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 eDNA-based approaches offer clear advantages over conventional field methods for monitoring rare and endangered species, accurate detection and quantification remains difficult and quality assessment is often poorly incorporated.

In this study, we developed and validated a novel 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 to define the limit of detection (LOD) and limit of quantification (LOQ), which were set at a concentration of respectively 0.07 copies per μl for the LOD and 0.14 copies per μl for the LOQ. A series of aquarium experiments revealed a significant and positive relationship between the number of individuals and the eDNA concentration measured. During a three 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 liter water, only twelve sites appeared to harbor relatively dense populations. The others, 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 harbored the species and therefore 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 that can be accommodated and higher sensitivity that is obtained.

Keywords: conservation, cryptic species, eDNA detection, ddPCR versus qPCR analyses, endangered, water sampling.

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.*, 2009). High mobility, as well as low density and/or cryptic behavior 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 non-detection error and its associated problems (i.e., so called false-negative detections) (Gu & Swithart 2004). In addition to their limited efficiency, conventional survey methods are laborious, expensive, and often invasive and harmful to the environment and its populations (UFC, 2014; 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, 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 (Jerde *et al.*, 2011; Thomsen *et al.*, 2012; Goldberg *et al.*, 2016; Lacoursière-Roussel *et al.*, 2016; Cristescu & Hebert, 2018; Coble *et al.*, 2019). Although the strength of this methodology is now widely accepted, there is an increasing need for rigorous, standardized and reliable estimates of sensitivity that allows 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 analyzed

(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 (McKee *et al.*, 2015; Goldberg *et al.*, 2016). Besides the aspect of water volume that is finally analyzed, one need to sample a water body in an integrated way to increase detection probabilities of rare or locally distributed species, since eDNA often shows a very patchy distribution in the water column, especially in lentic systems (Eichmiller *et al.* 2014; Dunker *et al.* 2016).

To be able to evaluate the outcome of any eDNA-based survey method, each of these aspects therefore needs to be incorporated.

In Europe, there are a number of declining species of freshwater fish of conservation concern. One of which, is the European weather loach or weatherfish, *Misgurnus fossilis* (Linnaeus, 1758), and is extremely difficult to monitor via conventional survey methods. Although its natural distribution range extends from northwestern France eastward to the Volga Basin (Fig. 1), the species is in steep decline, and has apparently disappeared from a large part of its former range (Meyer & Hinrichs, 2000; Hartvich *et al.*, 2010). 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 the modern-day European landscapes (Meyer & Hinrichs, 2000). As a consequence, the few remnant populations known and monitored, are often confined to

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drainage channels and ditches, where the practice of intensive weed removal and dredging pose additional threats to their survival. Further, 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, both in the number and size of its populations (Hanel & Lusk, 2005), which has been led to the species now being listed in the IUCN Red List of Threatened Species (Freyhof, 2011). Given the difficulty to monitor this species and the alarming decline throughout its range, a reliable eDNA monitoring method would be an important first step if conserving 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. Further we wanted to evaluate estimates of sensitivity that allows for quality assessment, better understanding of the eDNA results and finally 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, however, known to be sensitive to inhibitors (Doi *et al.*, 2015; Mauvisseau *et al.*, 2019a; Wood *et al.*, 2019). Many freshwater bodies are, however, loaded with organic compounds, either 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

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additionally assumed to have higher sensitivity and precision than qPCR (Hindson *et al.*, 2011; Doi *et al.*, 2015). Exploiting these advantages, we developed a new primers/probe assay for European weather loach that is compatible for 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 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 then tested our method, and compared it with conventional qPCR, on a large number of natural samples from 96 water bodies in Northern Belgium.

Materials and Methods

The use of fish from the breeding stock in the aquarium experiments at the 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 info about the other ethical questions).

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 (N-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 modeling (Belpaire *et al.*, 2016).

In 2017, 2018 and 2019, we sampled 26, 28 and 43 locations respectively (96 in total). All sampling was conducted between half of June and beginning of July across the three years. At the 96 sampling sites, a large number of 0.5 l subsamples (ranging between 20 up to 40 depending on the size of the water body under study) were taken around the ditch, canal, pond etc. This was 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

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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 re-sampling or disturbance of the water column. As filtering a sufficient amount of water is a prerequisite for maximizing detection probabilities (Wilcox *et al.*, 2016; Sepulveda *et al.*, 2019), 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 a 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, Suffolk, UK) as a biosafety precaution and to avoid potential DNA cross-contamination (U.S. Fish and Wildlife Service, 2017).

Set-up of aquarium experiment

We tested to which 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 either one, two, four, eight, 16 and 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 hrs, 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 lab.

Primers/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. Further, 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 (Quantus™ Fluorometer Promega, Leiden, Netherlands), that was first 1:100 diluted to obtain a starting point for seven steps series of 5 fold dilution series (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 (SD = 1.5) and was also used as positive control in the ddPCR analyses (see below). The LOD was then determined as the lowest concentration at which at least one positive detection was measured in each of the ten replicates (Tréguier *et al.*, 2014; Mauvisseau *et al.*, 2019b). We specified the LOQ as the lowest concentration in the dilution series in which 90% of the replicates fall into the SD (Forootan *et al.*, 2017).

DNA extraction and implementation of an Internal Positive Control (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, HEPA-filtered compartments with over pressure, in order 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 in order to evaluate extraction efficiency. This IPC contained approx. 30000 copies per μl 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. Then, each filter was recapped and shaken for five minutes 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 SXCAPSULE method (suitable for filters without preservation

buffer as in Spens *et al.* 2017). The only modification consisted of the addition of 2.0 μ l IPC to the lysis buffer containing 718 μ l ATL buffer and 80 μ l proteinase K.

DNA-extracts obtained from both filter types, were finally eluted to 100 μ L (see Appendix 4 for further details).

ddPCR analyses

Digital droplet PCR was conducted using a QX200 ddPCR system (Bio-Rad, Temse, Belgium) in 20 μ L. 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 contains 10 μ l Bio-Rad ddPCR supermix for probes (no dUTP), 750 nM of each primer, 375 nM of each probe (IDT, Leuven, Belgium), 4 μ l template DNA and was adjusted to the final volume of 20 μ l by adding 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 TouchTM 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 sec 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 the 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/ μ l diluted 1:100 for the *M. fossilis* target assay, or a 1:100 dilution of our IPC stock (approx. 30000 copies / μ l) for the IPC assay.

qPCR analyses

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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 those critically low levels. These eDNA extracts were analyzed 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 μ l, which included 4 μ l template eDNA, 10 μ l of Bio-Rad SsoAdvanced Universal Probes Supermix, 200 nM of each primer and 100nM of each probe. A final volume of 20 μ l was adjusted by adding DEPC water. Cycling conditions were 50 °C for 5 min, followed by 45 cycles of 95 °C for 30 sec and 56 °C for 1 min. A 1:10 dilution series of *M. fossilis* DNA (0,5 ng/ μ l, Quantus™ Fluorometer), ranging from 10^{-1} ng/ μ l⁻¹ to 10^{-4} ng/ μ l, 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 analyzed using the CFX Manager Software (Bio-Rad).

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, on the other hand, calculated to test for a linear and significant relationship between the measured number of copies per μ l resulting from the ddPCR analyses and the calculated number of copies in the serial dilution

experiment, at the one hand, and the total biomass of *M. fossilis* individuals used in the aquarium experiment, at 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:

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

with $C_{IPC \text{ observed}}$ being the obtained sample-specific copy number of IPC per μl measured via ddPCR on the final undiluted DNA extract, $C_{IPC \text{ calculated}}$ 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), and V_w being 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 liter 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):

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

Here, C_X is the number of target-eDNA copies per liter 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.

Results

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 (Fig. 2). Before 1950, 50 official records of the species across Flanders were found, whereas this number collapsed to only 4 observations during the last decade (between 2011-2019). Most of these observations were located in the eastern part of Flanders, within the river basins of the Demer, Dijle, Nete and Meuse (Fig. 3a). Since 2000, the species was only observed at seven single locations.

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 ($r = 0.977$, $P = 0.0042$) (Fig. 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 / μ l. The following dilution, 1/1562500, did not result in any amplification for any of the replicates (Fig. 4). The LOQ, on the other hand, was found to occur at a concentration of 0.14 copies / μ l (1/12500 dilution) (Fig. 4).

The aquarium experiment revealed a significant and positive relationship between the number of individuals and the number of copies that were detected per μ l DNA ($r = 0.959$; $P = 0.002$; Fig. 5). Here, the number of copies per μ l ranged from 4.2 copies / μ l when only one individual was included in the aquarium to a level of 549 copies / μ l when 32 individuals were present.

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/ μ l (see Appendix 5). The quantification efficiency (Q_e) of the samples was on average 1.11 l (ranging from 6.96 l 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 liter of water (within these positive samples), was on average 60.23 (ranging from 666.4 to 0.333 copies / l; Fig. 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 showed a $Q_e < 0.2$ l, and

therefore need to be omitted from further interpretation because of their substantial risk of a false-negative (see Table 2 and Appendix 5).

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 per μl , with detection rates ranging between two up to eight positive replicates per sample), only two samples showed a positive signal when analyzed via qPCR (see Table 3). Both samples resulted in a mean C_q value of 48.64 ± 5.10 and 35.82 ± 0.08 measured obtained from two and only one positive replicate respectively (Table 3).

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 (Kranenborg & de Bruin, 2009), and point to an urgent need for a method to assess current populations where conservation of this species can focus on (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 three year survey effort across Flanders, Belgium) were positive for this declining species. Although this, at first glance looks positive, only twelve sites were classed as harboring relative dense populations (i.e., each of the PCR replicates scored positive 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 100m transect. The remaining samples that scored positive 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 1 out of 8

replicates (12.5% detection probability in our case), is to 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 in eDNA studies of great concern as it may lead to over-optimistic or over-pessimistic results in case of respectively rare/protected or harmful/invasive species (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 the field and/or PCR blanks) gave a positive signal. Further, additional fishing efforts were carried out at six of the locations where a “weak” positive signal of *M. fossilis* ($< \text{LOQ}$) was detected, and resulted in the catch of the species at four locations (Fig. 6), albeit at very low densities (< 5 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

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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 at 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 on 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, in order to obtain an extraction efficiency 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 on 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 ($Q_e < 0.2$) 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 those 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 analyzed 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 two-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; Thomson *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.

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 already calculated that eDNA monitoring via qPCR was one-and a half times more cost-effective than electrofishing (Herder *et al.*, 2014; Sigsgaard *et al.*, 2015), with a three-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 less prone to variation caused by sampling intensity and expertise. Beyond confirmation of positive

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detections, spatially and temporally repeated eDNA sampling may be incorporated into an occupancy modeling framework to account for imperfect detection of rare species (MacKenzie *et al.*, 2002; Rees *et al.*, 2014). The efficient and standardized way of sampling makes eDNA a particularly powerful tool for broad-scale monitoring of species, for the detection of new populations and temporal monitoring of known, whether or not managed or reintroduced populations. Moreover, the combination of eDNA data with occupancy modeling could additionally allow robust identification of species habitat associations, estimation of colonization and extinction probabilities (MacKenzie *et al.*, 2003), over scales which are prohibitively labor and cost-intensive using conventional sampling approaches.

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Table 1. Primers and probes for targeting a 119 bp COI sequence of European weather loach (*Misgurnus 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
<i>M. fossilis</i> / COI	Mf-COI-F	Forward	CCCCCGACATAGCATTTCG	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)

Table 2. Decision table for further interpretation of eDNA samples based on the ddPCR measurement of the target DNA concentration and the Q_e (effective quantity) of analyzed eDNA based on the internal positive control (IPC) and filtered volume of water.

	Positive detection (> LOD)		Negative detection (< LOD)
Q_e / C_{rrd}	> $LOQ \Rightarrow$ <i>Quantitative interpretation</i>	< $LOQ \Rightarrow$ <i>Qualitative interpretation</i>	< LOQ
> Q_e threshold *	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 *	Target present but unreliable quantitative estimation and risk of underestimation	Target present but unreliable quantitative estimation and risk of underestimation	Target not detected, substantial risk for false-

(0/96 samples)

(3/96 samples)

negative interpretation

(7/96 samples)

* In this study the Q_e threshold was set on 0.2 L.

Table 3. Overview of the comparison of ddPCR *versus* qPCR analyses on a subsample of 15 positive samples in which *Misgurnus fossilis* was previously detected with ddPCR. 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 / μl over all replicates $\pm$ SD (from ddPCR) and the mean Cq $\pm$ SD.

sample	ddPCR			qPCR		
	replicates	prob	concentration	replicates	prob	Cq
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 legends

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

Fig. 2. Time series of the historical records of European weather loach (*Misgurnus fossilis*) before 1950 until Mid-2018 across Flanders (Belgium).

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

Fig. 4. Experimentally obtained limits of detection (LOD) and quantification limit using a five-fold dilution series with seven steps (e.g. 1/100, 1/500; 1/2500 etc.), with ten replicates each. The digital PCR log of measured concentration is along the y-axis, whereas the log of the calculated concentration is along the x-axis. The LOD is set at 0.07 copies / μ l and LOQ is set at 0.14 copies / μ l (1/12500 dilution). The starting dilution of this series (1/100) measured with ddPCR was 17.8 copies / μ l (SD = 1.5).

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

Fig. 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 μl DNA obtained from eight ddPCR replicates per sample (black circles). The experimentally obtained LOD (≤ 0.07 copies per μl , blue dotted line) and LOQ (≤ 0.014 copies per μl , 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.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6