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Reliable eDNA detection and quantification of the European weather loach (*Misgurnus fossilis*)

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Abstract

The European weather loach (*Misgurnus fossilis*) is a cryptic and poorly known fish species of high conservation concern. The species is experiencing dramatic population collapses across its native range to the point of regional extinction. Although environmental DNA (eDNA)-based approaches offer clear advantages over conventional field methods for monitoring rare and endangered species, accurate detection and quantification remain difficult and quality assessment is often poorly incorporated. In this study, we developed and validated a novel digital droplet PCR (ddPCR) eDNA-based method for reliable detection and quantification, which allows accurate monitoring of *M. fossilis* across a number of habitat types. A dilution experiment under laboratory conditions allowed the definition of the limit of detection (LOD) and the limit of quantification (LOQ), which were set at concentrations of 0.07 and 0.14 copies μl^{-1}, respectively. A series of aquarium experiments revealed a significant and positive relationship between the number of individuals and the eDNA concentration measured. During a 3 year survey (2017–2019), we assessed 96 locations for the presence of *M. fossilis* in Flanders (Belgium). eDNA analyses on these samples highlighted 45% positive detections of the species. On the basis of the eDNA concentration per litre of water, only 12 sites appeared to harbour relatively dense populations. The other 31 sites gave a relatively weak positive signal that was typically situated below the LOQ. Combining sample-specific estimates of effective DNA quantity (Q_e) and conventional field sampling, we concluded that each of these weak positive sites still likely harboured the species and therefore they do not represent false positives. Further, only seven of the classified negative samples warrant additional sampling as our analyses identified a substantial risk of false-negative detections (*i.e.*, type II errors) at these locations. Finally, we illustrated that ddPCR outcompetes conventional qPCR analyses, especially when target DNA concentrations are critically low, which could be attributed to a reduced sensitivity of ddPCR to inhibition effects, higher sample concentrations being accommodated and higher sensitivity obtained.

KEYWORDS
conservation, cryptic species, ddPCR *versus* qPCR analyses, eDNA detection, endangered, water sampling

1 | INTRODUCTION

Reliable detection remains a critical and often difficult component for conservation planning and monitoring of fish, especially when they are rare and elusive. Detection probability via conventional fishing methods (such as nets, pods, traps or electrofishing techniques) is often low (Radinger *et al*., 2019). High mobility, as well as low density and/or the cryptic behaviour of these organisms, can also lead to lower detection probabilities of many key target species (MacKenzie & Royle, 2005; Maxwell & Jennings, 2005; Britton *et al*., 2011; Porreca *et al*., 2013). This may in turn increase the risk of nondetection error and its associated problems (*i.e*., so called false-negative detections) (Gu & Swihart, 2004). In addition to their limited efficiency, conventional survey methods are laborious, expensive, and often invasive and harmful to the environment and its populations (Lintermans, 2015). Recent advances in molecular techniques allowing for the detection of environmental DNA (eDNA) can overcome these issues and have revolutionized species monitoring (Lodge *et al*., 2012). Aquatic eDNA-based methods rely on the collection and extraction of shed cellular material that is suspended in the water column and has been shown to significantly improve our ability to detect aquatic species in a variety of aquatic habitats (Coble *et al*., 2019; Cristescu & Hebert, 2018; Goldberg *et al*., 2016; Jerde *et al*., 2011; Lacoursière-Roussel *et al*., 2016; Thomsen *et al*., 2012). Although the strength of this methodology is now widely accepted, there is an increasing need for rigorous, standardized and reliable estimates of sensitivity that allow for comparison among methodologies and studies (Furlan *et al*., 2016; Wilcox *et al*., 2018). In general, the sensitivity of eDNA surveys is critically determined by the volume of water sampled in the field (Wilcox *et al*., 2018), the quantity of DNA that is finally analysed (which is also affected by the elution volume and amount of DNA extract that is ultimately used in the PCR reactions, *e.g*., Furlan *et al*., 2016; Dorazio & Erickson, 2018; Wilcox *et al*., 2018) and the occurrence of inhibitors that can possibly limit the amplification of DNA during PCR (Goldberg *et al*., 2016; McKee *et al*., 2015). Besides the aspect of water volume that is finally analysed, one needs to sample a water body in an integrated way to increase the detection probabilities of rare or locally distributed species since eDNA often shows a very patchy distribution in the water column, especially in lentic systems (Dunker *et al*., 2016; Eichmiller *et al*., 2014). To be able to evaluate the outcome of any eDNA-based survey method, each of these aspects needs to be incorporated.

In Europe, there are a number of declining species of freshwater fish of conservation concern. One of these is the European weather loach or weatherfish, *Misgurnus fossilis* (Linnaeus, 1758), which is extremely difficult to monitor via conventional survey methods. Although its natural distribution range extends from north-western France eastward to the Volga Basin (Figure 1), the species is in steep decline and has apparently disappeared from a large part of its former range (Hartvich *et al*., 2010; Meyer & Hinrichs, 2000). Drainage of wetlands and moist meadows with associated loss of high amplitudes in water level, oxygen saturation and water temperature are the main reasons for this decline (Lelek, 1987). Furthermore, *M. fossilis* is known to prefer soft-bottomed backwaters and periodically flooded pools with an abundantly submerged vegetation, conditions which are becoming increasingly scarce in modern-day European landscapes (Meyer & Hinrichs, 2000). As a consequence, the few remnant populations known and monitored are often confined to drainage channels and ditches, where the practice of intensive weed removal and dredging pose additional threats to their survival. Furthermore, the intensive agricultural use of pesticides is also thought to impact populations (Schreiber *et al*., 2017) to further decline (Spindler, 1997). Either separately or combined, these factors are responsible for the dramatic decline of this fish species across Europe, in both the number and size of its populations (Hanel & Lusk, 2005), which has led to the species now being listed in the IUCN Red List of Threatened Species (Freyhof, 2011). Given the difficulty of monitoring this species and the alarming decline throughout its range, a reliable eDNA monitoring method would be an important first step if conservation of the weather loach is going to be undertaken.

Here we aimed to develop and validate a novel eDNA-based method for accurate detection and quantification of *M. fossilis* DNA in natural samples. Furthermore, we wanted to evaluate estimates of sensitivity that allow for quality assessment, better understanding of the eDNA results and higher accuracy.

Although eDNA detection of this species has already been shown to be successful via conventional qPCR amplification and appears much more efficient than conventional monitoring methods (Sigsgaard *et al*., 2015), qPCR-based eDNA assays are known to be sensitive to inhibitors (Doi *et al*., 2015; Mauvisseau, Davy-Bowker, *et al*., 2019a; Wood *et al*., 2019). Many freshwater bodies are, however, loaded with organic compounds because they are turbid, have high dissolved organic compounds or are eutrophic, which often leads to PCR inhibition and a reduced efficiency of DNA polymerase in PCR reactions. As a result, qPCR assays are likely exposed to a substantial risk of false-negative detections. In this context, digital droplet PCR (ddPCR) has been shown to be less vulnerable to PCR inhibition and additionally assumed to have higher sensitivity and precision than qPCR (Doi *et al*., 2015; Hindson *et al*., 2011). Exploiting these advantages, we developed a new primer/probe assay for the European weather loach that is compatible with ddPCR detection, and validated this assay following appropriate specificity and sensitivity tests. An internal positive control (IPC) was additionally incorporated during extraction of the samples in order to obtain an estimate of extraction efficiency and

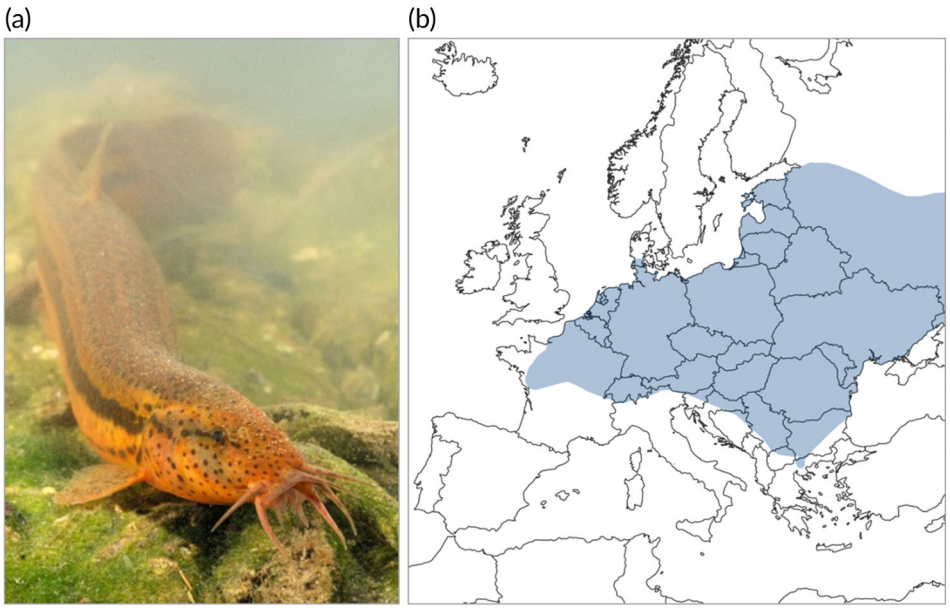

FIGURE 1 (a) Occurrence of the European weather loach (*M. fossilis*) (©Vilda/Rollin Verlinde) and (b) its native distribution area (adapted from Lelek, 1987)

to be able to infer PCR inhibition (Furlan *et al*., 2016). We also conducted a series of aquarium experiments to further validate our new ddPCR assay and to assess the relationship between eDNA concentration and fish abundance. Finally, we tested our method and compared it with conventional qPCR on a large number of natural samples from 96 water bodies in northern Belgium.

2 | MATERIALS AND METHODS

The use of fish from the breeding stock in the aquarium experiments at the Research Institute for Nature and Forest (INBO) complied with Belgian animal welfare laws, guidelines and policies as approved by the Animal Welfare Department within the Flemish public administration (see Appendix 1 for additional information about the other ethical questions).

2.1 | Historical records, selection of field sites and sample collection

As a first step, we generated an overview of the distribution range of the weather loach, *M. fossilis*, in Flanders (northern Belgium) by combining all known and/or officially registered localities where *M. fossilis* was historically found (such as visual observations, data of fyke netting, traps or electrofishing). This information allowed us not only to obtain a temporal overview and trend of these recordings, but also to select locations for water sampling and further eDNA analyses. Next, 75 locations were selected based on historical records of the species presence and 21 additional sites were chosen for further screening on the basis of environmental traits and habitat modelling (Belpaire *et al*., 2016).

In 2017, 2018, and 2019, we sampled 26, 28, and 43 locations, respectively (96 in total). Over the three years of surveying, all samples were collected between mid-June and the beginning of July. At the 96 sampling sites, a large number of 0.5 l subsamples (ranging from 20 up to 40 depending on the size of the water body under study) were taken around the ditch, canal, pond, *etc*. These were then pooled to obtain a single integrated and homogenous sample for each location. Water was sampled just below the water surface (±10 cm) using a long sterile sampling pole with a sterile Whirl-Pak bag (Sigma-Aldrich, Overijse, Belgium) attached at the end. In more high-flow environments, subsamples were always taken consecutively in an upstream direction to avoid resampling or disturbance of the water column. As filtering a sufficient amount of water is a prerequisite for maximizing detection probabilities (Sepulveda *et al*., 2019; Wilcox *et al*., 2016), we filtered the merged water samples through a large 0.22 μm enclosed polyethersulfone Steripak-GP sterile filter capsule (SPGPM10RJ, Merck Millipore, Darmstadt, Germany) on site. A Vampire sampler pump (Buerkle, Bad Bellingen, Germany) with disposable silicone tubing was used to filter substantial amounts of water until filter saturation was reached. The total amount of filtered water varied among sites, but was carefully measured and recorded for further calculation of target eDNA concentration. After filtering, the filter was air dried using the Vampire sampler pump, capped at both ends and immediately stored at −21°C in a BlueLine box (delta T, Fernwald, Germany) until DNA extraction. During each of the eDNA sampling campaigns, a field negative control was included using a 2 l deionized water sample to test for potential cross-contamination during filtering. This resulted in a total of 99 filters that were stored for further analyses. All reusable field material was decontaminated between sites with 2% Virkon S (Antec-DuPont International LTD, Sudbury, Suffolk, UK) as a biosafety precaution and to avoid potential DNA cross-contamination (US Fish and Wildlife Service, 2017).

2.2 | Set-up of aquarium experiment

We tested to what extent we can relate *M. fossilis* biomass to eDNA concentration in a controlled aquarium experiment at the INBO facility in Linkebeek (Belgium). Six 20 l aquariums were filled with ground

water from a draw-well at the end of May, and one, two, four, eight, 16, or 32 juvenile *M. fossilis* individuals were then added to each. The wet weight of the individuals assigned to each aquarium was determined before the onset of the experiment to give total biomass within each set up. All aquariums were allowed to equilibrate for 48 h before one 0.5 l water sample was taken. Using a sterile 60 ml Luer lock syringe, each water sample was subsequently filtered through a 0.45-µm enclosed PVDF Sterivex-HV filter capsule (SVHVL10RC, Merck Millipore, Darmstadt, Germany). After filtration, filters were air-dried, capped at both ends and stored at −21°C until further analyses in the laboratory.

2.3 | Primer/probe design and assay sensitivity

A species-specific set of primers and probe amplifying a 119 bp fragment of the Cytochrome C Oxidase subunit 1 gene (COI) of *M. fossilis* was designed using the Geneious Software (version 11.1.5) (https://www.geneious.com; Kearse *et al*., 2012) (see Table 1). We aligned all COI sequences from *M. fossilis* retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/nucleotide/) (see Appendix 2) and used the consensus sequence for designing the assay. The specificity of the assay was further assessed *in silico* using the primer-blast tool from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Results were confirmed by visual alignment of the assay against COI sequences from 45 generic fish species (Appendix 2) and seven closely related Cobitidae species (Appendix 3) known to be or likely to be present in the same ecosystems as the targeted organism. This was undertaken using the "multiple alignment" function on Geneious. Furthermore, an *in vitro* qPCR validation was conducted on tissue-derived extracts from six *Cobitis taenia* (Linnaeus, 1758) (spined loach) specimens and three invasive oriental loach (*Misgurnus bipartitus*) (Sauvage and Dabry de Thiersant, 1874) specimens.

To determine the relative sensitivity, limit of detection (LOD) and limit of quantification (LOQ) of the ddPCR primer/probe assay utilized, we conducted a serial dilution experiment with a positive control (*i.e*., DNA extracted from tissue sample of *M. fossilis*). The serial dilution was run with a total cellular DNA sample concentrated at 1 ng µl^{-1} (Quantus™ Fluorometer Promega, Leiden, Netherlands), that was first 1:100 diluted to obtain a starting point for a seven-step series of 5-fold dilutions (*e.g*., 1/100, 1/500, 1/2500, *etc*.). The series included 10 replicates of each dilution and 10 negative controls. The starting dilution of this series (1/100), measured with ddPCR, was 17.8 copies µl^{-1} (S.D. = 1.5) and this was also used as positive control in the ddPCR analyses (see below). The LOD was determined as the lowest concentration at which at least one positive detection was measured in each of the 10 replicates (Mauvisseau, Burian, *et al*., 2019b; Tréguier *et al*., 2014). We specified the LOQ as the lowest concentration in the dilution series in which 90% of the replicates fall into the S.D. (Forootan *et al*., 2017).

2.4 | DNA extraction and implementation of an IPC

All eDNA samples were stored and processed prior to PCR in a dedicated PCR-free building for low copy number template extractions, with controlled DNA-free, high-efficiency particulate air (HEPA)-filtered compartments with positive air pressure to avoid any contamination of eDNA samples. DNA extractions of both filter types (Sterivex and Steripak filters) were subjected to two different extraction protocols. In both protocols, an internal positive control (IPC) (KWR Watercycle Research Institute, Nieuwegein, Netherlands) was added in the first step of the extraction to evaluate extraction efficiency. This IPC contained approximately 30,000 copies µl^{-1} of a plasmid with a 149 bp insert sequence from Dengue virus type 2 (GenBank M29095.1) and can be quantified with the primers and probes shown in Table 1 with ddPCR as well as qPCR (NEN 6254, 2012).

One month prior to DNA extraction of the Steripak filters (field samples), 60 ml of sarkosyl preservation and lysis buffer (100 mM Tris, 100 mM EDTA, 10 mM NaCl, 1% sodium *N*-lauroylsarcosinate) were added via the inlet of each filter. Each filter was then recapped and shaken for 5 min at 800 rpm on a S50 shaker (CAT Ingenieurbüro, Staufen, Germany). Filters were stored in the dark at 22°C until extraction. Extraction and purification followed a modified protocol for Envirochek capsule filters (Civade *et al*., 2016) (see Appendix 4 for further details).

The frozen Sterivex filters from the aquarium experiment were extracted following a slightly modified version of the $SX_{CAPSULE}$ method (suitable for filters without preservation buffer as in Spens *et*

TABLE 1 Primers and probes for targeting a 119 bp COI sequence of European weather loach (*M. fossilis*) and a 149 bp plasmid insert sequence from Dengue virus type 2 (GenBank M29095.1) as internal positive control used in the present study

Target species/gene	Name	Primer/probe	Sequence (5′–3′) with modifications	Reference
M. fossilis COI	Mf-COI-F	Forward	CCCCCGACATAGCATTTCCG	Present study
	Mf-COI-R	Reverse	AACTGTTCAGCCTGTCCCAG	Present study
	Mf-COI-P	Probe	(6-FAM)CTCGTTCCTCCTTCTGCTGG(ZEN/IBFQ)	Present study
IPC/plasmid	IPC-D2-F	Forward	ATGACAGCCACTCCTCCG	NEN 6254 (2012)
	IPC-D2-R	Reverse	GGAACGAACCAAACAGTCTTC	NEN 6254 (2012)
	IPC-D2-P	Probe (for ddPCR)	(HEX)AGCAGAGACCCATTCCCTCAGAGC(ZEN/IBFQ)	NEN 6254 (2012)
	IPC-D2-P	Probe (for qPCR)	(Cy5)AGCAGAGACCCATTCCCTCAGAGC(IBRQ)	NEN 6254 (2012)

al., 2017). The only modification consisted of the addition of 2.0 μl of IPC to the lysis buffer containing 718 μl of ATL buffer and 80 μl of proteinase K. DNA extracts obtained from both filter types were finally eluted to 100 μl (see Appendix 4 for further details).

2.5 | ddPCR analyses

ddPCR was conducted using a QX200 ddPCR system (Bio-Rad, Temse, Belgium) in 20 μl. This system includes an Automated Droplet Generator, generating thousands of droplets from one ddPCR reaction containing the template DNA from any given sample. Each reaction contained 10 μl of Bio-Rad ddPCR supermix for probes (no deoxyuridine triphosphate), 750 nM of each primer, 375 nM of each probe (IDT, Leuven, Belgium) and 4 μl of template DNA, and was adjusted to the final volume of 20 μl by adding diethylpyrocarbonate (DEPC) water (Sigma-Aldrich, Overijse, Belgium). After droplet generation, the ddPCR 96-well plate (Bio-Rad, cat no. 12001925) was sealed with pierceable foil (Bio-Rad, cat no. 181-4040) and brought into a C1000 Touch™ Thermal Cycler with a 96-well Deep Reaction Module (Bio-Rad). PCR conditions were 10 min at 95°C, followed by 40 cycles of denaturation for 30 s at 94°C and extension at 56°C for 1 min, with a ramp rate of 2°C s^{-1}, followed by 10 min at 98°C and a hold at 12°C. The optimal primer annealing temperature of 56°C (*i.e*., with optimal separation between positive and negative droplets and highest amplitude of positive droplets) was determined using a PCR gradient, which was also the ideal annealing temperature for the IPC used in each of the samples. Following amplification, all samples were incorporated into a QX200 droplet reader (Bio-Rad) to visualize the total amount of target-positive and target-negative droplets. Calculation of eDNA copy numbers (per reaction volume) was performed by QuantaSoft software (v.1.7.4, Bio-Rad) and was estimated using the ratio between positive and negative droplets within a sample, using Poisson statistics (Miotke *et al*., 2015). Fluorescent thresholds for positive signals were determined according to QuantaSoft software instructions, and all droplets beyond this fluorescence threshold (Amplitude >3000) were counted as positive events and those below it as negative events. Purified DNA samples from all Steripak filters were run the first time with both the *M. fossilis* target assay and the IPC primers/probe assay in duplex using undiluted DNA template. The number of positive IPC droplets per sample was further utilized to obtain an estimate of effective quantity (Q_e, see below). In a next step, the same DNA samples were run a second time using seven replicates all on different PCR plates, but this time in simplex with only the *M. fossilis* target assay on undiluted eDNA template. Altogether this resulted in a total of eight ddPCR analyses that were run for each DNA sample. The DNA extracted from Sterivex filters was run similarly, but only including three replicates per sample. On each ddPCR plate, negative and positive controls were included. The negative (no-template) controls consisted of IDTE pH 8.0, whereas the positive controls consisted of either DNA extracted from *M. fossilis* tissue material at a concentration of 1 ng μl^{-1} diluted 1:100 for the *M. fossilis* target assay or a 1:100 dilution of our IPC stock (~30,000 copies μl^{-1}) for the IPC assay.

2.6 | qPCR analyses

To test the sensitivity of qPCR compared to ddPCR, we selected 15 locations that scored positive on *M. fossilis* via ddPCR, but had eDNA concentrations that were situated around the LOQ to be able to compare the sensitivity of both methods at these critically low levels. The eDNA extracts were analysed with the assay designed in this study, using eight qPCR replicates. qPCR reactions were performed on a Bio-Rad CFX96 Touch real-time PCR detection system in a total volume of 20 μl, which included 4 μl of template eDNA, 10 μl of Bio-Rad SsoAdvanced Universal Probes Supermix, 200 nM of each primer and 100 nM of each probe. A final volume of 20 μl was adjusted by adding DEPC water. Cycling conditions were 95°C for 5 min, followed by 45 cycles of 95°C for 30 s and 56°C for 1 min. A 1:10 dilution series of *M. fossilis* DNA (0,5 ng μl^{-1}, Quantus™ Fluorometer), ranging from 10^{-1} to 10^{-4} ng μl^{-1}, was used as a qPCR standard. Negative controls and positive tissue extracts were run alongside the samples. All samples were finally run eight times, diluted 1:2 to avoid inhibition, and in duplex with both the *M. fossilis* target assay and the IPC primer/probe assay. Data was analysed using the CFX Manager Software (Bio-Rad).

2.7 | Data analysis

To test for a significant trend in the number of *M. fossilis* observations over time, a Spearman rank correlation was calculated. A Pearson correlation was calculated to test for a linear and significant relationship between the measured number of copies per microlitre resulting from the ddPCR analyses and the calculated number of copies in the serial dilution experiment on the one hand, and the total biomass of *M. fossilis* individuals used in the aquarium experiment on the other.

From the total volume of water filtered and the obtained IPC recovery after extraction, we calculated the effective quantity of filtered water included in the DNA sample (Q_e) as:

$$Q_e = \left(\frac{C_{\text{IPC observed}}}{C_{\text{IPC calculated}}}\right) \times \left(\frac{1}{V_w}\right)$$

where $C_{\text{IPC observed}}$ is the obtained sample-specific copy number of IPC per microlitre measured via ddPCR on the final undiluted DNA extract, $C_{\text{IPC calculated}}$ is the number of copies of the positive IPC control included (from our IPC stock) before extraction and measured via ddPCR (on average 1099 ± 55 copies μl^{-1}) and V_w is the total amount of water filtered. Q_e can be used as a threshold or indicator of minimal effective quantity needed for reliable interpretation of positive and negative ddPCR detections (see Table 2). A Pearson correlation was calculated between Q_e and the total volume of water filtered to test for potential co-linearity between both variables.

To quantify the total number of *M. fossilis* copies present per litre of water, we converted the mean number of copies measured in each of the ddPCR replicates for each location according to the following formula (adapted from Agersnap *et al*., 2017 and Rusch *et al*., 2018):

$$C_X = \left[\frac{C_{rdd} \times \left(\frac{V_e}{V_r}\right)}{V_w}\right]$$

Where C_X is the number of target eDNA copies per litre of filtered water, C_{rdd} is the ddPCR calculation of eDNA copy numbers per reaction volume (20 μl), adjusted for a 10% loss during droplet generation, V_e is the total elution volume after extraction, V_r is the volume of eluted extract used in the ddPCR reaction and V_w is the volume of filtered water.

3 | RESULTS

3.1 | Historical records

Summation of the historical *M. fossilis* records over different time intervals revealed a significant decrease (r = −0.9856, P = 0.0006) in the number of observations over time (Figure 2). Before 1950, 50 official records of the species across Flanders were found, whereas this number collapsed to only four observations during the last decade (between 2011 and 2019). Most of these observations were located in the eastern part of Flanders, within the river basins of the Demer, Dijle, Nete and Meuse (Figure 3a). Since 2000, the species has only been observed at seven single locations.

3.2 | Sensitivity of the ddPCR assay and relationship between eDNA concentration and number of individuals

The laboratory tests of the dilution series showed that the decline in measured concentrations followed a nearly perfectly linear relation (r = 0.977, P = 0.0042) (Figure 4). The LOD (*i.e*., the dilution at which *M. fossilis* can still be detected in one of the eight replicates) was 1/312,500, with a measured concentration of 0.07 copies μl^{-1}. The following dilution, 1/1,562,500, did not result in any amplification for any of the replicates (Figure 4). The LOQ, on the other hand, was found to occur at a concentration of 0.14 copies μl^{-1} (1/12,500 dilution) (Figure 4).

The aquarium experiment revealed a significant and positive relationship between the number of individuals and the number of copies that were detected per microlitre of DNA (r = 0.959, P = 0.002; Figure 5). Here, the number of copies per microlitre ranged from 4.2 copies μl^{-1} when only one individual was included in the aquarium to 549 copies μl^{-1} when 32 individuals were present.

3.3 | DNA quality and detection and quantification of *M. fossilis* by ddPCR

From the 96 field samples, on average 5.0 ± 0.4 l of water was filtered through the Steripak filters (ranging from 0.5 l to a maximum of 25.0 l). Purified DNA extracts obtained from these samples yielded a mean DNA concentration of 92.0 ± 58.7 ng μl^{-1} (see Appendix 5). The quantification efficiency (Q_e) of the samples was on average 1.11 l (ranging from 6.96 to 0.03 l) and did not correlate with the volume of water filtered (r = −0.082, P = 0.426). Positive detection of *M. fossilis* DNA via ddPCR was found in 43 of the samples (44.8%). The calculated number of copies per litre of water (within these positive samples) was on average 60.23 (ranging from 666.4 to 0.333 copies l^{-1}; Figure 3b). However, 31 (72%) of the positive samples had a concentration that was situated below the LOQ. From the 53 samples that were negative (*i.e*., no *M. fossilis* DNA amplification), seven samples had Q_e < 0.2 l and therefore needed to be omitted from further interpretation because of their substantial risk of a false-negative (see Table 2 and Appendix 5).

3.4 | ddPCR *versus* qPCR

From the 15 selected samples in which we detected low eDNA concentrations of *M. fossilis* via ddPCR (concentrations ranging between 0.018 and 0.288 copies μl^{-1}, with detection rates ranging between two and eight positive replicates per sample), only two samples

TABLE 2 Decision table for further interpretation of eDNA samples based on the ddPCR measurement of the target DNA concentration and the Q_e of analysed eDNA based on the IPC and filtered volume of water

	Positive detection (>LOD)		Negative detection (<LOD)
Q_e/C_{rrd}	>LOQ = >quantitative interpretation	< LOQ = >qualitative interpretation	<LOQ
>Q_e threshold[a]	Target present and allows quantitative estimation (12/96 samples)	Target present but too rare for quantitative estimation (28/96 samples)	Target not detected, low risk of false-negative interpretation (true negative) (46/96 samples)
<Q_e threshold[a]	Target present but unreliable quantitative estimation and risk of underestimation (0/96 samples)	Target present but unreliable quantitative estimation and risk of underestimation (3/96 samples)	Target not detected, substantial risk for false-negative interpretation (7/96 samples)

[a]In this study the Q_e threshold was set at 0.2 l.

showed a positive signal when analysed via qPCR (see Table 3). Both samples resulted in mean C_q values of 48.64 ± 5.10 and 35.82 ± 0.08 obtained from two and only one positive replicate, respectively (Table 3).

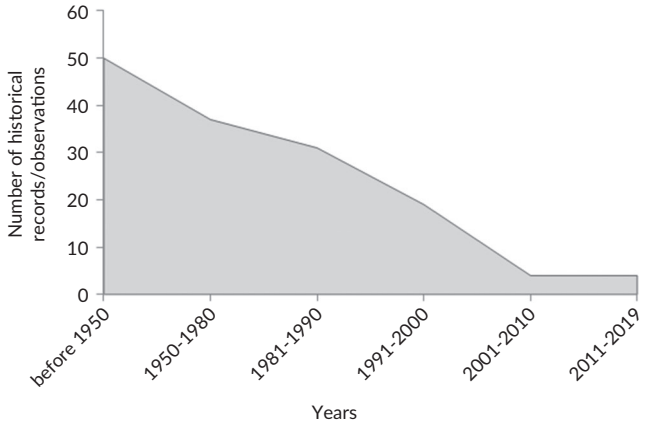

FIGURE 2 Time series of the historical records of the European weather loach (*M. fossilis*) before 1950 until mid-2018 across Flanders (Belgium)

4 | DISCUSSION

Historical records of *M. fossilis* (*i.e.*, fishing data and visual observations) in Flanders (Belgium) show a remarkable decline from 1950 onwards, with only seven documented sites where this species has been recorded since 2000. Considering that during the last decade the European weather loach has received increasing attention in terms of monitoring efforts, these observations suggest that the species is becoming extremely rare and even entering the brink of extinction (at least at the regional or national level assessed here). Indeed, this pattern appears to be the same across many other European countries, such as the Czech Republic (Lusk *et al*., 2004), Austria (Spindler, 1997), Croatia (Mrakovčić *et al*., 2008), Poland (Witkowski *et al*., 2009), Denmark (Sigsgaard *et al*., 2015) and the Netherlands (Kranenbarg & de Bruin, 2009), and points to an urgent need for a method to assess current populations where conservation of this species can be focused (Hartvich *et al*., 2010). Given the difficulties in detecting and monitoring this obscure fish species via conventional survey methods, eDNA monitoring is a likely candidate for a new and improved survey tool (Sigsgaard *et al*., 2015).

In this study, we highlight that 45% of the locations assessed (from the approximately 100 locations spanning a 3 year survey effort across Flanders, Belgium) were positive for this declining

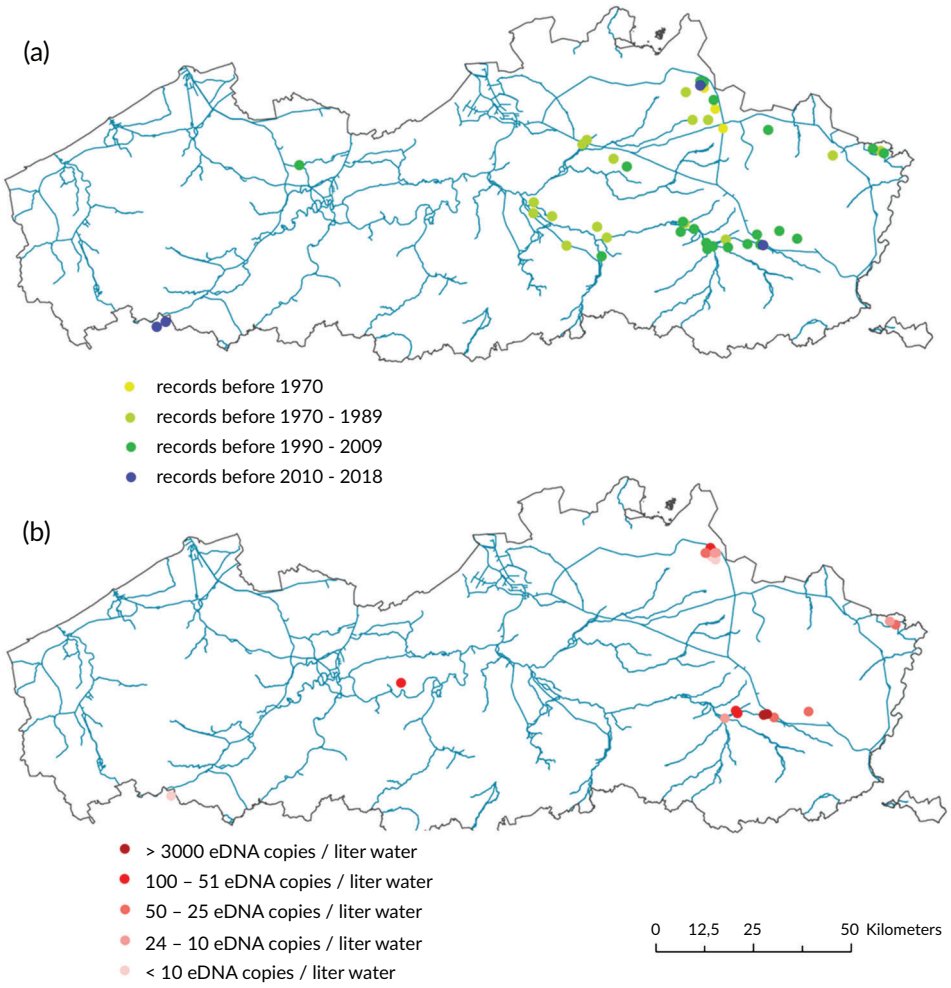

FIGURE 3 Distribution map of (a) historical observations and (b) locations with positive eDNA detection of the European weather loach (*M. fossilis*) in Flanders (Belgium). eDNA sampling occurred during 2017–2019, with different colours representing variable eDNA concentrations measured per litre of water via ddPCR

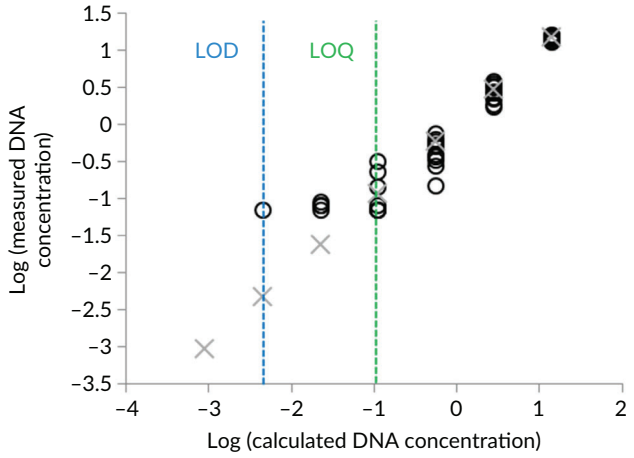

FIGURE 4 Experimentally obtained limits of detection (LOD) and quantification limit using a 5-fold dilution series with seven steps (*e.g.*, 1/100, 1/500, 1/2500, *etc.*), with 10 replicates each. The digital PCR log of measured concentration is along the *y* axis and the log of the calculated concentration is along the *x* axis. The LOD is set at 0.07 copies μl^{-1} and the LOQ is set at 0.14 copies μl^{-1} (1/12,500 dilution). The starting dilution of this series (1/100) measured with ddPCR was 17.8 copies μl^{-1} (S.D. = 1.5). ○ measured concentration, × expected concentration

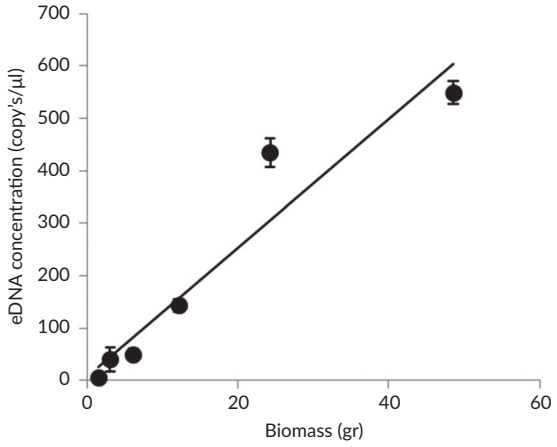

FIGURE 5 Relationship between the number of *M. fossilis* individuals and the measured eDNA concentration (number of copies per microlitre) via ddPCR obtained from a series of similar sized aquaria to which one, two, four, eight, 16 or 32 juvenile *M. fossilis* individuals were added. DNA was obtained from similar sized water samples (180 ml) taken 48 h after *M. fossilis* individuals were added to aquaria and filtered through a 0.45-µm enclosed PVDF Sterivex-HV filter capsule

species. Although at first glance this looks positive, only 12 sites were classed as harbouring relative dense populations (*i.e.*, each of the PCR replicates scored positively and resulted in high eDNA concentrations). For instance, electric fishing at one of these sites easily resulted in the catch of 25 individuals over a 100 m transect. The remaining samples that scored positively only had a few positive droplets, which were (in many cases) only restricted to a few ddPCR replicates. Although we acknowledge that the choice of a threshold for positive detection can vary greatly among studies and significantly influence the overall results, the majority of our "weak" positive detections clearly fell below a strict LOD that is in medical diagnostic applications set at a concentration that produces at least 95% positive replicates (Baker *et al.*, 2018). Such a stringent LOD threshold is expected to indicate a concentration of three molecules per reaction under error-free conditions (Ståhlberg & Kubista, 2014). For the majority of eDNA-based studies such a LOD is, however, likely to be a little strict as eDNA samples are naturally characterized by a substantial amount of noise. Maintaining such a strict threshold would therefore likely result in a large number of false-negative detections. Moreover, the detection of even one target molecule in one out of eight replicates (12.5% detection probability in our case) is in our view very informative, as it may reveal new locations that deserve further investigation. One of the main arguments for implementing high threshold values for LOD and LOQ is to avoid false-positive detections, which is of great concern in eDNA studies as it may lead to overoptimistic or overpessimistic results in case of rare/protected or harmful/invasive species, respectively (Darling & Mahon, 2011). This is an overall problem in many aquatic systems, as eDNA-based methods are generally expected to detect organisms at lower densities than conventional methods, making it difficult to invalidate the risk of false-positives and to directly compare the sensitivity of eDNA to conventional methods (Mahon *et al.*, 2013). We are, however, confined in our findings as none of the negative control samples (both field and/or PCR blanks) gave a positive signal. Furthermore, additional fishing efforts were carried out at six of the locations where a "weak" positive signal of *M. fossilis* (<LOQ) was detected and resulted in the catch of the species at four locations (Figure 6), albeit at very low densities (less than five individuals). These results indicate that when stringent LOD and LOQ tolerance thresholds are set, there is the possibility that populations of this target species may be overlooked. In this context, the inclusion of a quantitative estimate of sensitivity or reliability can be very helpful for the interpretation of such indistinct eDNA results.

In general, each methodology (ddPCR or qPCR) is characterized by a more or less fixed LOD and LOQ, depending on the chemistry of the reagents, the efficiency of the assay, the optimal annealing temperature, *etc.* In order to improve the sensitivity of an eDNA survey, one therefore needs to focus on other aspects that can critically affect the resolution of eDNA-based survey methods. An important component that significantly affects the outcome of an eDNA survey is the effective quantity of water used, which is in turn a function of the amount of water that is sampled/filtered and the efficiency with which the DNA is extracted from that sample (Wilcox *et al.*, 2018). Some studies also point on the importance of filtering a consistently large amount of water, as eDNA can occur in clumped aggregations (Turner *et al.*, 2014). This aspect can, in our view, largely be overcome by using merged and homogenized samples. Insights into the efficiency at which DNA is extracted can easily be obtained via the inclusion of an internal positive control, which also offers valuable information on the occurrence of inhibition (Furlan *et al.*, 2016). In this study, we used a plasmid as an IPC to obtain an extraction efficiency

TABLE 3 Overview of the comparison of ddPCR *vs.* qPCR analyses on a subsample of 15 positive samples in which *M. fossilis* was previously detected with ddPCR

	ddPCR			qPCR		
Sample	**Replicates**	**Prob**	**Concentration**	**Replicates**	**Prob**	**C_q**
1	8	0.25	0.018 ± 0.035	8	0	
2	8	0.75	0.103 ± 0.075	8	0	
3	8	0.5	0.288 ± 0.619	8	0	
4	8	0.5	0.0863 ± 0.129	8	0	
5	8	0.625	0.0813 ± 0.077	8	0	
6	8	0.75	0.0938 ± 0.072	8	0.250	48.64 ± 5.10
7	8	0.625	0.0713 ± 0.0610	8	0.125	35.82 ± 0.08
8	8	0.875	0.120 ± 0.082	8	0	
9	8	1	0.149 ± 0.073	8	0	
10	8	0.375	0.038 ± 0.054	8	0	
11	8	0.5	0.063 ± 0.071	8	0	
12	8	0.625	0.110 ± 0.117	8	0	
13	8	0.625	0.098 ± 0.111	8	0	
14	8	0.75	0.099 ± 0.075	8	0	
15	8	0.375	0.094 ± 0.174	8	0	

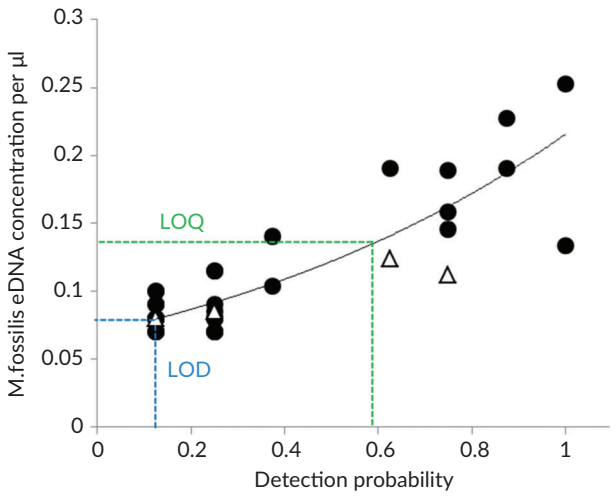

FIGURE 6 Relationship between the detection probability (*i.e.*, the ratio of positive replicates/total number of replicates) and the mean concentration of *M. fossilis* per microlitre of DNA obtained from eight ddPCR replicates per sample (black circles). The experimentally obtained LOD (≤0.07 copies μl^{-1}, blue dotted line) and LOQ (≤0.014 copies μl^{-1}, green dotted line) are plotted on the graph. White triangles represent locations where the species was effectively caught via electric fishing in the weeks/months after eDNA sampling. ● eDNA detection, △ eDNA detection + fishing

index that can allow for the calculation of quantification efficiency (Q_e) in combination with the total amount of water volume that is filtered. Documentation of such quantification efficiency estimates in eDNA studies would be very helpful to obtain insights into the sensitivity of a certain sample and to compare the outcome of different studies or methods in terms of quality assessment (see Wilcox *et al.*, 2018). On the basis of the Q_e obtained in this study, we set a threshold (Q_e < 0.2 l) that can be used for reliable interpretation of our eDNA data. In our case, seven out of 53 negative samples had an effective quantity below the threshold of 0.2 l, which led us to conclude that these samples might be exposed to a substantial risk of false-negative detection and need further attention.

The fact that in a number of our samples only one or two of the eight ddPCR replicates gave a positive result (see samples with a detection probability ≤0.125 in Appendix 5) illustrates that a large number of PCR replicates are preferential for the detection of extremely rare species or low DNA concentrations in a given system. Wilcox *et al.* (2016, 2018) indeed also highlighted the importance of the volume of sample that is finally analysed in the PCR as it can have profound effects on the outcome of an eDNA survey (*i.e.*, positive detection or not). In this context, ddPCR can offer another advantage over conventional qPCR as samples can, in many cases, be run under higher concentrations without any sign of inhibition. In this study, for instance, we were able to run the natural samples undiluted in the ddPCR reactions, whereas the IPC concentrations revealed that the same samples needed a 2-fold dilution prior to qPCR analyses to circumvent inhibition. Dilution of target DNA prior to analysis may ultimately reduce the effective quantity of DNA used and thus increase the risk of false-negative detections via qPCR when the target DNA is occurring in very low concentrations (Ficetola *et al.*, 2008; Hyman & Collins, 2012; Thomsen *et al.*, 2012). It may also explain the remarkable discrepancy in sensitivity of ddPCR compared to qPCR in the detection of *M. fossilis* in our natural samples.

4.1 | In conclusion

Our work clearly shows that for the detection of *M. fossilis*, ddPCR analyses offer a great advantage in the resolution of detection compared to both qPCR-analyses and conventional fishing techniques.

Previous work has already calculated that eDNA monitoring via qPCR is 1.5 times more cost-effective than electrofishing (Herder *et al*., 2014; Sigsgaard *et al*., 2015), with a 3-fold increase in detection rate (Kranenbarg *et al*., 2014). Besides these advantages, eDNA analyses are easier to carry out in a standardized way and thus are less prone to variation caused by sampling intensity and expertise. Beyond confirmation of positive detections, spatially and temporally repeated eDNA sampling may be incorporated into an occupancy modelling framework to account for imperfect detection of rare species (MacKenzie *et al*., 2002; Rees *et al*., 2014). The efficient and standardized method of sampling makes eDNA a particularly powerful tool for broad-scale monitoring of species, detection of new populations, or temporal monitoring of known populations. Moreover, the combination of eDNA data with occupancy modelling could additionally allow robust identification of species habitat associations and estimation of colonization and extinction probabilities (MacKenzie *et al*., 2003) over scales which are prohibitively labour- and cost-intensive using conventional sampling approaches.

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Note. The list includes the number of replicates that were run on both ddPCR and qPCR, and the detection probability (positive replicates/total number of replicates), the mean copy number per microlitre over all replicates ±S.D. (from ddPCR) and the mean C_q ± S.D.

APPENDIX 1 | Answers to the ethical questions of the journal

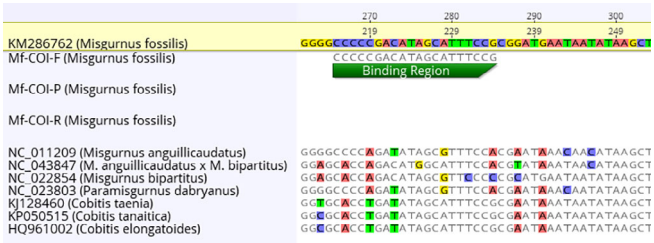
1 Were fishes collected as part of faunal surveys? *No, the study consisted of noninvasive eDNA sampling.*
2 Were fishes killed during or at the end of your experiment (*e.g.*, for tissue sampling)? *No.*
3 Were surgical procedures performed? *No.*
4 Did the experimental conditions severely distress any fishes involved in your experiments? *No.*
5 Did any procedures (*e.g.*, predation studies, toxicity testing) cause lasting harm to sentient fishes? *No.*
6 Did any procedure involve sentient, unanaesthetised animals that were subjected chemical agents that induce neuromuscular blockade, such as muscle relaxants? *No.*

APPENDIX 2 | List of species and related Genbank accession numbers utilized when developing and validating the species-specific primers and probe used in this study

Species	Accession number
Misgurnus fossilis (Linnaeus, 1758)	KM286763.1, KM286765.1, KM286764.1, KM286762.1, JQ011427.1, JQ011436.1, KR477226.1, KR477070.1, KR477225.1, KR477069.1, KR477224.1, KR477068.1, KR477223.1, KR477067.1
Alburnus alburnus (Linnaeus, 1758)	KJ552594.1
Alytes obstetricans (Laurenti, 1768)	JN379850.1
Ameiurus nebulosus (Lesueur, 1819)	KX909526.1
Anguilla anguilla (Linnaeus, 1758)	KX870811.1
Blicca bjoerkna (Linnaeus, 1758)	MF135910.1
Bufo bufo (Linnaeus, 1758)	JN379844.1
Carassius gibelio (Bloch, 1782)	KJ553038.1
Cyprinus carpio Linnaeus, 1758	KR861884.1
Epidalea calamita (Laurenti, 1768)	KJ128665.1
Esox lucius Linnaeus, 1758	KR477035.1
Gasterosteus aculeatus Linnaeus, 1758	KP823183.1
Gobio gobio (Linnaeus, 1758)	HQ961007.1
Gymnocephalus cernua (Linnaeus, 1758)	HQ960571.1
Hyla arborea (Linnaeus, 1758)	KP697937.1
Ichthyosaura alpestris (Laurenti, 1768)	KP697926.1
Lepomis gibbosus (Linnaeus, 1758)	KJ553934.1
Leuciscus burdigalensis Valenciennes, 1844	KC354965.1
Leuciscus idus (Linnaeus, 1758)	MF135890.1, HM560271.1
Lissotriton helveticus (Razoumovsky, 1789)	GQ374501.1
Lissotriton vulgaris (Linnaeus, 1758)	KJ128675.1

Species	Accession number
Lithobates catesbeianus (Shaw, 1802)	EF525860.1
Lota lota (Linnaeus, 1758)	KR477219.1, KM286761.1, KM286759.1
Neogobius melanostomus (Pallas, 1814)	KP247496.1
Pelobates fuscus (Laurenti, 1768)	KP697819.1
Pelophylax esculentus (Linnaeus, 1758)	KP697836.1
Pelophylax lessonae (Camerano, 1882)	KP697908.1
Perca fluviatilis Linnaeus, 1758	KR477235.1
Ponticola kessleri (Günther, 1861)	KC501152.1
Proterorhinus semilunaris (Heckel, 1837)	KR477085.1
Pseudorasbora parva Temminck & Schlegel, 1846	KJ554179.1
Rana arvalis Nilsson, 1842	KP697924.1
Rana temporaria Linnaeus, 1758	FN813803.1
Rhodeus amarus (Bloch, 1782)	KJ554098.1
Rutilus rutilus (Linnaeus, 1758)	KJ554419.1
Salamandra salamandra (Linnaeus, 1758)	EF525911.1
Salmo trutta Linnaeus, 1758	KM287101.1, KX594647.1
Sander lucioperca (Linnaeus, 1758)	HQ960672.1
Scardinius erythrophthalmus (Linnaeus, 1758)	KM287129.1
Squalius cephalus (Linnaeus, 1758)	KC354982.1
Tinca tinca (Linnaeus, 1758)	EU716109.1
Triturus carnifex (Laurenti, 1768)	EF525962.1
Triturus cristatus (Laurenti, 1768)	EF525975.1
Umbra pygmaea (DeKay, 1842)	KM287172.1

3 | Multiple sequence alignment against the *Misgurnus fossilis* COI gene of the new assay primers and probe showing mismatches with sequences of seven closely related cobitidae species



4 | Extraction protocol used for eDNA extraction from Steripak filters

The PCR-free room for eDNA extraction was equipped with temperature-controlled HEPA filtered (H13) positive air pressure and overnight ultraviolet C (UV-C) treatment of benches and hoods. Before entering this extraction room, people had to put on protective clothing including a disposable body suit, mask, hair net, overshoes and first pair of gloves in an antechamber. On entering the extraction room, a second pair of gloves was worn. Before and after each manipulation all benches, hoods and all nonsterile material entering the room was carefully decontaminated with bleach containing minimum 3% NaOCl (50% commercial bleach) followed by 70% alcohol (Goldberg *et al*., 2016). All sample manipulations were performed in a class 100 cabinet inside the extraction room with H14 filter and UV-C air recirculation (Angelantoni, Massa Martana, Italy). One month after 60 ml of sarkosyl buffer (100 mM Tris, 100 mM EDTA, 10 mM NaCl, 1% sodium *N*-lauroylsarcosinate) was added to each filter, filters were shaken for 15 min at 800 rpm on a S50 shaker (CAT Ingenieurbüro, Staufen, Germany) and the buffer was transferred into two subsamples of 15 ml. The remaining half of the buffer was left in the capsule and stored again as a back-up. Consecutively, we added 33 ml of absolute ethanol, 2 ml of 5 M sodium chloride and 2.5 μl of plasmid DNA containing ~75,000 copies of an IPC to each tube. Next, each tube was inverted five times and stored overnight at −21°C. The following morning, frozen tubes were centrifuged at 15000 *g* for 15 min at room temperature. Supernatant was discarded and tubes with the remaining pellets were briefly put upside down on a tissue. In the following step, 720 μl of ATL buffer and 20 μl of proteinase K from the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was added to the first centrifuge tube and pulse vortexed vigorously to bring the pellet in complete suspension. This mixture was then transferred to the second centrifuge tube obtained from the same filter, and the

protocol was repeated. The resulting suspension of both tubes was transferred to a new 5 ml DNA LoBind tube (Eppendorf, Aarschot, Belgium) and incubated for 2 h in a SI-1400 rotating incubator at 56° and 5 rpm (Scientific Industries, New York, USA). The resulting lysate was centrifuged for 1 min at 6000 *g* to remove most of the particulate debris and 740 µl of supernatant was transferred to a new 5 ml DNA LoBind tube to which equal amounts of AL buffer and ice-cold ethanol was added. Samples were further processed using the Qiagen DNeasy Blood and Tissue Kit as per the manufacturer's instructions. Final elution was done in 100 µl of tris-EDTA (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) following already described protocol for Sterivex capsule filters (Spens *et al*., 2017). Briefly, both spin columns, four at a time, and TE were preheated at 70°C. After application of TE on the membrane spin columns were incubated 10 min at room temperature before centrifugation. Eluate was re-eluted again in the same way in a 1.5 ml DNA LoBind tube to maximize DNA yield and concentration. Samples underwent further purification with the DNeasy PowerClean Cleanup Kit (Qiagen) as per the manufacturer's instructions and were finally eluted again at 70°C in 100 µl of TE.

5 | Overview of the ddPCR results for *Misgurnus fossilis* at each sampling site

The list of sampling sites includes year of sampling, water type, volume of water filtered, effective quantity (Q_e) analysed, detection probability (ratio of positive replicates/total number of replicates) and the number of eDNA copies per litre filtered of water

Site no.	Year of sampling	Site name	Water type	Volume (l)	Q_e (l)	Detection probability	Copies per litre of water
1	2018	Hasselt (Herkenrode), site 2	Ditch	9.5	1.61	1.00	666.41
2	2018	Hasselt (Herkenrode), site 1a	Ditch	6.25	1.61	1.00	622.66
3	2019	Hasselt (Herkenrode), site 16	Ditch	3.5	1.73	1.00	376.49
4	2019	Hasselt (Herkenrode), site 10	Ditch	2	0.91	1.00	325.40
5	2019	Hasselt (Herkenrode), site 14	Pond/lake	2	0.86	0.88	248.31
6	2019	Hasselt (Herkenrode), site 1b	Ditch	1.8	2.22	1.00	68.32
7	2017	Kinrooi, site 1	Ditch	2	0.24	0.88	49.69
8	2017	Arendonk (Het Goorken), site 4	Ditch	2	0.39	1.00	30.79
9	2017	Arendonk (Het Goorken), site 5	Ditch	2	0.41	0.75	18.11
10	2019	Herk-de-Stad (Schulensbroek), site 29	Ditch	7.5	1.46	0.13	17.50
11	2017	Kinrooi, site 3	Stream	4	0.53	0.63	14.84
12	2017	Berlare, site 1	Pond	5.4	2.58	0.88	13.85
13	2017	Arendonk (Het Goorken), site 3	Pond	1.8	0.32	0.75	10.47[a]
14	2019	Hasselt (Herkenrode), site 3	Ditch/pond	8	0.09	0.25	10.29[a]
15	2019	Hasselt (Herkenrode), site 19	Ditch/pond	1.9	0.69	0.38	9.68[a]
16	2017	Genk (Het Wik), site 1	Pond	3.7	0.28	0.75	9.38[a]
17	2019	Hasselt (Herkenrode), site 21	Stream	8	2.26	1.00	8.92[a]
18	2019	Hasselt (Herkenrode), site 7	Ditch	10	0.39	0.25	8.71[a]
19	2019	Hasselt (Herkenrode), site 13	Ditch	4.2	1.61	0.75	8.48[a]
20	2019	Diest (Webbekoms Broek), site 5b	Pond	2	0.23	0.13	7.04[a]
21	2017	Hasselt (Kuringen), site 1	River	5.9	2.10	0.63	6.57[a]
22	2018	Herk-de-Stad (Schulensbroek), site 11	Ditch/pond	2.5	0.75	0.38	6.56[a]
23	2019	Hasselt (Herkenrode), site 11	Pond/ditch	3.1	0.20	0.13	6.25[a]
24	2017	Fretin (France)	Canal	1.7	0.34	0.25	6.25[a]
25	2018	Herk-de-Stad (Schulensbroek), site 5	Ditch	2.5	0.55	0.25	5.00[a]
26	2017	Arendonk (Het Goorken), site 8	Ditch	4.1	0.19	0.25	5.00[a]
27	2018	Herk-de-Stad (Schulensbroek), site 12	Ditch/canal	2	0.61	0.13	4.58[a]
28	2019	Diest (Webbekoms Broek), site 15	Ditch	25	0.65	0.25	3.43[a]
29	2018	Diest (Webbekoms Broek), site 1a	Ditch	12.5	0.95	0.25	3.33[a]
30	2017	Arendonk (Het Goorken), site 9	Ditch	4	0.20	0.13	2.50[a]
31	2019	Hasselt (Herkenrode), site 20	River	0.7	1.25	0.25	2.30[a]

Site no.	Year of sampling	Site name	Water type	Volume (l)	Q_e (l)	Detection probability	Copies per litre of water
32	2019	Herk-de-Stad (Schulensbroek), site 28	Ditch	15	1.30	0.13	2.27[a]
33	2019	Diest (Webbekoms Broek), site 1b	Ditch	6	0.60	0.13	2.19[a]
34	2018	Herk-de-Stad (Schulensbroek), site 10	Pond	7	0.59	0.13	1.67[a]
35	2019	Herk-de-Stad (Schulensbroek), site 30	Ditch	4	1.23	0.13	1.43[a]
36	2018	Herk-de-Stad (Schulensbroek), site 17	Ditch/canal	5	1.56	0.25	1.25[a]
37	2018	Herk-de-Stad (Schulensbroek), site 18	Ditch/canal	3.75	1.69	0.25	0.87[a]
38	2019	Diest (Webbekoms Broek), site 12	Ditch	0.85	1.41	0.13	0.83[a]
39	2019	Hasselt (Herkenrode), site 8	Ditch/canal	0.71	1.86	0.13	0.68[a]
40	2018	Herk-de-Stad (Schulensbroek), site 4	Pond/lake	3	3.28	0.13	0.56[a]
41	2018	Herk-de-Stad (Schulensbroek), site 20	Pond/ditch	3	6.96	0.13	0.45[a]
42	2019	Hoogstraten (Meerle)	River	3.6	4.89	0.25	0.35[a]
43	2019	Postel (Watering)	Pond/lake	2	2.07	0.13	0.33[a]
44	2019	Herk-de-Stad (Schulensbroek), site 26	Ditch/canal	4.4	5.27	0.00	0.00
45	2018	Herk-de-Stad (Schulensbroek), site 9	Pond/lake	6.25	3.27	0.00	0.00
46	2019	Herk-de-Stad (Schulensbroek), site 23	Ditch	3.7	3.19	0.00	0.00
47	2019	Hasselt (Herkenrode), site 18	Ditch	1.65	2.54	0.00	0.00
48	2018	Herk-de-Stad (Schulensbroek), site 16	Ditch/canal	8	1.89	0.00	0.00
49	2018	Herk-de-Stad (Schulensbroek), site 8	Ditch	1.5	1.80	0.00	0.00
50	2018	Herk-de-Stad (Schulensbroek), site 19	Ditch/canal	4	1.73	0.00	0.00
51	2019	Hasselt (Herkenrode), site 6	Lake/ditch	5	1.71	0.00	0.00
52	2017	Wasquehal (France)	Canal	5.8	1.70	0.00	0.00
53	2017	Herk-de-Stad (Schulensbroek), site 3	River	4.4	1.68	0.00	0.00
54	2018	Diest (Webbekoms Broek), site 8	Stream	2	1.61	0.00	0.00
55	2019	Herk-de-Stad (Schulensbroek), site 27	Pond/lake	7	1.49	0.00	0.00
56	2019	Hasselt (Kuringen), site 2	Ditch	6	1.36	0.00	0.00
57	2019	Diest (Webbekoms Broek), site 10	Ditch	10	1.30	0.00	0.00
58	2017	Berlare, site 2	Lake	5.5	1.10	0.00	0.00
59	2019	Hasselt (Herkenrode), site 12	Ditch	5.8	1.02	0.00	0.00
60	2019	Hasselt (Herkenrode), site 5	Stream	2.9	0.98	0.00	0.00
61	2019	Diest (Webbekoms Broek), site 14	Ditch	11.5	0.92	0.00	0.00
62	2019	Hasselt (Herkenrode), site 9	Ditch	2	0.92	0.00	0.00
63	2018	Diest (Webbekoms Broek), site 3	Canal	10	0.90	0.00	0.00
64	2017	Arendonk (Het Goorken), site 1	Ditch/canal	3.9	0.89	0.00	0.00
65	2017	Kinrooi, site 2	Stream	4	0.87	0.00	0.00
66	2017	Genk (Het Wik), site 2	Pond	4.1	0.84	0.00	0.00
67	2017	Berlare, site 3	Pond	6	0.81	0.00	0.00
68	2017	Herk-de-Stad (Schulensbroek), site 1	Lake	5.7	0.79	0.00	0.00
69	2017	Wambrechies (France)	Canal	6	0.69	0.00	0.00
70	2018	Diest (Webbekoms Broek), site 2	Ditch	2.25	0.69	0.00	0.00
71	2018	Herk-de-Stad (Schulensbroek), site 7	Ditch	8	0.61	0.00	0.00
72	2019	Herk-de-Stad (Schulensbroek), site 31	Ditch	0.85	0.58	0.00	0.00
73	2018	Herk-de-Stad (Schulensbroek), site 13	Ditch/canal	1.5	0.57	0.00	0.00
74	2018	Diest (Webbekoms Broek), site 4	Ditch/pond	1.75	0.55	0.00	0.00
75	2019	Diest (Webbekoms Broek), site 13	Ditch	1.9	0.54	0.00	0.00
76	2019	Hasselt (Herkenrode), site 17	Ditch	7.4	0.51	0.00	0.00
77	2017	Herk-de-Stad (Schulensbroek), site 2	Pond/lake	5.2	0.50	0.00	0.00
78	2019	Herk-de-Stad (Schulensbroek), site 24	Ditch	8	0.43	0.00	0.00

(Continues)

Site no.	Year of sampling	Site name	Water type	Volume (l)	Q_e (l)	Detection probability	Copies per litre of water
79	2018	Diest (Webbekoms Broek), site 7	Ditch	13	0.43	0.00	0.00
80	2017	Arendonk (Het Goorken), site 2	Ditch/canal	2.9	0.42	0.00	0.00
81	2019	Hasselt (Herkenrode), site 4	Stream	3.1	0.42	0.00	0.00
82	2019	Diest (Webbekoms Broek), site 11	Ditch/canal	2.4	0.40	0.00	0.00
83	2018	Herk-de-Stad (Schulensbroek), site 21	Pond	4	0.39	0.00	0.00
84	2019	Herk-de-Stad (Schulensbroek), site 22	Pond	1.1	0.37	0.00	0.00
85	2019	Herk-de-Stad (Schulensbroek), site 25	Ditch/pond	5.7	0.36	0.00	0.00
86	2019	Diest (Webbekoms Broek), site 9	Ditch	3.9	0.36	0.00	0.00
87	2018	Herk-de-Stad (Schulensbroek), site 14	River	0.5	0.34	0.00	0.00
88	2017	Arendonk (Goorken), site 6	Pond	6.6	0.26	0.00	0.00
89	2017	Genk (Het Wik), site 4	Pond	6	0.25	0.00	0.00
90	2018	Diest (Webbekoms Broek), site 6	Ditch	11.5	0.18	0.00	0.00[b]
91	2019	Hasselt (Herkenrode), site 15	Ditch/pond	2.8	0.18	0.00	0.00[b]
92	2017	Arendonk (Het Goorken), site 7	Ditch	4.8	0.14	0.00	0.00[b]
93	2018	Herk-de-Stad (Schulensbroek), site 6	Ditch	18	0.11	0.00	0.00[b]
94	2018	Herk-de-Stad (Schulensbroek), site 15	Ditch	3	0.08	0.00	0.00[b]
95	2017	Genk (Het Wik), site 3	Pond	3.9	0.03	0.00	0.00[b]
96	2018	Diest (Webbekoms Broek), site 5a	Pond	7.5	0.03	0.00	0.00[b]

[a]Target present but too rare for reliable quantitative estimation because <LOQ.
[b]Q_e < 0.2 l with risk of underestimating positives and false-negative interpretation.