio crassus* in the lower Rhône River.

Regarding regional surveys at the scale of large rivers, traditional surveys (including shell-only data) found 28 species, while eDNA revealed 32 species in the Rhône River and 25 vs 30 in the Meuse River.

As regards Sphaeriidae, eDNA analysis over the whole of the Rhône and the whole of the Meuse has made it possible to detect 1.11 and 1.17 times more species than the available data, respectively.

Of the 15 inventory sites where we were able to compare data acquired by eDNA detection with data acquired by one-off surveys using traditional methods, (i) the number of species detected by eDNA analysis was always greater than or equal to that detected by traditional methods -excepted for one site- and (ii) all species observed alive by traditional methods were well recovered in eDNA analyses (Fig. 4). Only in a

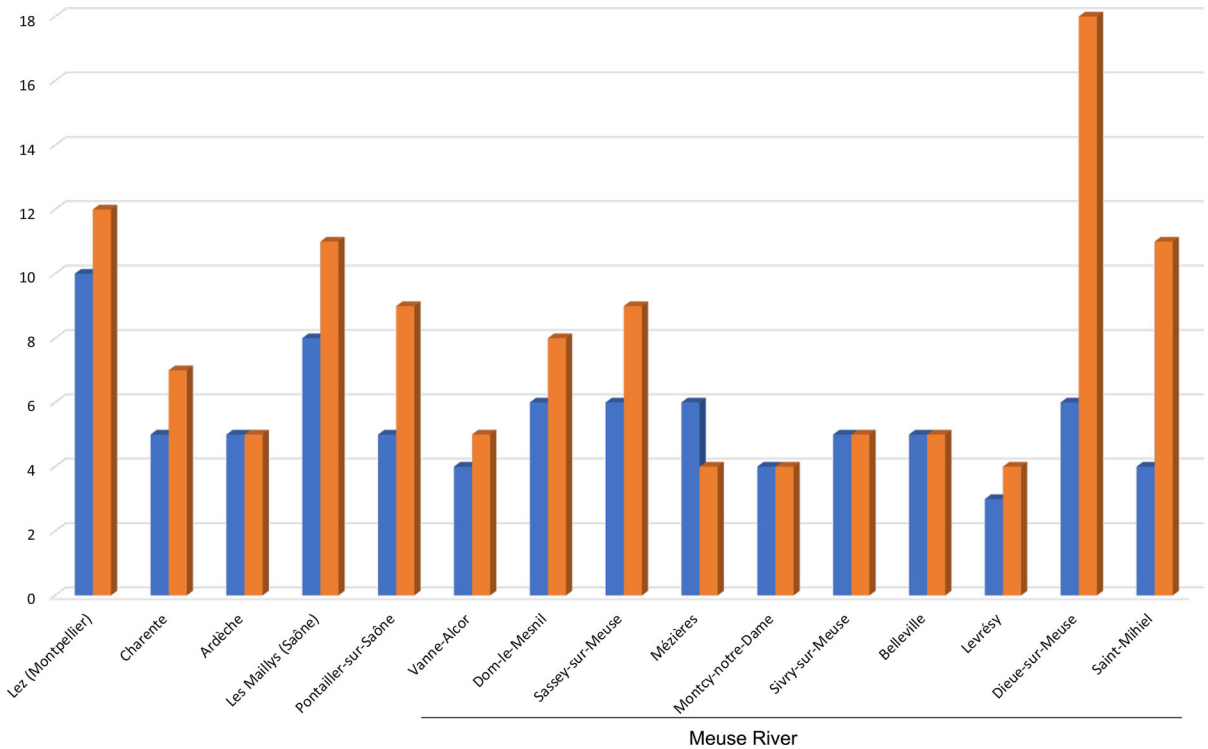

Fig. 4 Comparison of species richness detected with traditional methods (blue bars) and eDNA analysis (orange bar). Comparison is based on large species only, excluding Sphaerids, excepted for the Lez Dieue-sur-Meuse and Saint-Mihiel sites

few cases, species were observed as shells only and were not detected by eDNA analysis (noticeably in site Mézières, the only site were more species were found with traditional methods than with eDNA analyses). We assume that in these cases, the species in question were extirpated from the site by the time the eDNA was collected, or that for small species, the shells may have been drained by the current at the sampling site from upstream. Considering only the large species (i.e. excluding Sphaeriidae) for which detection rates by traditional methods are assumed to be quite high, the detection rate of the species by eDNA analysis is on average 1.33 times higher than the detection rate by traditional methods. If only specimens observed alive during traditional inventories are considered, the detection rate by eDNA analysis is 2.11 times higher.

On the three sites of one-off surveys for which we have data concerning Sphaeriidae (Lez, Dieue-sur-Meuse and Saint-Mihiel), the number of species detected by eDNA is 1.6 (Lez) to 6 (Saint-Mihiel) times higher than the number of species detected using traditional methods.

Discussion

Primers and in silico validation

Regarding the Unionida, our primers amplified a 16S fragment able to distinguish all the valid species of the Western Palearctic. It also amplifies male mtDNA, at least for some of the species. As for the Venerida, some of the haplotypes were shared between different species for the genera *Corbicula*, *Dreissena*, *Euglesa* and *Sphaerium*. For the *Corbicula* species, taxonomy is still unclear and GenBank specimen determination may be hazardous. There are two haplotypes available for *C. fluminalis*. One of them seems to be found only in this species and differs from the other *Corbicula* species' haplotypes from one base pair only. Since the other one is shared with *C. fluminea s. l.*, and because of taxonomical uncertainty, we consider the *Corbicula* species complex cannot be distinguished. The only haplotype of *Dreissena blanci* seems to be shared with some specimens identified as *D. presbensis* and *D. stankovici*. Providing there was no misidentification when these sequences were submitted to GenBank (as *D. blanci* may also occur in lakes Prespa and Ohrid), these three species cannot be sorted apart.

Two specimens collected in the Great Lakes and identified as *S. corneum* by Klymus et al. (2017, GenBank reference numbers KY426905 and KY426906) had a haplotype usually found in *S. nucleus* (ex. Sharma et al., 2013; Petkevičiūtė et al., 2018). However, Klymus et al. (2017) do not give any information about the way specimens were identified and very little morphological characters allow discriminating *S. corneum* and *S. nucleus* (Petkevičiūtė et al., 2018). Moreover, this haplotype is closer to that of *S. ovale* as proposed by Prié & Lecaplain *in prep*. More molecular data are needed to make sure that the haplotypes we use here in our reference database are really diagnostic for each species.

Regarding the iridescent pea mussel *Euglesa pulchella*, our analyses are based on the unique sequence produced by Mouthon & Forcellini (2017). This sequence is very close to the different haplotypes of the short-ended peaclam *Euglesa subtruncata*, from which it differs only by a few base pairs in the short fragment of the 16S gene that we are amplifying (one to three base pair depending on *E. subtruncata*'s haplotypes). Based on this single sequence, our results suggest a larger range in France than that given by Mouthon (2018). The reliability of our results is corroborated by the fact that we found the species in the places where it is already known according to Mouthon (2018): the basins of the upstream Rhône (where it was observed until 2012 upstream of the Doubs, river Drugeon), the Meuse and the Seine (where it is known only from fossils records). The additional data provided by eDNA analysis only adds a few stations on the Mediterranean coastal rivers and one on the Garonne River drainage. If the haplotype we use in our reference database was shared with some of *E. subtruncata*'s haplotypes, we should have found it just about everywhere, as *E. subtruncata* is a common and widespread species. However, it remains possible that our results on this species will be challenged in the future by more in-depth studies on the different haplotypes of *E. subtruncata* and *E. pulchella*.

Taxon and ecological conditions sampling

Most Western Palearctic taxa could be successfully detected in the field. Detection distances probably vary among taxa, population abundance and other parameters such as flow conditions, but also on eDNA

detection. As has been stressed by Stoeckle et al. (2017), successful eDNA detection relies on site-specific environmental parameters. In presence of sediment, the pumping time was lowered because the filtration capsule tended to clog up. In most rivers suitable to *Margaritifera margaritifera*, i.e., on a siliceous substrate with acidic waters and often a high forest cover, humic substances concentration were high and pumping time was also low. Given that these environmental conditions cumulate two inhibitory parameters (humic substances concentrations and siliceous sediment particles) and a lower amount of water analyzed, eDNA detection success was supposed to be low. However, our results showed that *M. margaritifera* was indeed detected in sampled sites where it was known to occur, and a high diversity of associated Sphaerid species was also revealed by eDNA. While detection distances are probably lower in these rivers, our methods remain operational.

Traditional vs eDNA surveys

The analysis of eDNA has proven its effectiveness for presence/absence compared to inventories using traditional methods. In all our sampling tests, eDNA analysis allowed detecting all the species that were known from traditional sampling, and often many more. In a few cases, a species was detected by traditional methods but not with eDNA analysis. In these cases, the data collected by traditional methods always concerned shells only. We hypothesize that the species was no longer living on the site at the time of the eDNA sampling. Low-abundance species can be masked by eDNA of high-abundance species resulting in false negatives (Evans et al., 2015). In our dataset, Venerida species were generally very unbalanced with *Corbicula* often yielding hundreds of thousands of reads, whereas *Euglesa* spp. were detected from only from a few tens of reads. In such circumstances, it is very likely that detection is lower for the Sphaerids. However we have regularly detected rare and minute species even in *Corbicula*-filled places, suggesting that our method is good enough for surveying all bivalves species even in presence of super-abundance species.

Although eDNA expertise's cost (about 1000 Euros per site all included if outsourced to private consultancy) may seem prohibitive, it must be put into perspective with the much higher cost of a team of professional malacologists, including professional scuba divers (for the Naiads) who should survey kilometers of river bottom and time-consuming sediment sorting (for the Sphaerids).

Moreover, eDNA analysis is efficient for small and difficult to identify species (Sphaerids in particular). The use of eDNA analysis in France resulted in a significant increase in Sphaerids data collected in the INPN database (Fig. 5).

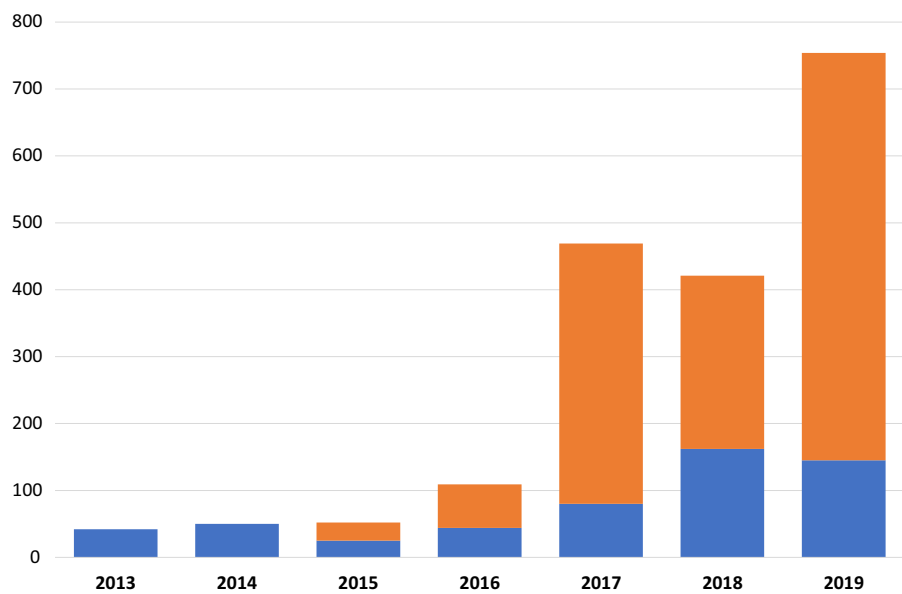

Fig. 5 Yearly Sphaerids data inputs in the INPN database (France). Blue: data collected with traditional methods; orange: contribution of eDNA analysis-based surveys. Figured eDNA data come from this study only (other eDNA data are not included). eDNA freshwater bivalves metabarcoding tests were performed between 2015 and 2017; commercial services were offered as early as mid-2018

Focus on invasive and unnoticed threatened species

Some species were detected out of their known range. As could be expected, introduced and invasive species were found more widespread than available data suggested. The quagga mussel *Dreissena rostriformis bugensis* was previously known only from a few rivers in northeastern France (Bij de Vaate & Beisel, 2011; Marescaux et al., 2012; Wagner, 2014). In 2016, it was identified by the use of eDNA in the lower section of the Rhône River, a result which could be confirmed by scuba diving sampling and sediment dredging using a boat and a standard triangular dredge (Prié & Fruget, 2017). In 2017, it was found with eDNA analysis near the town of Montpellier in southern France and in the Seine River to the east of Paris. These results are congruent with the expansion routes predicted for this species. They are also congruent with the absence data in other sampling sites.

The ridgebeak peaclam *Euglesa compressa* was revealed by eDNA samples in the Ardèche River in summer 2016, just before the publication by Mouthon and Forcellini (2017) of the identification of this alien species in France. eDNA analyses also lead to its discovery in the Seine River in 2017.

These results provide further evidence to support the use of eDNA for bivalve surveys, including for invasive species not initially targeted by the sampling protocol or not known to occur in a previously studied river. It is also valuable for the monitoring of invaders' colonization, not only because it is a powerful tool to detect their presence (including hard to detect veliger larva for example), but also because it provides trustful absence data.

The analysis of eDNA also revealed a wider expansion for the thick-shelled river mussel *Unio crassus* (Fig. 6), a species of conservation concern (categorized as "Endangered" according to IUCN red list, Lopes-Lima et al., 2014) and listed in the European Habitat Directive annex II and IV, and then protected by law in most European countries). This species was only known from the upper Rhône tributaries, the Loire and Seine drainages and minor drainages in northeastern France. eDNA analyses allowed finding it in the upstream Dronne and Charente Rivers, two watersheds lying south of the Loire watershed, the south-westernmost known occurrence in Europe, results which could be confirmed by direct observation (Charneau et al., 2019). Even more surprising, the species was found downstream the Rhône River, while only ancient data witness its presence in the Camargue area. This result highlights the potential of eDNA to reveal unnoticed species in the large rivers ecosystems, where traditional methods generally fail due to harsh environmental conditions (depth, current, turbidity, and navigation).

On the other hand, some species are not considered by conservation measures due to a supposedly wide distribution range. Among them, *Sphaerium solidum* is a thick-shelled peaclam for which quite a lot of data is available in France. Indeed, as the name suggests, this species' shell is thick enough to persist in the environment many years after the animal's death. It is therefore quite often mentioned in freshwater bivalves' surveys. It is a downstream specialist, found in large rivers, deep in well-oxygenated water, preferably on sandy substrates. No data at all could be collected by eDNA analysis for this species on the 250 or so sites sampled within its supposed range (inferred from available data) and presenting a priori favorable biotopes (downstream ecosystems on calcareous, sandy-muddy substrates). Although the national INPN database includes 28 stations in France, there are only three relatively recent data on living individuals (Choisy-au-Bac in the Aisne in 2009 and two stations downstream the Seine River in 2006, Bij de Vaate et al., 2007). According to eDNA data, this species seems to be almost, if not totally, extirpated from France. Welter-Schultes (2012) considers it "almost extinct in the European Union". We have less dramatic but similar results with other downstream specialists such as the River orb mussel *Sphaerium rivicola* and the Depressed river mussel *Pseudanodonta complanata* which were detected only in a few samples while distribution maps based on historical and shell-only data provide a wide distribution range in France. Most of the attention is directed to upstream specialists such as the freshwater pearl mussel which is considered a target species of conservation (Geist, 2010) and the only species currently included in a European CEN standard (Boon et al., 2019). Species occurring more downstream seem to be much more threatened, as can be expected considering the multiple threats facing the lower watersheds compared to the upper ecosystems. Another example of unbalanced conservation efforts.

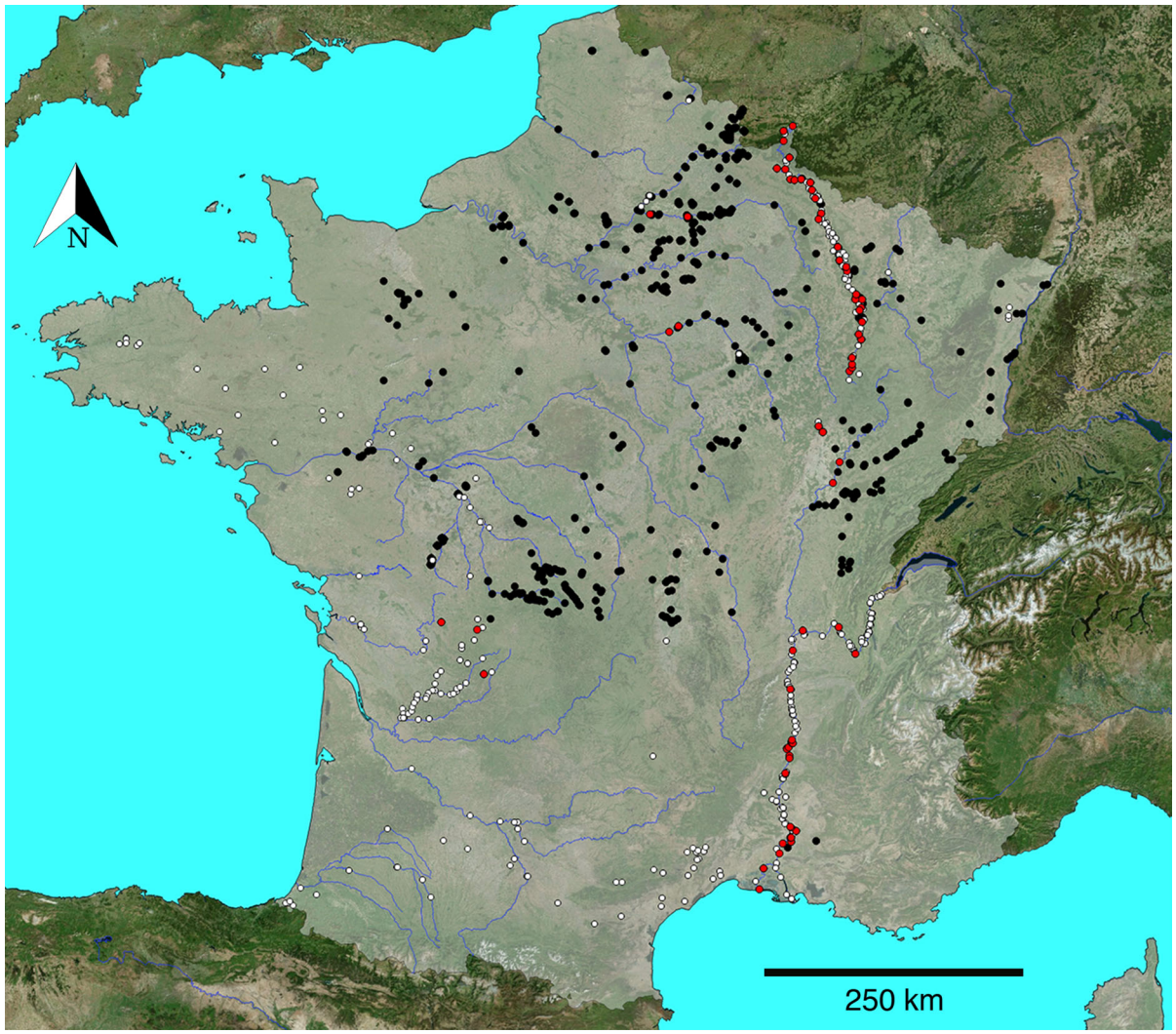

Fig. 6 *Unio crassus* in France. White dots: eDNA sampling sites with absence; red dots: eDNA sampling sites with presence; black dots: all available data prior to eDNA deployment, with no limit of time. The data acquireddownstream the Rhône River and in the south-west is completely new

Conclusion

The analysis of eDNA is a paradigm shift for hydrobiologists, comparable to the deployment of ultrasound detectors for bats surveys at the beginning of the century. For freshwater bivalves, the results presented here provide a new picture of species distribution and subsequent conservation issues, allowing consideration of species that were not being addressed because they are difficult to access (either because they are difficult to harvest or because they are difficult to identify). The use of eDNA analysis is non-intrusive, while traditional survey generally have an impact on the bottom of the rivers (trampling when using viewing glasses, sediment disturbance for the Sphaerids' surveys, see Stoeckle et al., 2016). Moreover, the identification of many freshwater bivalve species is based on the examination of internal characteristics, which supposes to kill the animal. eDNA-based surveys solve the problem of the taxonomic expertise needed when using traditional methods, as it relies on a barcoding approach. It is also limits the spread of invasive introduced pathogens (e.g., Chytrid *Batrachochytrium dendrobatidis* Powell, 2007 or crayfish plague *Aphanomyces astaci* Schikora, 1906) since the equipment used is sterile.

It also limit the human risks linked to prospecting in watercourses, a point that is not negligible, particularly in a professional context. Finally, eDNA is easy to sample in the field, which means that non-specialists—such as river technicians or students—can do the field work after a very short training, and the protocols can easily be standardized.

This progress does not run counter to traditional inventory methods since eDNA analysis does not provide neither precise location of populations nor population size (data that are needed, noticeably in the context of impact studies). The eDNA analysis is a first step toward the deployment of field research, giving a list of species to be searched for in a given studied area. Environmental DNA analysis is comparable to a smell: it detects a presence (or not), a general direction, but the essential remains to be discovered (where exactly, how many individuals?). In the same way that flair presides over tracking, eDNA analysis is a formidable prelude to field inventories.

Our multi-species detection approach allow in a single water sample to characterize all the bivalve fauna living in a given water shed, including small Sphaerids species. This makes eDNA surveys more valuable because a lot more data is collected as "by catch", making it possible to also characterize faunal associations. It also allows for detection of species that were never detected before, including the early detection of potentially invasive species.

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