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ORIGINAL ARTICLE

Using quantitative eDNA analyses to accurately estimate American bullfrog abundance and to evaluate management efficacy

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Abstract

Biological invasions contribute now more than ever to the global homogenization of fauna and flora. Large-scale monitoring programs are, therefore, needed to detect incipient invasions and to evaluate management interventions. As conventional monitoring methods are constrained by large costs, environmental DNA (eDNA)-based methods are increasingly recognized as valuable monitoring tools. However, accurately estimating species abundance from eDNA concentrations in natural systems remains challenging and consequently hinders their integration in management applications. Here, we used droplet digital PCR (ddPCR) in eDNA surveys to estimate the abundance of invasive American bullfrogs (*Lithobates catesbeianus*). We first introduced bullfrog tadpoles in natural ponds to assess the relationship between abundances and eDNA concentrations under field conditions. Next, we combined eDNA sampling with fyke netting in naturally colonized ponds to investigate whether bullfrog eDNA concentrations can estimate bullfrog capture success and conventional abundance measures obtained via depletion sampling. Finally, we evaluated eradication measures by comparing bullfrog eDNA concentrations before and after fyke netting. We found a strong linear relationship between the numbers of introduced tadpoles and eDNA concentrations ($r^2 = 0.988$). Bullfrog eDNA concentrations were not only linearly related to the catch-per-unit-effort ($r^2 = 0.739$), but also to conventional abundance estimates ($r^2 = 0.716$), particularly when eDNA concentrations were standardized for pond area ($r^2 = 0.834$) and volume ($r^2 = 0.888$). Bullfrog tadpoles were only captured when eDNA concentrations exceeded 1.5 copies μl^{-1}, indicating that quantitative eDNA analyses enable the localization of breeding ponds. We found a significant reduction in eDNA concentrations after fyke netting proportional to the number of captured bullfrogs. These results demonstrate that eDNA quantification is a reliable tool that accurately estimates bullfrog abundance in natural lentic systems. We show that quantitative eDNA analyses can complement the toolbox of natural

resource managers and facilitate the coordination of eradication campaigns targeting alien invasive species.

KEYWORDS
alien invasive species, conservation genetics, depletion sampling, droplet digital PCR, environmental DNA quantification, fyke netting, *Lithobates catesbeianus*, monitoring biological invasions

1 | INTRODUCTION

Alien invasive species disrupt indigenous communities and exacerbate global biodiversity loss at unprecedented rates (Pyšek et al., 2020). Freshwater ecosystems are disproportionally affected, which endanger the large number of species that these ecosystems accommodate (De Meester et al., 2005; Reid et al., 2019). Improved monitoring programs and the development of early warning–rapid response systems are vital to safeguard these fragile ecosystems from the impact of biological invasions (Simberloff et al., 2013). Such monitoring programs not only enable the detection of incipient invasions, but can also provide estimates on species abundance that in turn can tailor management actions (Vilà & García-Berthou, 2010). However, conventional field-based monitoring techniques are costly and laborious when they are conducted over large spatial and temporal scales, and often cause damage to the environment and its associated species (Mueller et al., 2017).

Molecular survey techniques are, therefore, rapidly gaining ground serving as efficient biomonitoring tools, particularly for aquatic species that are cryptic or occur in low numbers (Carim et al., 2020; Sepulveda et al., 2020). Because all living organisms leave traces of genetic material enclosed in gametes, metabolic waste, or shed tissue in their environment, the capture of this environmental DNA (eDNA) and subsequent amplification of genetic markers can reveal their presence, which eliminates the need for visual observation (Ficetola et al., 2008). In addition to eDNA-based species detection, abundance estimates can also be inferred from quantitative eDNA analyses (Brys, Halfmaerten, et al., 2021; Everts et al., 2021; Spear et al., 2021). Even though eDNA concentrations are strongly related to species abundance in experimental systems, a considerable proportion of variance remains unaccounted for in field conditions (Yates et al., 2019), which can be attributed to both abiotic (temperature, UV, pH, suspended organic matter, hydrodynamics, etc.) and biotic influences (taxonomic group, life stage, reproduction, habitat use, etc.). Whereas these factors are typically standardized in experimental studies, they are expected to blur the functional link between eDNA concentration and species abundance in natural systems (Harrison et al., 2019; Yates et al., 2021).

An additional issue that is frequently overlooked in the existing debate on the applicability of quantitative eDNA analyses is that their validation in natural systems is generally conducted with indirect measures of species abundance (Spear et al., 2021). Indeed, because the exact number of individuals related to a particular eDNA signal is often not known, eDNA concentrations are benchmarked against proxies for species abundance inferred from visual transect surveys (Plante et al., 2021), acoustic surveys (Plough et al., 2021), or catch and effort data (Dougherty et al., 2016). However, these indirect measures are often biased (Hubert et al., 2012; Maunder & Punt, 2004; Peterson et al., 2004) which introduces undesirable noise in the association between eDNA concentrations and species abundances (Shelton et al., 2019). On the other hand, studies that related eDNA concentrations to absolute abundance estimates determined by capture–mark–recapture (Pochardt et al., 2020; Spear et al., 2021) and depletion sampling (Sepulveda et al., 2021) have generally reported correlations well above the average field study (Yates et al., 2019). However, these estimates are based on assumptions that are seldom fully met under field conditions (Cowx, 1983; Peterson et al., 2004). The inherent inaccuracy of these benchmarks unjustifiably hampers the integration of this molecular tool into management applications, and hence impedes proper evaluation of the efficacy of management interventions or the ultimate success of eradication programs (Hansen et al., 2018; Rojahn et al., 2021; Shelton et al., 2019; Yates et al., 2021).

In this study, we used droplet digital PCR (ddPCR) in eDNA surveys to estimate American bullfrog (*Lithobates catesbeianus* [Shaw, 1802]; hereafter referred to as bullfrog) abundance in natural lentic systems in Belgium and investigated how quantitative eDNA analyses can be integrated in monitoring and eradication programs of this invasive species. Previous research has shown that eDNA concentrations were closely related to bullfrog tadpole abundances under controlled conditions (Everts et al., 2021), but the extent to which this relation holds in natural water bodies remains unclear. In the present study, we therefore (i) introduced variable numbers of bullfrog tadpoles in natural ponds to investigate the relationship between eDNA concentrations and their abundance under field conditions. (ii) Next, we combined eDNA sampling with fyke netting in a large variety of ponds that were naturally colonized by bullfrogs to assess whether bullfrog eDNA concentrations were correlated with standardized catches and conventional abundance estimates. (iii) Finally, we related eDNA concentrations before and after fyke netting to the total number of captured bullfrogs to investigate the effectiveness of the eradication efforts applied.

2 | METHODS

2.1 | Study species

Bullfrogs are native to the eastern United States, but have been introduced to the western United States and Canada, South America, Asia, and Europe (Ficetola et al., 2007). This large frog species exerts a severe pressure on indigenous amphibian communities through fierce resource competition, gape-limited predation, and the transmission of novel pathogens, and is therefore ranked among the most destructive invaders worldwide (Lowe et al., 2000). Bullfrogs first arrived in Belgium in the 1990s, and nowadays have occupied an area of more than 500 km² in Flanders (Northern Region of Belgium; Figure 1a). Apart from a number of satellite populations, a large metapopulation stretching over hundreds of water bodies is situated along the river Grote Nete. In order to prevent further spread, bullfrogs have been combatted with increasing effort since 2012, mostly using double fyke nets that effectively capture bullfrog tadpoles (Louette et al., 2013).

2.2 | eDNA quantification to estimate bullfrog abundance

The functional link between bullfrog tadpole abundances and eDNA concentrations under natural conditions was examined in three similar-sized natural ponds (Table 1) that were located within the distribution range of bullfrogs in Belgium ("experimental pond" 1, 2, and 3; Figure 1b, c). An exploratory eDNA sampling indicated that bullfrogs were absent in each of these ponds before the onset of the experiment. Living bullfrog tadpoles larger than 50 mm but preceding the development of forelimbs were acquired from an ongoing management program and were temporally stocked in large sentinel nets that were installed in a nearby pond (Figure 2a–e).

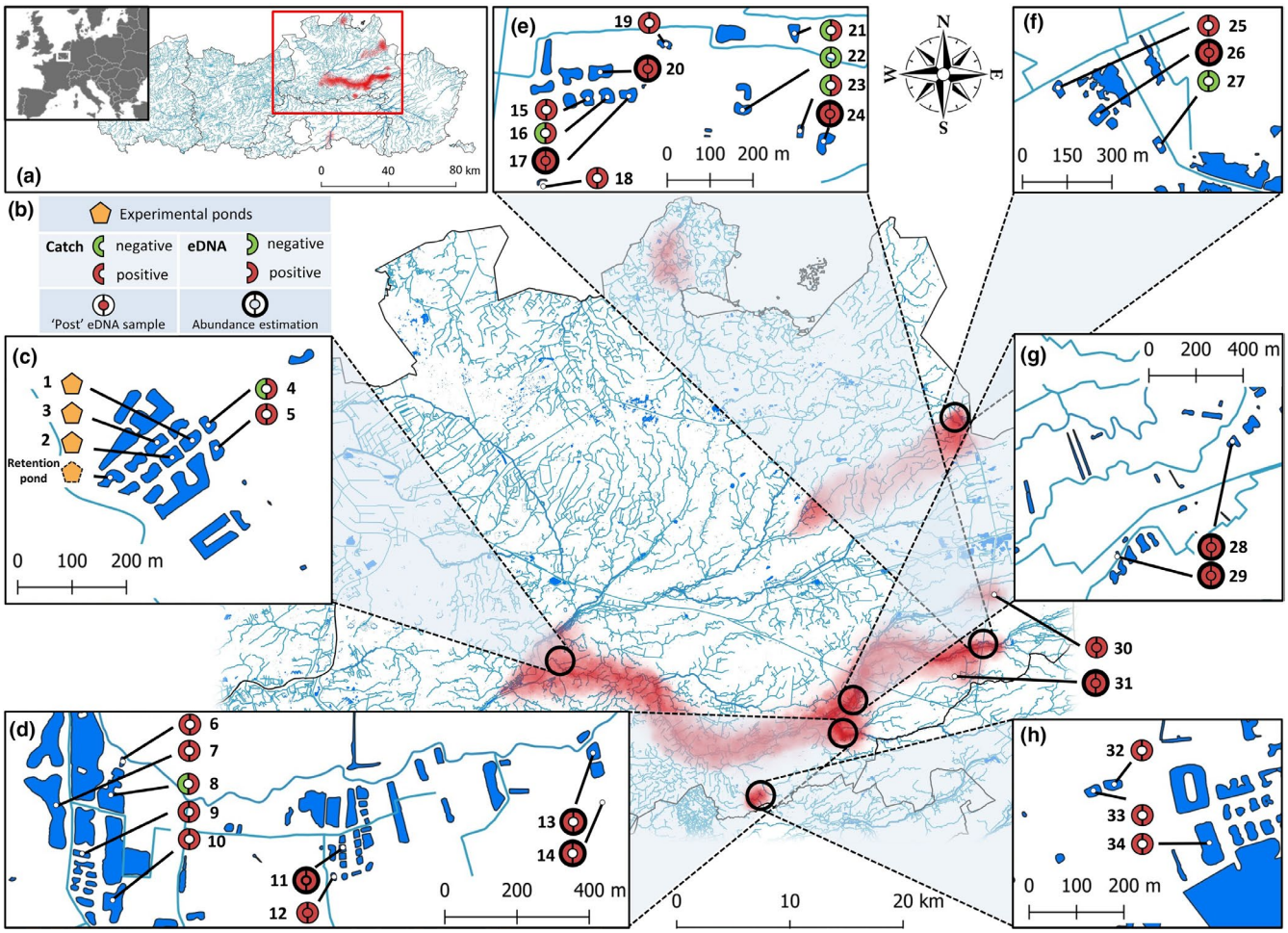

FIGURE 1 (a) The distribution of the invasive American bullfrog in Belgium. The study area is indicated by the red rectangle, and is magnified in (b). All ponds included in this work are pinpointed and associated with a numbered symbol. Experimental ponds are represented by orange pentagons in (c). Management ponds are associated with discs and were targeted for eDNA sampling and parallel fyke netting. (c–h) The left and right half of this disc represent the outcome of fyke netting and the 'pre fyke netting' eDNA samples, and green and red indicate the absence and presence of bullfrogs as indicated by the respective methods. Discs with a red core signify ponds where a 'post fyke netting' eDNA sampling was carried out. Discs with a black circumference are ponds where a conventional abundance estimate was calculated from depletion sampling data

TABLE 1 Characteristics of the sampled water bodies

Pond ID	Latitude (°N)	Longitude (°E)	Pond surface area (m^2)	Pond volume (m^3)	No. of catch days	No. of double fykes	Total catch	CPUE	Abundance estimate (±*SE*)
Experimental ponds									
1	51.1381	4.6080	927.301	1587.508					
2	51.1380	4.6070	891.090	1536.886					
3	51.1378	4.6074	947.590	1258.489					
Management ponds									
4	51.1384	4.6084	576.970	905.500	8	5	1	0.0250	**
5	51.1380	4.6086	698.780	1089.00	8	5	2	0.050	**
6	51.0775	4.9697	269.897	246.125	4	5	33	1.650	**
7	51.0763	4.9670	10116.820	11721.670	11	10	13765	125.136	**
8	51.0767	4.9693	5300.670	7989.00	4	5	17	0.850	**
9	51.0752	4.9680	658.610	503.840	4	5	0	0	**
10	51.0740	4.9693	3796.466	1530.00	4	5	2	0.100	**
11	51.0753	4.9783	341.371	602.100	4	5	16	0.800	16 ± 0.523
12	51.0746	4.9780	245.434	230.127	4	5	2	0.100	**
13	51.0776	4.9882	1364.778	1074.232	4	5	9	0.450	9 ± 0.622
14	51.0767	4.9886	610.000	263.300	4	5	13	0.650	13 ± 0.937
15	51.1450	5.1483	882.505	813.405	3	4	1	0.083	**
16	51.1451	5.1490	978.790	1147.000	3	4	0	0	**
17	51.1451	5.1497	966.4304	738.288	3	4	19	1.583	21 ± 3.109
18	51.1433	5.1468	100.516	19.546	4	1	6	1.500	**
19	51.1462	5.1510	471.088	*	4	5	6	0.300	**
20	51.1455	5.1488	1624.765	2404.000	3	5	514	34.267	902 ± 115.963
21	51.1462	5.1553	817.814	796.305	4	3	0	0	**
22	51.1447	5.1535	845.066	*	4	5	0	0	**
23	51.1443	5.1554	385.3283	343.167	4	5	0	0	**
24a	51.1441	5.1562	1274.512	2948.000	4	5	2042	102.100	2234 ± 24.119
24b	51.1441	5.1562	1274.512	2948.000	4	5	391	19.550	660 ± 87.06
25	51.3272	5.1054	1407.524	1708.078	3	5	1	0.0670	**
26a	51.3264	5.1073	2820.421	6001.834	8	10	3242	40.250	3270 ± 5.975
26b	51.3264	5.1073	2820.421	6001.834	4	10	632	15.800	836 ± 43.833
27	51.3255	5.1102	1353.087	*	4	5	0	0	**
28	51.0996	4.9817	1598.833	2029.000	4	5	4987	249.350	7384 ± 195.260
29	51.0954	4.9748	283.670	133.718	3	1	519	173.000	745 ± 57.543
30	51.1867	5.1584	1089.037	674.223	8	10	3693	46.163	**
31	51.1234	5.1185	850.834	1137.411	4	5	2309	115.450	2376 ± 10.816
32	51.0330	4.8591	725.2633	634.944	4	5	103	5.150	**
33	51.0329	4.8584	643.174	595.450	4	5	35	1.750	***
34	51.0319	4.8620	3409.877	6388.407	8	10	1074	13.425	***

Note: Total pond volumes were not quantified for ponds marked with one asterisk (*) because they were either too shallow for sonar measurements or no bullfrog eDNA was detected. No abundance estimates were calculated for ponds marked with two asterisks (**) because the underlying assumptions were violated or because no bullfrogs were caught.

Between the 30th of June and the 23rd of July 2021, a series of exactly known numbers of living bullfrog tadpoles with an average biomass of 14.06 ± 1.34 (*SEM*) g were set free in each experimental pond (Figure 2f). The series started with a low number of bullfrog tadpoles, upon which incrementally more individuals were added right after eDNA sampling was carried out (described below). In this way, a first eDNA sampling round was conducted in experimental pond 1 after the introduction of 25 bullfrog tadpoles, a

FIGURE 2 (a) Captured bullfrog tadpoles were transported to the experimental ponds in large reservoirs (b) that were equipped with a continuous oxygen supply (white arrow). The tadpoles were subsequently introduced in large sentinel nets installed in a retention pond (c, d) which was enclosed with an amphibian screen (white arrow). The desired number of tadpoles was removed from the nets (e), weighed (f), and introduced to the experimental ponds

second after 25 more tadpoles were added to arrive at 50 individuals, and a final sampling round after 200 additional tadpoles were introduced to reach a total number of 250 individuals. Similarly and simultaneously, a total number of 25, 50, 100, and 250 tadpoles were successively stocked in experimental pond 2, and 1, 5, and 1000 tadpoles in experimental pond 3. The further spread of bullfrogs in the region was prevented by a 150-cm tall amphibian screen enclosing each pond (see Figure 2c, d), while several traps were installed within the fenced area to passively capture metamorphosed escapees.

eDNA sampling was conducted after a 5-day acclimatization period following each increase in abundance. As DNA particles can be patchily distributed in the water column, especially in lentic systems (Brys, Haegeman, et al., 2021), pond coverage was maximized by means of an integrated sampling strategy. Every 5 m along the perimeter of each pond, a subsample was taken by gradually filling a 0.5-L sterile sampling bag with pond water. Because the sampling bag was attached to a telescopic sampling pole, pond water within a 5-m radius of each subsampling stage was included in every subsample. The upper 10 cm of the water column was targeted to maximize the capture of eDNA particles (Moyer et al., 2014). In this way, twenty to thirty 0.5-L subsamples were collected and ultimately pooled to obtain one homogenous water sample per sampling round. This sampling strategy provided a good spatial coverage given the relatively small sizes of the studied ponds (Table 1). This process was repeated twice per experimental pond and per introduced abundance. The pooled, homogenized water samples were then filtered on enclosed disk filters with an integrated 5-μm glass fiber prefilter and a 0.8 μm PES membrane (NatureMetrics, Surrey, England) using a Vampire sampler pump (Buerkle, Bad Bellingen, Germany) with disposable silicone tubing. Water was pumped through the filters until clogging (1.896 ± 0.221 L), and the filters were stored at −21°C in anticipation of the molecular analyses. Sterile nitrile gloves were worn during the entire sampling process, and reusable field material was decontaminated with 2% Virkon S (Antec DuPont, Suffolk, UK).

Following eDNA sampling, the water turbidity of each pond was measured with a 2100Q Portable Turbidity meter (Hach®). Simultaneously, water pH and conductivity measurements were carried out with a WTW MultiLine® Multi 3620 IDS SET KS1. Since a given number of individuals is expected to result in lower eDNA

concentrations in a large water volume than in smaller water volumes, eDNA concentrations can only be appropriately related to the number of individuals when a spatial metric, such as the total surface area or water volume of a pond is taken into account. Therefore, a 999 CXI HDSI multibeam (Humminbird®) sonar device was used to generate bathymetric maps of the studied ponds from which the total water volume (m^3) was calculated (Figure 3a, b; Appendix S2). Pond surface areas were calculated from the coordinates of the perimeter of the studied ponds, which were recorded with a handheld GPS. Average daily ambient temperature measurements from a nearby weather station were used to verify that there were no systematic changes in water temperature that could have affected the experiment (Appendix S1).

2.3 | eDNA quantification to evaluate bullfrog management

To assess the relationship between natural bullfrog abundances and eDNA concentrations, eDNA sampling was coupled with an intensive fyke netting campaign in 31 'management ponds' spread over the entire distribution range of bullfrogs in Belgium (Figure 1; Table 1). A

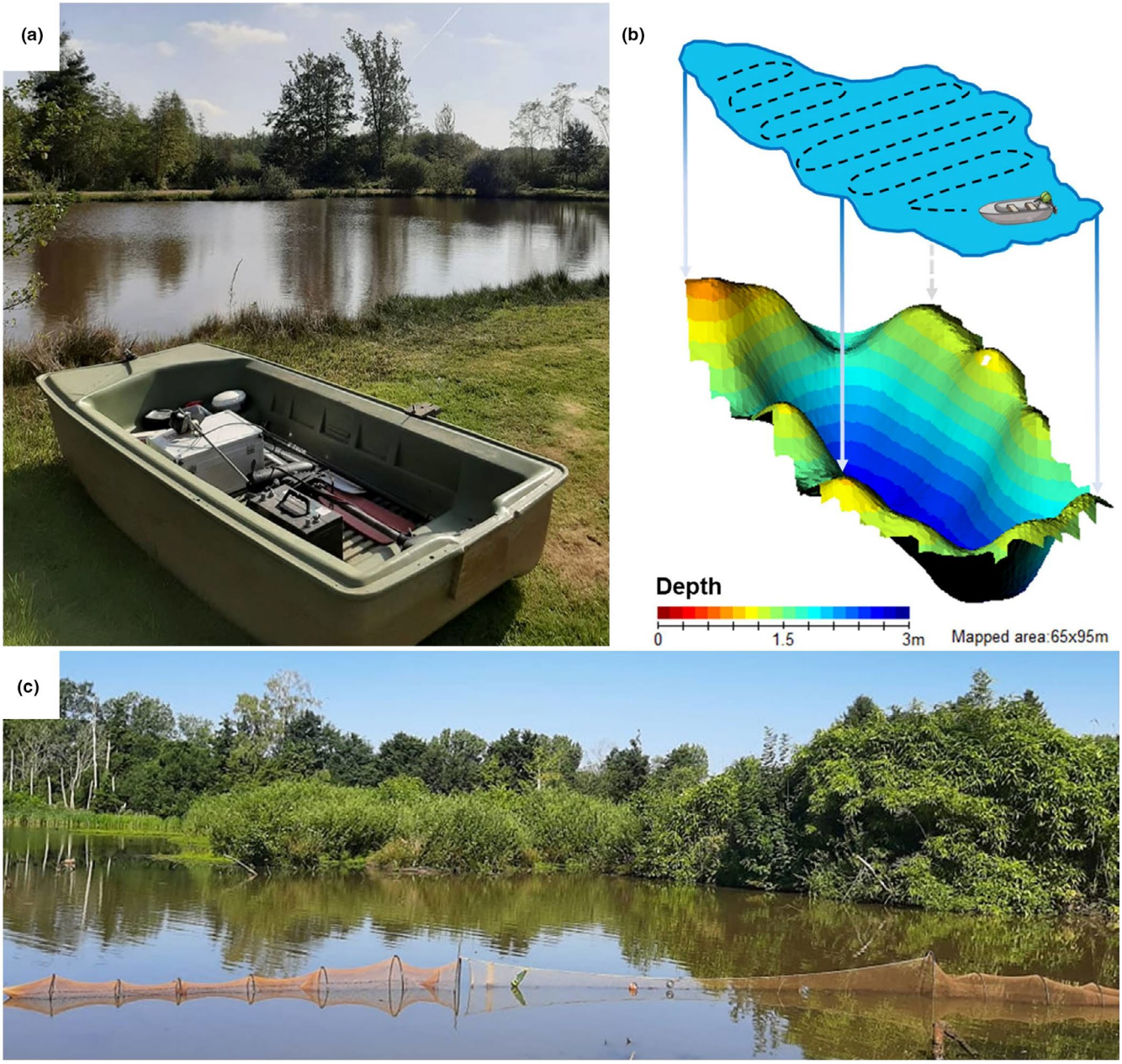

FIGURE 3 (a) The small rowing boat loaded with equipment for sonar measurements to quantify the volume of ponds. (b) The boat was navigated in a zig-zag pattern over the pond (black dotted line) while the sonar device continuously measured the depth of the pond. These data were then used to generate three-dimensional bathymetric maps of the sampled ponds (pond 26 in this case), from which the total pond volume was derived (detailed description in Appendix S2). (c) A double fyke net installed parallel to the pond bank

second independent fyke netting and coupled eDNA sampling round was carried out in ponds 24 and 26 one month later (pond 24b and 26b hereafter). All fyke netting actions were part of the LIFE framework of the European union (LIFE18 NAT/BE001016). Fyke netting and eDNA sampling were conducted between the 10th of May and the 20th of August 2021, which comprises the period that bullfrogs are active and thus maximizes detection and capture success (Everts et al., 2021; Hubert et al., 2012). One hour before installing the fyke nets, one eDNA sample was collected per pond following the same protocol as described above ('pre fyke netting' eDNA samples). To investigate the consistency of quantitative eDNA analyses in natural systems, two 'pre fyke netting' samples serving as biological replicates were collected in ponds 15, 16, 17, and 20. Bullfrogs were captured using double fyke nets with a mesh size of 8 mm, consisting of two fykes interconnected with a 7-meter-long leader net, each with an initial hoop of 80 × 90 cm followed by three narrowing funnels in each fyke (Figure 3c). Double fyke nets were installed parallel to and 2 meters from the bank for an average period of 5 days. Every day, by-catch was released and the total number of captured bullfrogs was recorded.

To investigate whether bullfrog eDNA concentrations decreased proportional to the total number of removed individuals, a second eDNA sampling round was carried out 7 days after removing the fyke nets ('post fyke netting' eDNA samples) in 12 management ponds (Figure 1). Because bullfrog eDNA in lentic systems is completely degraded within 1 week (Brys, Haegeman, et al., 2021), the proposed 7 days was assumed to be sufficient for the connection between eDNA concentrations and lowered bullfrog abundances to be restored. Active breeding of bullfrogs within this period was expected to disturb this relationship, but could only be excluded from ponds 17 and 20. Here, heavy machinery cleared the surrounding vegetation for the construction of large fences prior to eDNA sampling and fyke netting, which strongly suggests that juvenile and adult bullfrogs had been driven away, leaving only bullfrog tadpoles. In these ponds, a second 'post fyke netting' eDNA sample was collected 3 days after removing the fyke nets to assess whether eDNA concentrations already reached an equilibrium with the reduced tadpole abundance. Lastly, water turbidity, pH, and conductivity were measured, and total pond areas and volumes were quantified as described above (Appendix S2). Altogether, 33 fyke netting rounds were conducted and 51 eDNA samples were collected (37 'pre fyke netting' and 14 'post fyke netting' samples in 33 and 12 water bodies, respectively). Overall, an average volume of 1.714 ± 0.162 L pond water was filtered per sample.

2.4 | Molecular analyses

The laboratory protocol and the primer/probe assays used were comprehensively described and validated in Everts et al. (2021). Briefly, 1 ml of a lysis buffer was added via the inlet of each filter ($n = 71$). This buffer included a known concentration of an exogenous DNA fragment serving as an internal positive control (IPC) to assess PCR inhibition and indicate potential problems in the laboratory workflow. The IPC used here was a plasmid containing a 149 bp Dengue virus type 2 insert sequence (GenBank M29095.1). Following overnight incubation at 56°C, the lysis solution was collected from the filters, and DNA was extracted with Qiagen's DNeasy Blood & Tissue Kit. All eDNA samples were eluted in 200 µl Tris–EDTA. A volume of 150 µl of this DNA extract was then purified with Qiagen's DNeasy PowerClean Cleanup Kit and was finally eluted in 100 µl Tris–EDTA. The concentration of both bullfrog eDNA and IPC was simultaneously quantified by ddPCR in duplicate technical measurements (once undiluted and once 1:2 diluted) following Everts et al. (2021). Biological replicates of experimental ponds 1 and 2 at the abundance of 25 tadpoles, and experimental pond 3 at the abundance of 1 and 5 tadpoles got lost during the workflow, resulting in a total of 68 eDNA samples that were finally analyzed.

2.5 | Statistical analyses

The total number of bullfrog eDNA copies per microliter per liter filtered water (C_X) was calculated as follows:

$$C_X = \frac{C_{PCR} \times \frac{V_{PCR}}{V_R} \times \frac{V_E}{V_L} \times DF}{CF_{PC} \times V_W} \times CF_{Spatial},$$

where C_{PCR} is the measured bullfrog eDNA concentration in each ddPCR reaction (number of copies μl^{-1}), V_{PCR} is the total ddPCR reaction volume (20 µl), V_R is the volume of the eluted extract that was included in the PCR reaction volume (4 µl), V_E is the total elution volume (in µl) after eDNA extraction, V_L is the lysis volume (in µl) collected from each filter, DF is the dilution factor of a DNA extract (for a 1:2 dilution, DF = 2), CF_{PC} is a correction factor of 1.5 for purified samples (since an initial volume of 150 µl is finally eluted to 100 µl), and V_w is the total volume of filtered water (in L). When eDNA concentrations were related to absolute abundance estimates, eDNA concentrations were standardized via multiplication with the total pond area or volume ($CF_{Spatial}$). $CF_{Spatial}$ was set to one when eDNA concentrations were related to catch-per-unit-effort calculations, since this is a relative abundance measure. The obtained bullfrog eDNA concentrations were averaged among both technical replicates.

A type I linear regression was used to quantify the linear relationship between eDNA concentrations corrected for pond volume and the number of bullfrog tadpoles introduced in the experimental ponds, using the *lm* function. To assess whether experimental and management ponds differed in water quality and size, water turbidity, pH, conductivity, total pond area, and total pond volume were compared between ponds with two-sided Mann–Whitney *U* tests using the function *wilcox.test*.

For each management pond, a standardized measure for bullfrog capture was obtained by dividing the total number of captured bullfrogs (regardless of life stage) by the product of the number of catch days and the number of double fyke nets used. This measure corresponds to the catch-per-unit-effort (CPUE; Table 1), and was related to bullfrog eDNA concentration using a type I linear regression.

Because CPUE is an indirect abundance measure, the associated eDNA concentrations were not corrected for pond volume.

Conventional abundance estimates were obtained via depletion sampling. This technique utilizes the relation between the size of a population and the number of bullfrogs that were removed on multiple occasions without replacement. Conventional abundance estimates were calculated only for management ponds in which the daily CPUE decreased successively with the cumulative catch (n = 12), because this suggests accordance to the assumptions that (i) the catch effort applied considerably affects the population size and (ii) the population is not so large that the catch of one individual interferes with that of another (Table 1). Furthermore, since an equal catch probability for all individuals was assumed, and because tadpoles comprised the largest proportion of the catches in these 12 ponds, only tadpole catches were used (Cowx, 1983). Maximum weighted likelihood abundance estimates were calculated using *removal* function of the *FSA* package (Derek et al., 2021). A type I linear regression was used to relate the obtained abundance estimates to bullfrog eDNA concentrations that were corrected for total pond areas and volumes.

Differences in bullfrog abundances and eDNA concentrations before and after fyke netting were tested with a paired t-test using the *t.test* function. To comply with its assumptions, eDNA concentrations corrected for total pond volumes were log-transformed. Additionally, the total number of captured bullfrogs was related to the difference in bullfrog eDNA concentration from samples taken before and after fyke netting using a type I linear regression. All statistical tests were carried out with the *stats* package in RStudio version 4.1.1 (RStudio Team, 2022).

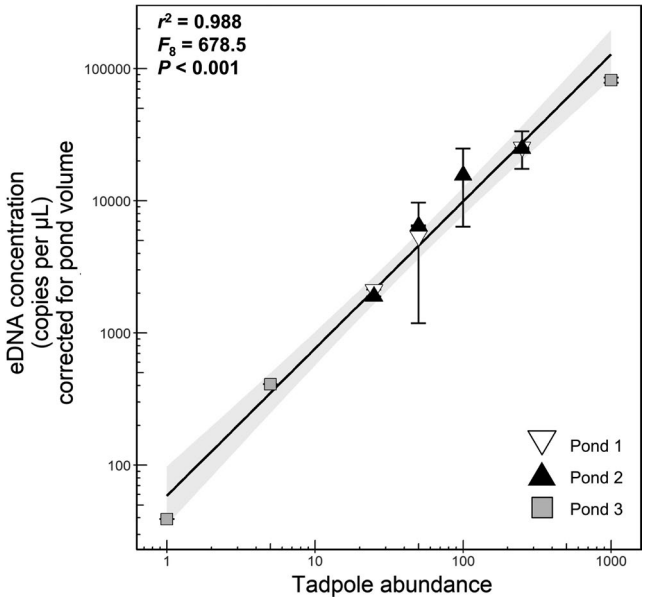

FIGURE 4 The linear relationship between introduced numbers of bullfrog tadpoles and eDNA concentrations in three natural ponds (legend in the bottom right corner). eDNA concentrations were expressed as numbers of copies μl^{-1}, and were corrected for pond volume. Each point corresponds to the average bullfrog eDNA concentration per pond and the associated error bars represent the standard error on duplicate biological replicates. The type I linear regression line is plotted in black, and the shaded area around the regression line indicates the 95% confidence interval. The corresponding test statistics are given in the top left corner. Note that both axes are logarithmic

3 | RESULTS

3.1 | Data quality

Negative and positive technical ddPCR controls showed no signs of contamination or amplification failure, respectively. More than 7000 accepted droplets were generated (17,241 ± 291 droplets) and the IPC was successfully quantified in all ddPCR reactions, indicating that the generated data were of high quality. Based on the highly similar bullfrog eDNA concentrations between both technical replicates (average standard error of 15.7% of the mean) and among the duplicate biological replicates from the experimental ponds and management ponds 15, 16, 17, and 20 (average standard error of 15.8% of the mean), bullfrog eDNA concentrations quantified using this field and lab protocol were deemed robust.

3.2 | eDNA quantification to estimate bullfrog abundance

Bullfrog eDNA was detected in all replicates and for all introduced abundances. The measured eDNA concentrations corrected for pond volume were significantly and positively correlated with the numbers of introduced bullfrogs ($F_8 = 678.500$, $r^2 = 0.988$, $p < 0.001$; Figure 4). Ambient temperature remained constant over the experimental period (18.00 ± 0.36°C; Appendix S1).

3.3 | eDNA quantification to evaluate bullfrog management

In two of the 33 management ponds, no bullfrog eDNA was detected nor were any bullfrogs captured. In five of the remaining 31 ponds, bullfrog eDNA was detected, but no bullfrogs were captured (Figure 1). Consequently, the relationship between CPUE and bullfrog eDNA concentrations was assessed in 26 ponds and was found to be significant ($F_{24} = 67.980$, $r^2 = 0.739$, $p < 0.001$; Figure 5). Management and experimental ponds only differed slightly in water conductivity ($W = 84$, $p = 0.0151$; 665.000 ± 216 and 317.195 ± 36.380 µS/cm for experimental and management ponds, respectively), but not in turbidity ($W = 42$, $p = 0.689$; 33.175 ± 12.720 and 43.308 ± 17.541 FNU), pH ($W = 39$, $p = 0.590$; 7.980 ± 0.336 and 8.172 ± 0.157), total pond area ($W = 22$, $p = 0.885$; 922.000 ± 16.500 and 1533.000 ± 340.000 m^2), or total pond volume ($W = 31$, $p = 0.532$; 1460.960 ± 102.285 and 2120.58 ± 488.515 m^3).

Conventional abundance estimates ranged from a minimum of 9 ± 0.622 to a maximum of 7384 ± 195.260 individuals (Table 1) and were significantly related to 'pre fyke netting' bullfrog eDNA

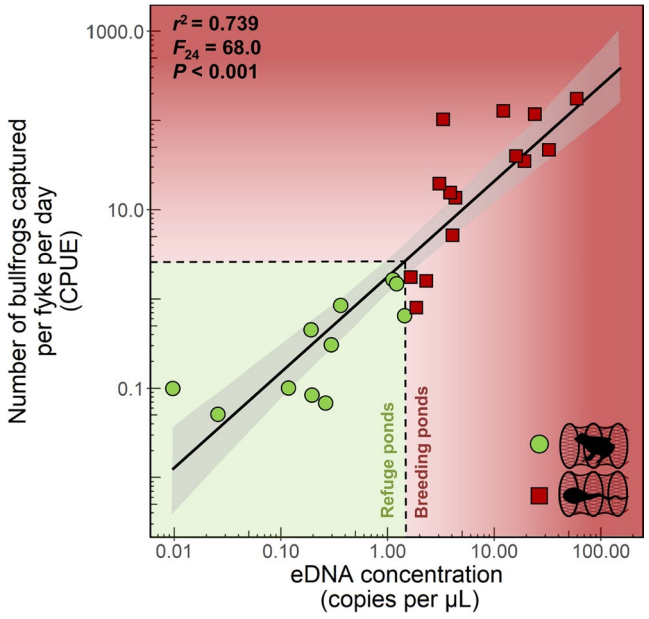

FIGURE 5 The linear relation between the catch-per-unit-effort and bullfrog eDNA concentration (number of copies μl^{-1}) before fyke netting. Green circles and red squares represent ponds where only juveniles and mainly tadpoles were captured, respectively. Test statistics of the type I linear regression are given in the top left corner, and because both axes are logarithmic, ponds with zero values were omitted ($n = 7$). The shaded area around the regression line indicates the 95% confidence interval. Only juvenile bullfrogs were captured in ponds with bullfrog eDNA concentrations lower than 1.5 copies μl^{-1} (green zone of graph), whereas mainly tadpoles were captured in ponds with bullfrog eDNA concentrations above this threshold (black dotted lines), hence representing breeding ponds (red zone of the graph)

concentrations both uncorrected ($F_{10} = 25.4$, $r^2 = 0.716$, $p < 0.001$) and corrected for total pond area ($F_{10} = 56.25$, $r^2 = 0.834$, $p < 0.001$) and total pond volume ($F_{10} = 79.110$, $r^2 = 0.888$, $p < 0.001$, Figure 6). Bullfrog abundances after fyke netting (i.e., the difference between the conventional abundance estimate and the total catch) were significantly lower than before fyke netting ($t_{19} = 2.892$, $p = 0.009$). The associated reductions in eDNA concentrations were already noticeable 3 days after the removal of bullfrogs by fyke netting (Figure 7). Pairwise comparisons of 'pre' and 'post fyke netting' samples showed significant reductions in bullfrog eDNA concentrations ($t_{23} = 10.003$, $p < 0.001$, Figure 7) that were significantly related to the total catch ($F_{10} = 11.1$, $r^2 = 0.526$, $p = 0.008$). In some ponds, however, eDNA concentrations after fyke netting were only slightly lower and in one pond even higher than before fyke netting.

4 | DISCUSSION

Although eDNA-based detection methods have been recognized as valuable complementary tools to manage biological invasions (Carim et al., 2020; Rojahn et al., 2021; Sepulveda et al., 2020),

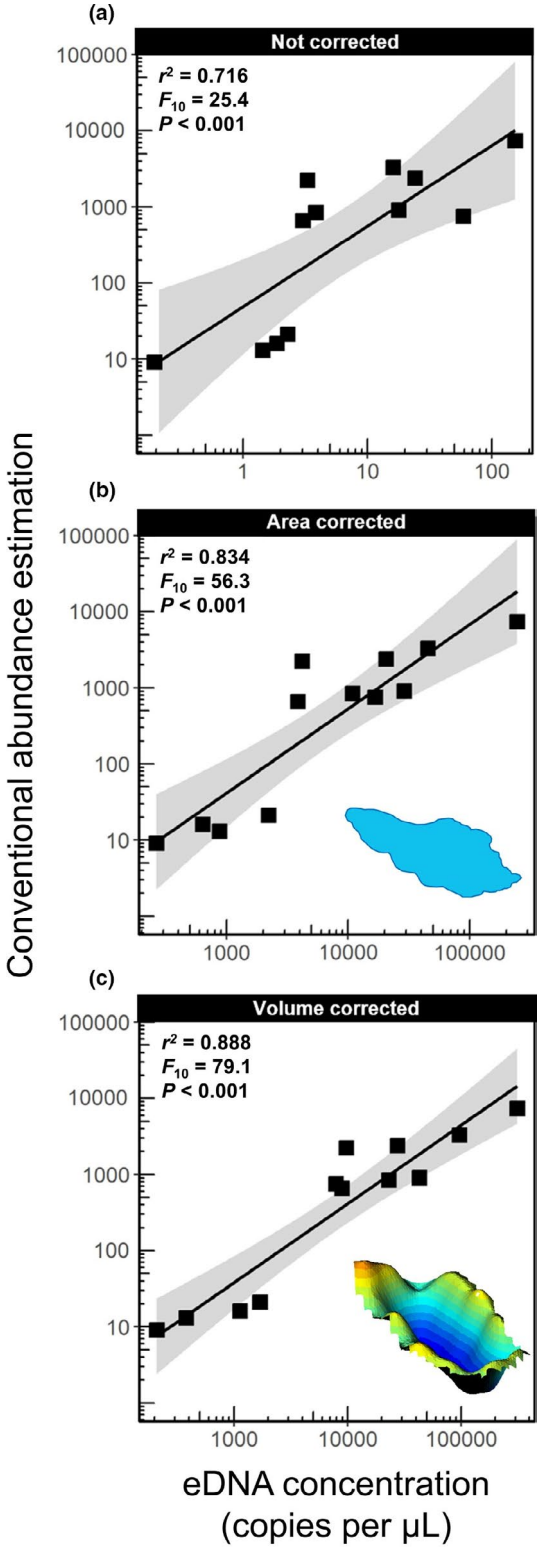

FIGURE 6 The relation between conventional abundance estimates based on depletion sampling and bullfrog eDNA concentrations (number of copies μl^{-1}), both uncorrected (a) and corrected for (b) pond area (1319.208 ± 293.321 m^2) and (c) pond volume (2196.143 ± 858.911 m^3). Test statistics of type I linear regressions are given in the top left corner. Note that both axes are logarithmic

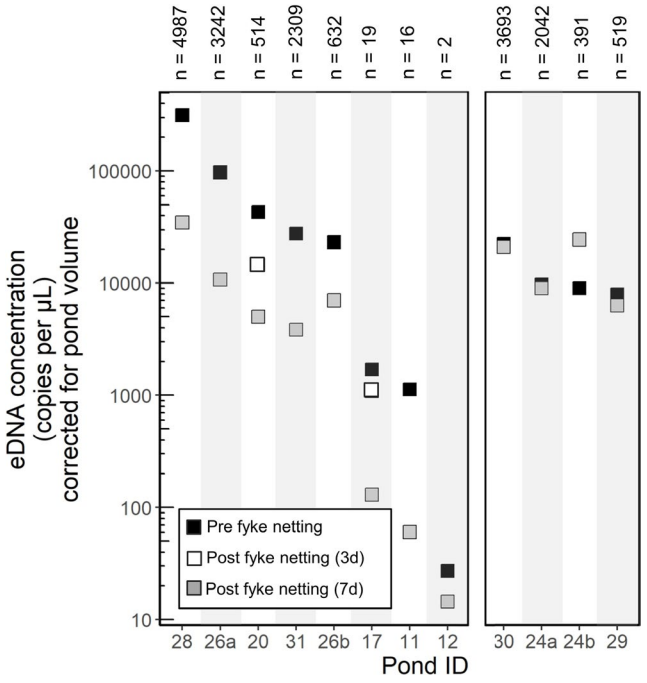

FIGURE 7 Bullfrog eDNA concentrations (number of copies μl^{-1}) were quantified before (black), 3 days after (white), and 7 days after (grey) fyke netting. Ponds in which eradication efforts appeared to have impacted bullfrog abundances are plotted in the left panel, whereas further efforts should be undertaken for the ponds in the right panel. The total number of bullfrogs captured per pond are given at the top of the graph. Note that the y-axis is logarithmic

the applicability of quantitative eDNA analyses to estimate target species abundance remains disputed (Hansen et al., 2018; Yates et al., 2021). Here, we show that bullfrog eDNA concentrations in natural ponds were strongly linearly related to the number of introduced bullfrog tadpoles (Figure 4), indicating that quantitative eDNA analyses can be a powerful method to infer target species abundance. An increasing number of studies have revealed promising linear relationships between eDNA concentrations and conventional abundance estimates of aquatic target species in natural systems (Pochardt et al., 2020; Salter et al., 2019; Spear et al., 2021), but none of these outperformed the strong correlation reported here. Several explanations can be put forward as to why this is the case.

First, lentic systems, such as the ponds studied here, are particularly suited for eDNA quantification compared to lotic and marine systems, as their exposure to currents that complicate the relationship between eDNA concentration and species abundance is minimal (Fremier et al., 2019). Moreover, lentic systems represent closed, clearly delineated, and relatively small ecosystems, which favor repeatable, quantitative, and representative sampling (De Meester et al., 2005). Second, amphibians are an excellent taxonomic group for eDNA-based monitoring, as they abundantly release DNA into the water column via the excretion of mucus containing ample amounts of DNA (Adams et al., 2019). Third, while eDNA concentrations are generally quantified with quantitative PCR (qPCR), there are several indications that it underperforms in terms of resilience to PCR inhibitors (Doi et al., 2015), its accuracy (King et al., 2022), and its sensitivity (Brys, Halfmaerten, et al., 2021) in comparison with a ddPCR approach as was used here. Finally, the applied quantitative eDNA analyses were validated appropriately because bullfrog tadpole abundances were known exactly and eDNA concentrations were standardized among ponds on a total pond volume basis. Only one other study quantified eDNA concentrations of exactly known abundances of adult painted turtles (*Chrysemys picta*) in natural systems while correcting for varying pond sizes (Adams et al., 2019). In contrast, most earlier studies related eDNA concentrations to indirect abundance measures (Plante et al., 2021; Plough et al., 2021; Sepulveda et al., 2021). However, the positioning and timing of the sampling efforts, life stage of the focal species, weather conditions, water temperature, water turbidity, macrophyte cover, and geomorphological features of the water body interactively affect the extent to which these conventional proxies are related to actual abundances (Louette et al., 2013; Peterson et al., 2004), thereby introducing excess variance into the relation with eDNA concentrations (Peterson et al., 2004; Shelton et al., 2019).

Our results further showed that bullfrog eDNA concentrations were linearly related to the number of bullfrogs that were subsequently captured per unit of effort that was applied (Figure 5). Moreover, an eDNA concentration of 1.5 copies μl^{-1} was found to be a clear threshold that separated ponds where only bullfrog juveniles were captured from those where bullfrog tadpoles were captured (hence breeding ponds). This threshold reflects a spatial discrepancy in habitat use between bullfrog life stages. Intraspecific competition and cannibalism by adults force juvenile bullfrogs to leave their natal ponds and disperse toward suboptimal water bodies serving as refuge ponds and driving the spread of the invasion. Oppositely, since bullfrog tadpoles, unlike many other amphibians, generally metamorphose after 2 years (Bury & Whelan, 1984), successful reproduction is restricted to permanent stagnant waters (ponds and small lakes). The observed lower bullfrog eDNA concentrations in refuge relative to breeding ponds can be attributed to the combination of a lower density and a more terrestrial habitat use of bullfrog juveniles relative to tadpoles (Figure 5). According to demographic models, such refuge ponds have to be targeted with the highest priority for effective control of bullfrog populations (Govindarajulu et al., 2005). In practice, however, the capture of juvenile bullfrogs requires considerably more effort than that of tadpoles (Louette et al., 2013). Combined with the rapid recolonization of ponds by juvenile bullfrogs, which quickly offsets successful removal, targeting and depleting strategically located breeding ponds serving as dispersal hubs might be more effective. In any case, we show that quantitative eDNA analyses can be used not only to estimate the number of bullfrogs expected to be captured with a given effort, but also to locate bullfrog breeding ponds, which altogether is extremely valuable information to support bullfrog eradication programs.

In order to optimize the efficacy and resource allocation of management programs targeting invasive species, insights into absolute abundances are critical (Lampo & Bayliss, 1996; Pochardt et al., 2020;

Spear et al., 2021). Capture–mark–recapture or depletion sampling are conventionally used for these purposes, but are laborious, invasive, and subject to multiple stringent assumptions that restrict their applicability (Cowx, 1983; Lampo & Bayliss, 1996). We show that bullfrog eDNA concentrations were strongly correlated with conventional abundance estimates obtained by depletion sampling, but only when eDNA concentrations were standardized according to total pond volumes (Figure 6). Standardizing eDNA concentrations according to other spatial metrics such as the total surface area also increased the proportion of explained variance in absolute abundance estimates, albeit to a lesser extent. However, whereas eDNA concentrations reflect the total population abundance, conventional abundance estimates are confined to one particular life stage (tadpoles in this case) because an equal capture probability is assumed (Cowx, 1983; Lampo & Bayliss, 1996). The remaining variance that was not explained by total pond area- or volume-corrected eDNA concentrations (17% or 11%, respectively) can therefore, at least partly, be attributed to this discrepancy (Spear et al., 2021). The observed difference between the two whole-pond corrections in their relation to conventional abundance estimates was limited (Figure 6b, c) because the studied ponds were relatively similar in terms of pond depth (1.437 ± 0.205 m; Table 1). Even though a total surface area-based correction of eDNA concentrations is easier to obtain and is increasingly finding its way into eDNA quantification studies, a total volume-based correction might be preferred when water bodies differ strongly in size and depth. However, these whole-water body spatial metric corrections (be it on the basis of surface area or volume) are only appropriate when the target species is randomly distributed throughout the water body and/or the spatial coverage of the sampling strategy is high, as was the case in the ponds included in this work. Oppositely, if the target species mainly occurs in nearshore areas, for instance, eDNA concentrations should be corrected based on the surface area or water volume of that specific area. Nonetheless, these findings indicate that quantitative eDNA analyses offer a highly accurate alternative to conventional abundance estimations that is applicable in a broader set of conditions, and highlights the importance of correcting eDNA concentrations for pond volumes.

Following fyke netting, bullfrog eDNA concentrations decreased significantly and proportionally to the number of captured bullfrogs (Figure 7). Although this decrease was already considerable 3 days after fyke netting, bullfrog eDNA concentrations were not yet in equilibrium with the reduced larval abundance, as was measured 7 days after removal. Notably, even the removal of two individuals was reflected in a reduced bullfrog eDNA concentration (Figure 7), which underlines the sensitivity of quantitative eDNA analyses. These results thus not only confirm the prompt degradation of eDNA in natural lentic systems (Brys, Haegeman, et al., 2021), but also show that the effectiveness of eradication efforts can be appropriately evaluated with quantitative eDNA analyses. In some ponds, however, eDNA concentrations only decreased slightly or even increased after removing considerable numbers of bullfrogs. This is most likely because only a proportion of the population was captured, and/or ongoing bullfrog reproduction outweighed the number of bullfrogs that were captured and removed. In pond 24, for instance, eDNA concentrations decreased only slightly after the first capture round (pond 24a), and increased after the second capture round 1 month later (pond 24b; Figure 7). Here, clear signs of active reproduction were observed, such as egg deposits and calling males, suggesting that local eDNA concentrations were spiked in between the 'pre' and 'post fyke netting' eDNA samples. Even though active reproduction of bullfrogs could not be excluded from the majority of other ponds, our results suggest that the management efforts undertaken reduced bullfrog population sizes in most cases, yet insufficiently for complete local bullfrog eradication.

Altogether, our findings highlight that quantitative eDNA analyses accurately and reliably estimate bullfrog abundance in natural lentic systems. We demonstrate that eDNA concentrations can be used to not only locate bullfrog breeding ponds, but also to estimate the catch that can be expected for a given unit of effort and to evaluate the efficacy of management efforts. Quantitative eDNA analyses can thus be considered valuable additions to the nature resource manager's toolbox for coordinating the management of bullfrogs.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

T.E. and R.B. conceived the study design and experimental set-up. T.E., R.B., N.D.R., and C.V.D. collected field samples, and S.N. performed the molecular analyses. T.E. analysed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication. T.E., R.B., S.D., A.D.V, and C.V.D received funding for this work. S.D. and A.D.V. were responsible for the eradication program.

DATA AVAILABILITY STATEMENT

All data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.qz612jmhm

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REFERENCES

Adams, C. I. M., Hoekstra, L. A., Muell, M. R., & Janzen, F. J. (2019). A brief review of non-avian reptile environmental DNA (eDNA), with a case study of painted turtle (*Chrysemys picta)* eDNA under field conditions. *Diversity*, *11*(4), 50. https://doi.org/10.3390/d11040050

Brys, R., Haegeman, A., Halfmaerten, D., Neyrinck, S., Staelens, A., Auwerx, J., & Ruttink, T. (2021). Monitoring of spatio-temporal occupancy patterns of fish and amphibian species in a lentic aquatic system using environmental DNA. *Molecular Ecology*, *30*(13), 3097–3110. https://doi.org/10.1111/mec.15742

Brys, R., Halfmaerten, D., Neyrinck, S., Mauvisseau, Q., Auwerx, J., Sweet, M., & Mergeay, J. (2021). Reliable eDNA detection and quantification of the European weather loach (*Misgurnus fossilis*). *Journal of Fish Biology*, *98*(2), 399–414. https://doi.org/10.1111/jfb.14315

Bury, R. B., & Whelan, J. A. (1984). Ecology and management of the bullfrog. (vol. *155*). U.S. Fish and Wildlife Service.

Carim, K. J., Bean, N. J., Connor, J. M., Baker, W. P., Jaeger, M., Ruggles, M. P., McKelvey, K. S., Franklin, T. W., Young, M. K., & Schwartz, M. K. (2020). Environmental DNA sampling informs fish eradication efforts: Case studies and lessons learned. *North American Journal of Fisheries Management*, *40*, 488–508. https://doi.org/10.1002/nafm.10428

Cowx, I. G. (1983). Review of the methods for estimating fish population size from survey removal data. *Fish Management*, *14*(2), 67–82. https://doi.org/10.1111/j.1365-2109.1983.tb00057.x

De Meester, L., Declerck, S., Stoks, R., Louette, G., Van De Meutter, F., De Bie, T., Michels, E., & Brendonck, L. (2005). Ponds and pools as model systems in conservation biology, ecology and evolutionary biology. *Aquatic Conservation: Marine and Freshwater Systems*, *15*, 715–725. https://doi.org/10.1002/aqc.748

Derek, H. O., Doll, J. C., Wheeler, P., & Dinno, A. (2021). FSA: Fisheries Stock Analysis (R package version 0.9.1). Retrieved from https://github.com/droglenc/FSA

Doi, H., Takahara, T., Minamoto, T., Matsuhashi, S., Uchii, K., & Yamanaka, H. (2015). Droplet digital polymerase chain reaction (PCR) outperforms real-time PCR in the detection of environmental DNA from an invasive fish species. *Environmental Science and Technology*, *49*(9), 5601–5608. https://doi.org/10.1021/acs.est.5b00253

Dougherty, M. M., Larson, E. R., Renshaw, M. A., Gantz, C. A., Egan, S. P., Erickson, D. M., & Lodge, D. M. (2016). Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. *Journal of Applied Ecology*, *53*(3), 722–732. https://doi.org/10.1111/1365-2664.12621

Everts, T., Halfmaerten, D., Neyrinck, S., De Regge, N., Jacquemyn, H., & Brys, R. (2021). Accurate detection and quantification of seasonal abundance of American bullfrog (*Lithobates catesbeianus*) using ddPCR eDNA assays. *Scientific Reports*, *11*, 11282. https://doi.org/10.1038/s41598-021-90771-w

Ficetola, G. F., Coïc, C., Detaint, M., Berroneau, M., Lorvelec, O., & Miaud, C. (2007). Pattern of distribution of the American bullfrog *Rana catesbeiana* in Europe. *Biological Invasions*, *9*, 767–772. https://doi.org/10.1007/s10530-006-9080-y

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, *4*(4), 423–425. https://doi.org/10.1098/rsbl.2008.0118

Fremier, A. K., Strickler, K. M., Parzych, J., Powers, S., & Goldberg, C. S. (2019). Stream transport and retention of environmental DNA pulse releases in relation to hydrogeomorphic scaling factors. *Environmental Science and Technology*, *53*(12), 6640–6649. https://doi.org/10.1021/acs.est.8b06829

Govindarajulu, P., Altwegg, R., & Anholt, B. R. (2005). Matrix model investigation of invasive species control: Bullfrogs on Vancouver island. *Ecological Applications*, *15*(6), 2161–2170. https://doi.org/10.1890/05-0486

Hansen, B. K., Bekkevold, D., Clausen, L. W., & Nielsen, E. E. (2018). The sceptical optimist: challenges and perspectives for the application of environmental DNA in marine fisheries. *Fish and Fisheries*, *19*(5), 751–768. https://doi.org/10.1111/faf.12286

Harrison, J. B., Sunday, J. M., & Rogers, S. M. (2019). Predicting the fate of eDNA in the environment and implications for studying biodiversity. *Proceedings of the Royal Society B: Biological Sciences*, *286*, 20191409.

Hubert, W. A., Pope, K. L., & Dettmers, J. M. (2012). Passive capture techniques. In A. V. Zale, D. L. Parrish, & T. M. Sutton (Eds.), *Fisheries techniques* (3rd ed., pp. 223–265). American Fisheries Society.

King, A. C., Krieg, R., Weston, A., & Zenker, A. K. (2022). Using eDNA to simultaneously detect the distribution of native and invasive crayfish within an entire country. *Journal of Environmental Management*, *302*, 113929. https://doi.org/10.1016/j.jenvman.2021.113929

Lampo, M., & Bayliss, P. (1996). Density estimates of cane toads from native populations based on mark-recapture data. *Wildlife Research*, *23*(3), 305–315. https://doi.org/10.1071/WR9960305

Louette, G., Devisscher, S., & Adriaens, T. (2013). Control of invasive American bullfrog *Lithobates catesbeianus* in small shallow water bodies. *European Journal of Wildlife Research*, *59*(1), 105–114. https://doi.org/10.1007/s10344-012-0655-x

Lowe, S., Browne, M., Boudjelas, S., & De Poorter, M. (2000). 100 of the world's worst invasive alien species.

Maunder, M. N., & Punt, A. E. (2004). Standardizing catch and effort data: A review of recent approaches. *Fisheries Research*, *70*, 141–159. https://doi.org/10.1016/j.fishres.2004.08.002

Moyer, G. R., Díaz-Ferguson, E., Hill, J. E., & Shea, C. (2014). Assessing environmental DNA detection in controlled lentic systems. *PLoS One*, *9*, e103767. https://doi.org/10.1371/journal.pone.0103767

Mueller, M., Pander, J., Knott, J., & Geist, J. (2017). Comparison of nine different methods to assess fish communities in lentic floodplain habitats. *Journal of Fish Biology*, *91*(1), 144–174. https://doi.org/10.1111/jfb.13333

Peterson, J. T., Thurow, R. F., & Guzevich, J. W. (2004). An evaluation of multipass electrofishing for estimating the abundance of stream-dwelling salmonids. *Transactions of the American Fisheries Society*, *133*, 462–475. https://doi.org/10.1577/03-044

Plante, F., Bourgault, P., Dubois, Y., & Bernatchez, L. (2021). Environmental DNA as a detection and quantitative tool for stream-dwelling salamanders: A comparison with the traditional active search method. *Environmental DNA*, *3*(6), 1128–1141. https://doi.org/10.1002/edn3.233

Plough, L. V., Bunch, A. J., Lee, B. B., Fitzgerald, C. L., Stence, C. P., & Richardson, B. (2021). Development and testing of an environmental DNA (eDNA) assay for endangered Atlantic sturgeon to assess its potential as a monitoring and management tool. *Environmental DNA*, *3*(4), 800–814. https://doi.org/10.1002/edn3.186

Pochardt, M., Allen, J. M., Hart, T., Miller, S. D. L., Yu, D. W., & Levi, T. (2020). Environmental DNA facilitates accurate, inexpensive, and multiyear population estimates of millions of anadromous fish. *Molecular Ecology Resources*, *20*(2), 457–467. https://doi.org/10.1111/1755-0998.13123

Pyšek, P., Hulme, P. E., Simberloff, D., Bacher, S., Blackburn, T. M., Carlton, J. T., Dawson, W., Essl, F., Foxcroft, L. C., Genovesi, P., Jeschke, J. M., Kühn, I., Liebhold, A. M., Mandrak, N. E., Meyerson, L. A., Pauchard, A., Pergl, J., Roy, H. E., Seebens, H., … Richardson, D. M. (2020). Scientists' warning on invasive alien species. *Biological Reviews*, *95*, 1511–1534. https://doi.org/10.1111/brv.12627

Reid, A. J., Carlson, A. K., Creed, I. F., Eliason, E. J., Gell, P. A., Johnson, P. T. J., Kidd, K. A., MacCormack, T. J., Olden, J. D., Ormerod, S. J., Smol, J. P., Taylor, W. W., Tockner, K., Vermaire, J. C., Dudgeon, D.,

& Cooke, S. J. (2019). Emerging threats and persistent conservation challenges for freshwater biodiversity. *Biological Reviews*, *94*, 849–873. https://doi.org/10.1111/brv.12480

Rojahn, J., Pearce, L., Gleeson, D. M., Duncan, R. P., Gilligan, D. M., & Bylemans, J. (2021). The value of quantitative environmental DNA analyses for the management of invasive and endangered native fish. *Freshwater Biology*, *66*(8), 1619–1629. https://doi.org/10.1111/fwb.13779

RStudio Team. (2022). *RStudio: Integrated development environment for R*. RStudio. Retrieved from http://www.rstudio.com/

Salter, I., Joensen, M., Kristiansen, R., Steingrund, P., & Vestergaard, P. (2019). Environmental DNA concentrations are correlated with regional biomass of Atlantic cod in oceanic waters. *Communications Biology*, *2*(1), 1–9. https://doi.org/10.1038/s42003-019-0696-8

Sepulveda, A. J., Al-Chokhachy, R., Laramie, M. B., Crapster, K., Knotek, L., Miller, B., Zale, A. V., & Pilliod, D. S. (2021). It's complicated. Environmental DNA as a predictor of trout and char abundance in streams. *Canadian Journal of Fisheries and Aquatic Sciences*, *78*(4), 422–432. https://doi.org/10.1139/cjfas-2020-0182

Sepulveda, A. J., Nelson, N. M., Jerde, C. L., & Luikart, G. (2020). Are environmental DNA methods ready for aquatic invasive species management? *Trends in Ecology and Evolution*, *35*(8), 668–678. https://doi.org/10.1016/j.tree.2020.03.011

Shelton, A. O., Kelly, R. P., O'Donnell, J. L., Park, L., Schwenke, P., Greene, C., Henderson, R. A., & Beamer, E. M. (2019). Environmental DNA provides quantitative estimates of a threatened salmon species. *Biological Conservation*, *237*, 383–391. https://doi.org/10.1016/j.biocon.2019.07.003

Simberloff, D., Martin, J. L., Genovesi, P., Maris, V., Wardle, D. A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M., Pyšek, P., Sousa, R., Tabacchi, E., & Vilà, M. (2013). Impacts of biological invasions: What's what and the way forward. *Trends in Ecology and Evolution*, *28*(1), 58–66. https://doi.org/10.1016/j.tree.2012.07.013

Spear, M. J., Embke, H. S., Krysan, P. J., & Vander Zanden, M. J. (2021). Application of eDNA as a tool for assessing fish population abundance. *Environmental DNA*, *3*(1), 83–91. https://doi.org/10.1002/edn3.94

Vilà, M., & García-Berthou, E. (2010). Monitoring biological invasions in freshwater habitats. In C. Hurford, M. Schneider, & I. Cowx (Eds.), *Conservation monitoring in freshwater habitats: A practical guide and case studies* (pp. 91–100). Springer. https://doi.org/10.1007/978-1-4020-9278-7

Yates, M. C., Cristescu, M. E., & Derry, A. M. (2021). Integrating physiology and environmental dynamics to operationalize environmental DNA (eDNA) as a means to monitor freshwater macro-organism abundance. *Molecular Ecology*, *30*(24), 6531–6550. https://doi.org/10.1111/mec.16202

Yates, M. C., Fraser, D. J., & Derry, A. M. (2019). Meta-analysis supports further refinement of eDNA for monitoring aquatic species-specific abundance in nature. *Environmental DNA*, *1*, 5–13. https://doi.org/10.1002/edn3.7

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