Management of Biological Invasions (2023) Volume 14, Issue 2: 201–220

Special Issue: Proceedings of the 22nd International Conference on Aquatic Invasive Species
Guest editor: Mattias Johansson

Research Article

The American bullfrog exposed: distribution, invasion fronts, and spatial configuration of invasion hubs revealed by eDNA-based monitoring and environmental assessments

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Editors' Note: This study was contributed in relation to the 22nd International Conference on Aquatic Invasive Species held in Ostend, Belgium, April 18–22, 2022 (https://icais.org). This conference has provided a venue for the exchange of information on various aspects of aquatic invasive species since its inception in 1990. The conference continues to provide an opportunity for dialog between academia, industry and environmental regulators.

Citation: Everts T, Van Driessche C, Neyrinck S, Jacquemyn H, Brys R (2023) The American bullfrog exposed: distribution, invasion fronts, and spatial configuration of invasion hubs revealed by eDNA-based monitoring and environmental assessments. *Management of Biological Invasions* 14(2): 201–220, https://doi.org/10.3391/mbi.2023.14.2.02

Received: 30 July 2022
Accepted: 13 October 2022
Published: 6 February 2023

Thematic editor: Mattias Johansson

Abstract

The American bullfrog (*Lithobates catesbeianus* [Shaw, 1802]) is one of the hundred most destructive Alien Invasive Species (AIS) worldwide that has invaded more than 40 countries across 4 continents. In Belgium, bullfrogs have occupied a large area in a relatively short period of time despite a decade of intensive management interventions. Acquiring better insights into the distribution, abundance, and spatial spread of this invasive frog species is an important first step towards a successful management strategy. In this study, we sampled 382 permanent water bodies and combined environmental DNA (eDNA)-based analyses using quantitative droplet digital PCR (ddPCR) with assessments of habitat characteristics to generate an overview of the present distribution of bullfrogs in Flanders (Northern Region of Belgium) and the type of water bodies they have invaded. Our results revealed a fragmented distribution pattern covering an area of 364.76 km² that consisted of eight metapopulations located in five different river valleys, suggesting the occurrence of multiple anthropogenically-mediated introductions. Bullfrogs appeared to be firmly established in the valley of the Grote Nete, where invaded waterbodies have been found along 72% of the length of this river, divided into three distinct metapopulations. Unlike refuge sites, bullfrogs were found to be highly selective in their choice of breeding sites, which were characterised by abundant emergent vegetation and sparse tree cover along the shoreline. The division of the vast occupied area into well-defined, accurately delineated metapopulations facilitates the identification of functional management units. Furthermore, the obtained knowledge of the patterns of range expansions and the spatial configuration and associated environmental features of breeding sites can be used to prioritise management interventions in strategically located invasion hubs. Overall, we conclude that eDNA-based monitoring combined with environmental assessments provide important information that can be used to manage widespread aquatic AIS more effectively.

Key words: aquatic invasive species management, droplet digital PCR, eDNA applications, freshwater systems, habitat suitability, invasion dynamics, pond-breeding amphibian

Introduction

Freshwater ecosystems cover no more than one percent of the Earth's surface but accommodate ten percent of all species (Dudgeon et al. 2006). However, these ecosystems are currently suffering from various anthropogenic stressors, including the introduction of non-indigenous species that turn out to be invasive (Gallardo et al. 2016). Global financial costs related to Alien Invasive Species (AIS) in aquatic environments amount to at least 23 billion USD annually and are expected to increase over time (Cuthbert et al. 2021). Effective management of biological invasions threatening these fragile aquatic ecosystems is therefore ecologically and economically paramount. While prevention and early-detection-rapid-response strategies have received considerable attention, many conservation managers are confronted with the occurrence of widespread invaders, in which a different set of objectives and strategies are involved, but for which hands-on management recommendations are still largely lacking (Vander Zanden and Olden 2008; Kamath et al. 2016; Green and Grosholz 2021).

Insights into the spatial distribution of AIS and patterns of secondary spread are essential in devising successful containment strategies (Vimercati et al. 2019; Araya-Donoso et al. 2022; Greenhalgh et al. 2022). However, conventional aquatic species surveillance can be labour-intensive and costly when conducted on vast spatiotemporal scales and when a priori knowledge of occurrence and ecological habitat preferences is unavailable (Mueller et al. 2017; Vimercati et al. 2019; Da Silva Neto et al. 2020). Moreover, detecting AIS at low densities requires an intensive monitoring effort at a certain location to the detriment of other locations (Hayes et al. 2005). An alternative to monitor aquatic environments involves the amplification and analysis of DNA isolated from environmental samples (Ficetola et al. 2008). Targeted environmental DNA (eDNA)-based species detection (eDNA barcoding) is a highly sensitive, non-destructive molecular monitoring technique that is often less financially demanding than its conventional counterparts (Dejean et al. 2012; Smart et al. 2016; Fediajevaite et al. 2021; Keller et al. 2022). Not only can even a single individual be detected in a water body as large as an Olympic swimming pool, but also the relative density of the target species can be inferred from eDNA concentrations (Brys et al. 2021; Everts et al. 2021, 2022). Consequently, eDNA-based analyses can be a valuable source of information for designing effective biological invasion management programmes. For instance, eDNA barcoding was recently used to quantify habitat requirements of the invasive African clawed frog (*Xenopus laevis* [Daudin, 1802]) in France (Vimercati et al. 2019), to delineate the distribution range of the invasive signal crayfish (*Pacifastacus leniusculus* [Dana, 1852]) in the United Kingdom (Greenhalgh et al. 2022), to track expanding invasion fronts of the invasive European

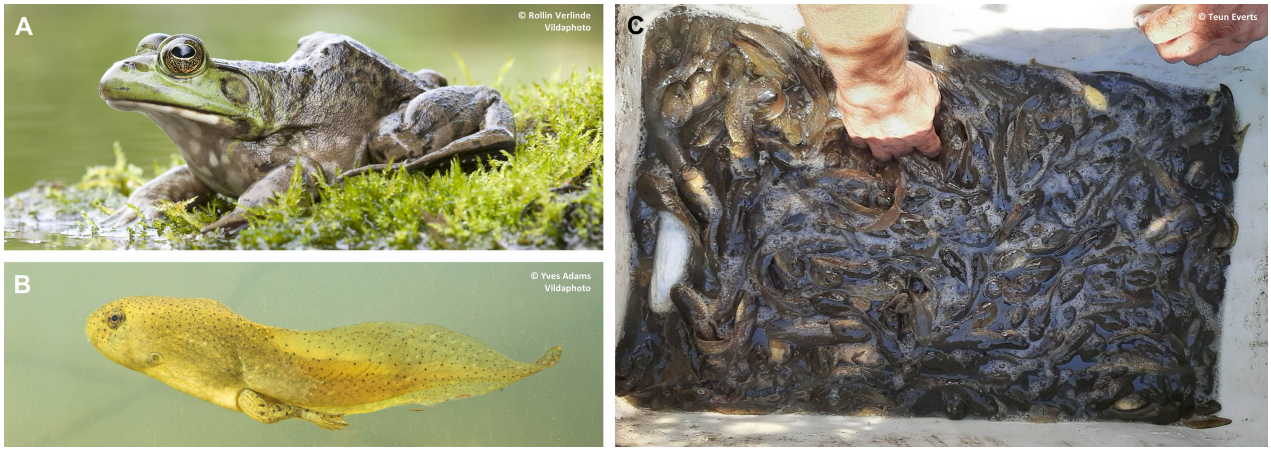

Figure 1. (A) An adult and (B) a tadpole of the American bullfrog. (C) Targeting breeding sites for removal efforts using fyke nets generally result in large numbers of captured tadpoles. Photographs by Rollin Verlinde (A), Yves Adams (B), and Teun Everts (C).

green crab (*Carcinu maenas* [Linnaeus, 1758]) in North America (Keller et al. 2022), and to evaluate eradication success of invasive American bullfrog (*Lithobates catesbeianus* [Shaw, 1802]) populations in Belgium (Everts et al. 2022). However, explicit formulations of specific management strategies to counter widespread freshwater AIS based on quantitative eDNA analyses remain scarce.

The American bullfrog (bullfrog hereafter) is a prime example of an invasive species that causes considerable management challenges worldwide (Figure 1a, b). From the eastern USA, this large anuran species was exported to the western USA, western Canada, South America, Asia, and Europe for commercial frog farming and pet trade throughout the nineteenth and twentieth century (Kraus 2008). Once released into natural systems, non-indigenous bullfrogs proved to be highly invasive and posed a major threat to indigenous amphibian communities through resource competition, predation, and the transmission of pathogens previously absent in the introduced regions (Miaud et al. 2016; Yap et al. 2018; Bissattini et al. 2019). As the American bullfrog is highly elusive, mobile (annual movement of up to 1600 meters; Smith and Green 2005), and fertile (clutch size of up to 25000 eggs; Bury and Whelan 1984), colonisation can start unnoticed and proceed rapidly, making effective control challenging (Snow and Witmer 2010). Consequently, the import of bullfrogs from outside the European Union was banned since 1997 (EEC Regulation 338/97). Furthermore, a European regulation prohibited the possession and trade of bullfrogs, and obliged member states to take stringent measures to counter bullfrog establishment and spread from 2016 onwards (EU Regulation 1143/2014). Here, bullfrogs are currently widespread in Belgium, France, Germany, and Italy, and management interventions can barely keep up with the pace of the invasion (Tsiamis et al. 2017). Integrated management approaches that contribute to preventing further spread and reducing the invaded area are therefore urgently needed (Snow and Witmer 2010; Groffen et al. 2019).

Bullfrogs are generalist omnivores inhabiting a variety of eutrophic water bodies (Bury and Whelan 1984). Even though bullfrogs can be found in permanent ponds, seasonally drying ponds, ditches, and rivers, breeding is restricted to permanent water bodies, as the tadpoles of this warm-adapted species require at least two years of development before metamorphosis in maritime temperate climates (Bury and Whelan 1984). Because newly metamorphosed bullfrogs typically emigrate from their natal water bodies to escape competition and cannibalism of conspecific adults (Bury and Whelan 1984; Gahl et al. 2009), these breeding sites serve as local invasion hubs that drive invasive spread (Adriaens et al. 2013). Since bullfrogs often fail to establish at recently colonised sites (Sepulveda 2018) and are challenging to capture once having reached the post-metamorphic stage (Everts et al. 2022), tadpole removal from breeding sites can be expected to be more effective in slowing further spread and containing the invasion (Figure 1c), regardless of the resulting lower impact on population growth rates (Govindarajulu et al. 2005). Importantly, a complete depletion of breeding sites should be pursued, as partial removal allows a resurgence of the local population sizes due to density-dependent population dynamics (Bury and Whelan 1984). Annual culling of at least 75% of the bullfrog tadpole population was found to be sufficient for long-term population control (Gray 2009). In this way, bullfrogs were recently successfully removed from the Yosemite Valley (Kamoroff et al. 2020). Identifying and mapping these breeding sites can therefore significantly improve large-scale management strategies (Florance et al. 2011; Mizumoto et al. 2022).

In this study, we combined quantitative droplet digital PCR (ddPCR) eDNA-based analyses with environmental assessments to generate insights into the distribution and spread of the American bullfrog in Flanders (Northern Region of Belgium). First, we conducted a large-scale eDNA survey to determine the spatial distribution of bullfrogs. Second, we used quantitative eDNA data and assessments of habitat characteristics to determine its ecological habitat preferences and the environmental features associated with breeding sites. Finally, by combining the acquired insights, we propose specific management interventions aimed at reducing the further spread of this invasive species.

Materials and methods

Study area

The first registered observation of the American bullfrog in Belgium was in Wallonia (Southern Region of Belgium) and dates back to 1992 (Adriaens et al. 2013). Because the few populations that had established in Wallonia are now considered extinct, this study focuses on Flanders. Here, the first observation originates from 1997 and was recorded in the vicinity of a fish farm located near the upper reaches of the river Grote Nete. This farm is

believed to have imported living bullfrogs for the commercial pet trade, and escapees are assumed to have spread to lower reaches of the river valley. At the same time, living bullfrogs were additionally sold on a pet market. Deliberate releases and unintentional escapes are suspected to have led to multiple metapopulations scattered across Flanders. At present, bullfrogs are most common in the Campine Region (province of Antwerp), a diluvial sand region in the northeast of Belgium that consists of fragments of marshes, heaths, meadows, and pine forests interspersed with intensively cultivated and urbanised areas. Here, bullfrogs have been observed near the rivers Mark, Wamp, and Dyle, but are most abundant in the valley of the Grote Nete. This area consists of a large number of ponds and a network of ditches draining into the rivers Grote Nete, Molse Nete, and Grote Laak from approximately 70 metres above sea level in the east to 14 metres in the west (Figure 2). This valley is assigned as a Special Area of Conservation for some Habitat Directive species, including the great crested newt (*Triturus cristatus* [Laurenti, 1768]), spined loach (*Cobitis taenia* [Linnaeus, 1758]), and European brook lamprey (*Lampetra planeri* [Bloch, 1784]).

Data collection

eDNA data

Based on the putative bullfrog distribution, as determined by occurrence records published in open-access databases, past management interventions, and preliminary surveys, 382 permanent water bodies were selected for eDNA sampling in order to map the spatial distribution of bullfrogs in Flanders. These water bodies were sampled in 2020, 2021, and 2022 between the 3rd of May and the 30th of September. This time window corresponds to the period that bullfrogs are reproductively active, which maximizes eDNA-based detection rates (Everts et al. 2021). Because aqueous eDNA particles are concentrated near submerged individuals and thus unevenly distributed in lentic systems (Brys et al. 2021), habitat coverage was maximized with an integrated sampling strategy. Water samples were collected using a 0.5 L sampling bag attached to a telescopic sampling pole. Samples were collected every 5 meters along the perimeter of each water body within a 5 meter radius by gradually filling the sampling bag with water from the upper 10 cm of the water column. All 0.5 L samples were pooled to acquire one homogenous water sample of 10 to 15 L for every water body. Using a Vampire sampler pump (Buerkle, Bad Bellingen, Germany) and disposable silicon tubing, this pooled water sample was then guided over an enclosed disk filter (1 filter per water body) comprised of an integrated 5 µm glass fibre prefilter and a 0.8 µM PES membrane (NatureMetrics, Surrey, England) until the filters clogged (1.654 ± 1.194 L). The filters were immediately stored at −21 °C in a BlueLine box (delta T, Fernwald, Germany) for optimal conservation during transportation to the

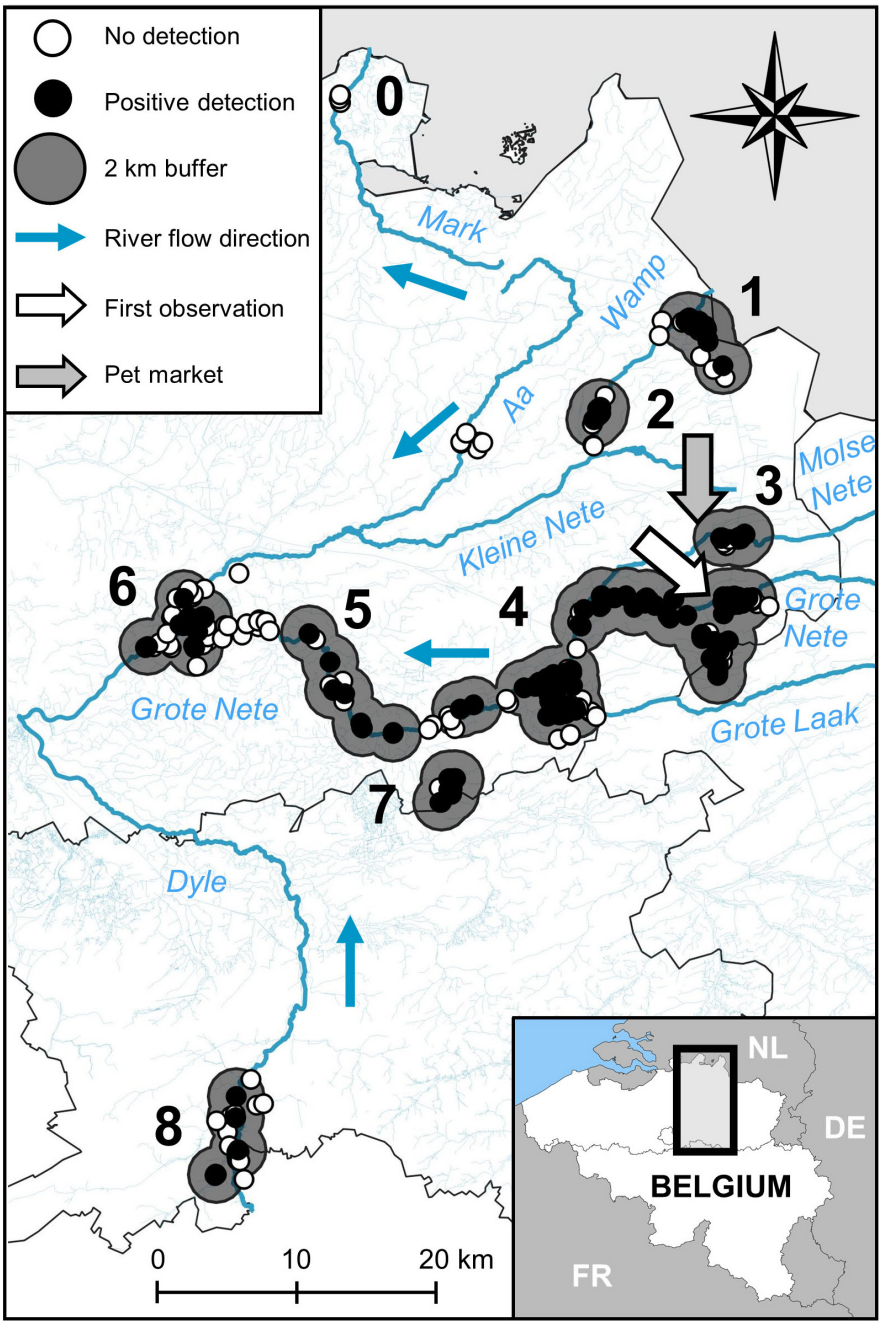

Figure 2. The present spatial distribution of American bullfrogs in Flanders based on an extensive eDNA survey of 382 water bodies. White and black dots represent bullfrog-negative (n = 193) and bullfrog-positive (n = 189) water bodies. The Area Of Occupancy (AOO) based on buffers of 2 km radius around each bullfrog detection was used to quantify the spatial distribution. Eight extant metapopulations in five river catchments were identified, and the successful eradication of metapopulation 0 was confirmed. The white arrow indicates the location of the fish farm that was expected to have imported bullfrogs in Belgium, and where the first official bullfrog observation in Flanders was recorded. The grey arrow corresponds to the location of the pet market where living bullfrogs were sold. The major natural water ways, their names, and their flow directions are given in blue. Note that dots are superimposed.

laboratory. Sterile nitrile gloves were worn at all times and reusable field material was decontaminated with 2% Virkon S (Antec DuPont, Suffolk, UK). At the end of each sampling day, 2 litres of deionized water was filtered in the field following an identical protocol to probe for genetic cross-contamination.

Table 1. The chemical, biotic, and geographical habitat variables that were quantified in bullfrog-free (n = 30), bullfrog refuge (n = 30), and bullfrog breeding sites (n = 30) to determine the habitat preferences of American bullfrogs in Flanders. The mean and standard deviation for each variable are given per colonisation status.

Code	Description	Colonisation status		
		Bullfrog-free	Bullfrog refuge	Bullfrog breeding
Chemical				
ACID	pH value of the water	7.39 ± 1.04	7.62 ± 0.99	7.58 ± 0.85
COND	Electrolytic conductivity of the water (in µS/cm)	369.64 ± 209.52	384.44 ± 179.22	270.61 ± 127.41
DO	Dissolved oxygen content of the water (in %)	90.94 ± 37.34	97.17 ± 46.64	96.46 ± 21.15
Biotic				
*TREE	Tree cover along the shoreline	4.10 ± 1.45	3.40 ± 1.40	1.80 ± 0.76
*SHRUB	Shrub cover along the shoreline	3.77 ± 1.57	3.17 ± 1.26	3.17 ± 1.39
*EMER VEG	Emergent vegetation	1.67 ± 0.99	1.57 ± 0.82	4.40 ± 1.19
*FLOAT VEG	Free floating vegetation	2.40 ± 1.43	2.13 ± 1.50	2.47 ± 1.50
*SUBM VEG	Submerged vegetation	1.60 ± 1.10	2.07 ± 1.57	2.23 ± 1.50
Geographical				
CIRCUM	Pond circumference (in meters) measured in QGIS	219.28 ± 138.69	174.58 ± 136.61	232.79 ± 170.39
AREA	Pond area (in square meters) measured in QGIS	2337.03 ± 2429.74	1251.44 ± 1283.11	2836.21 ± 3728.94
PER	Perimeter to area ratio (perimeter/$\sqrt{\text{area}}$)	4.70 ± 0.93	5.06 ± 2.23	4.73 ± 0.97

* Variables visually estimated on an ordinal scale (1 = 0%, 2 = 1–5%, 3 = 6–25%, 4 = 26–50%, 5 = 51–75%, and 6 = 76–100%).

Environmental data

Following eDNA sampling, 11 habitat variables were quantified for a subset of 90 permanent water bodies (30 bullfrog-free sites, 30 refuge sites, and 30 breeding sites; Table 1). The water oxygen content, pH, and electrolytic conductivity were determined with a WTW CellOX® oxygen electrode, a WTW Sentix®41 pH-combination electrode, and a Mettler Toledo® 721 conductometer, respectively. In addition, tree cover, shrub cover, emerging vegetation, floating vegetation, and submerged vegetation were visually estimated and determined on an ordinal scale (0%, 1–5%, 6–25%, 26–50%, 51–75%, and 76–100%). Finally, the circumference, area and perimeter to surface ratio were determined for each water body using Geographical Information Systems (QGIS 3.22.9) with a georeferenced digital database of permanent lentic waters in Flanders (Leyssen et al. 2020).

DNA extraction and molecular analyses

A previously developed and validated droplet digital PCR (ddPCR) primer/probe assay was used to detect bullfrog eDNA and estimate their density based on eDNA concentrations (Everts et al. 2021, 2022). Briefly, 1 ml of a lysis buffer including an internal positive control (IPC) was applied to each filter. An IPC is an exogenous DNA fragment with a known concentration serving as an assessor of PCR inhibition or failure, and thus as a signaller of issues in the laboratory workflow. This IPC was a plasmid containing a 149 bp Dengue virus type 2 insert sequence (GenBank M29095.1). After incubating all filters overnight at 56 °C, the lysis solution was collected from the filters and the DNA was extracted with Qiagen's DNeasy Blood and Tissue Kit and finally 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 eluted in 100 µl Tris–EDTA. The concentration of both bullfrog eDNA and IPC in each sample was simultaneously quantified by ddPCR following Everts et al. (2021) in replicated technical measurements. The number of technical replicates per sample ranged from 2 to 10 depending on the location within the presumed distribution area. Because detection probabilities are generally lower when species are less abundant (Dougherty et al. 2016), more technical replicates were included for samples at the periphery of the distribution, where fewer bullfrogs were expected to occur than in the core.

Data analysis

The total number of bullfrog eDNA copies per microliter DNA extract obtained from one litre filtered water was calculated following Everts et al. (2022). Bullfrogs were considered absent from a water body if no bullfrog amplification was observed (bullfrog colonisation status = 0). Bullfrog presence was inferred when at least one technical replicate contained one or more positive ddPCR droplets. Based on previous insights obtained from seasonal eDNA patterns in natural ponds (Everts et al. 2021) and netting experiments under controlled settings in natural systems (Everts et al. 2022), 4 copies μl^{-1} per litre filtered water was generally considered as a conservative threshold for separating refuge sites (< 4 copies μl^{-1}; bullfrog colonisation status = 1) from breeding sites (> 4 copies μl^{-1}; bullfrog colonisation status = 2).

Based on the presence-absence data, the Area Of Occupancy (AOO) was calculated to quantify the present spatial distribution of bullfrogs in Flanders. This measure was originally developed by the International Union for Conservation of Nature (IUCN) to monitor the ecological status of ecosystems (IUCN 2022). To do so, a 2 km radius buffer corresponding to a distance slightly exceeding the maximum annual displacement of bullfrogs (1.6 km; Smith and Green 2005) was constructed around each positive eDNA detection. Overlapping buffers were merged and the total surface area was calculated. The resulting number of polygons was interpreted as the number of separate bullfrog metapopulations and the length of the river Grote Nete within these merged buffers was measured.

To identify the habitat characteristics associated with bullfrog refuge sites (bullfrog colonisation status 0 and 1 were binary coded) and breeding sites (bullfrog colonisation status 0+1 and 2 were binary coded), Generalized Linear Models (GLMs) with a logit link and binomial error distribution were constructed. Multicollinearity among the predictor variables was assessed with a correlation matrix. Because the Spearman correlation coefficient was equal to or higher than 0.7 for all combinations of the geographical variables, only the perimeter to area ratio of sampled water bodies was retained as this area-adjusted measure of edge habitat was

expected to be better predictor of bullfrog breeding sites in comparison with the other geographical variables. Log-transformations were conducted when the assumption of residual normality was violated. Models with all possible combinations of the 9 retained variables were constructed ($n = 2^9 = 512$) and compared based on the Akaike information criterion corrected for small sample sizes (AIC_c). A model-averaging approach was used to estimate the importance of each predictor variable based on all constructed models. The best performing model for predicting each dependent variable was checked for variance inflation and overdispersion, and its fit was evaluated using Nagelkerke pseudo R^2. All statistical analyses were carried out using R version 4.2.0 (RStudio Team 2022). The functions *lm* and *glm* from the *stats* package were used to build GLM's, the *nagelkerke* function from the *rcompanion* package to evaluate model performance, and the *dredge* function from the *MuMIn* package for model selection and model averaging.

Results

Spatial distribution and invasion fronts

Bullfrog eDNA was detected in 189 out of the 382 sampled water bodies (Figure 2). A total area of 364.76 km² was found to be occupied by bullfrogs in Flanders. Eight separate metapopulations located in five different river valleys could be delineated: one large metapopulation along the river Grote Nete (metapopulation 4 = 148.96 km²; Figure 2) and seven smaller metapopulations (metapopulation 1 = 30.24 km², 2 = 16.60 km², 3 = 19.32 km², 5 = 51.56 km², 6 = 40.96 km², 7 = 21.41 km², and 8 = 35.71 km²; Figure 2). Water bodies colonised by bullfrogs were found along a 61.35 km stretch of the Grote Nete (37.89 km, 18.32 km and 5.14 km in metapopulations 4, 5, and 6, respectively), covering 72% of the total length of the river. Here, the invaded area consisted of three metapopulations (metapopulations 4, 5, and 6; Figure 2) that were separated by a large number of water bodies in which no bullfrog eDNA was detected. Upstream range expansion from the first recorded bullfrog observation was only 6.50 km, while downstream range expansion consisted of a stretch of 54.85 km along the river. From the 382 sampled water bodies, 31 were located at the upstream periphery of metapopulation 4 to more precisely delineate the upstream invasion front in the Grote Nete valley. Here, 11 and 4 water bodies were found to be refuge sites and breeding sites, respectively (Figure 3a). No bullfrog eDNA was detected in five consecutive water bodies located approximately 1 km from the most upstream identified breeding site, suggesting the presence of an invasion front. A southward expansion of the distribution range in the upstream section of the Grote Nete valley was detected, independent of any river system (Figure 3b). Based on 22 of the total 382 sampled water bodies, a new invasion front was identified in 2021, 5 km from the invasion front previously determined in 2020. Here,

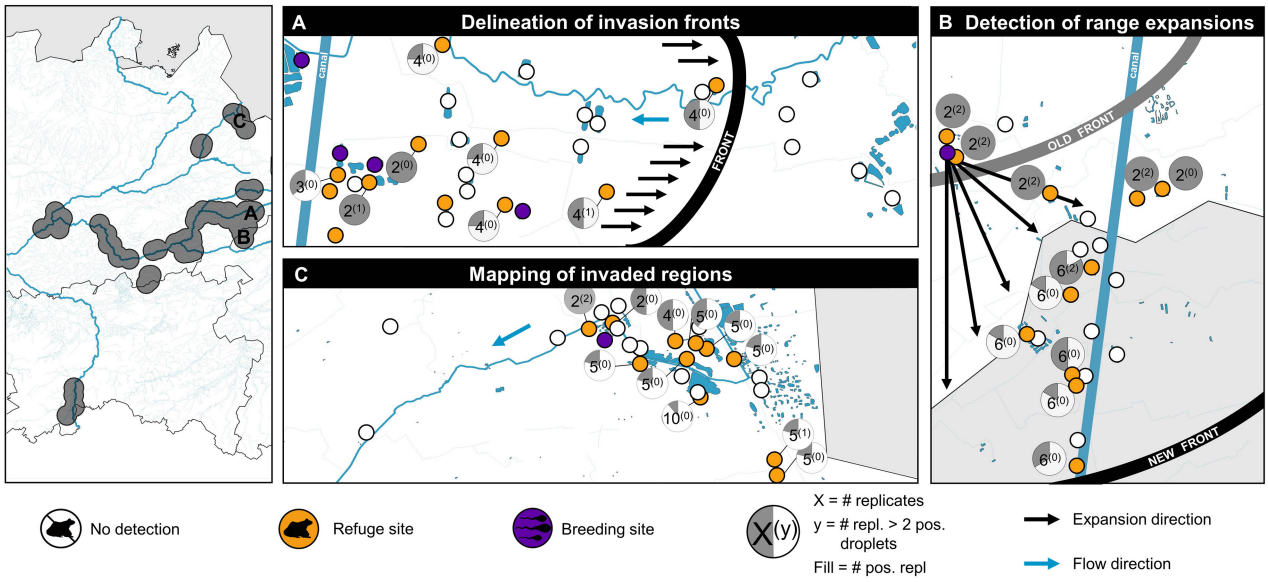

Figure 3. Field situations demonstrating how the spatial configuration of eDNA concentrations in the landscape can provide valuable insights into the distribution and invasive spread. The location of each field situation within the invasion range of bullfrogs in Flanders is given in the left panel. Circles in each of the three right panels correspond to the location of sampled water bodies (which are represented by blue polygons), and their colour corresponds to the presence and abundance of bullfrogs according to the legend below. The total number of replicates that were analysed, the number of replicates containing bullfrog DNA and the number of replicates with more than two bullfrog-positive ddPCR droplets are represented by pie charts, but only for sites with concentrations lower than 1 copy μL^{-1}. (A) The upstream invasion front of metapopulation 4 around the river Grote Nete was clearly identified by multiple negative eDNA samples. (B) An eDNA screening near a previously identified invasion front suggested a recent range expansion of metapopulation 4 that can be assumed to have been driven by a newly established breeding site located near the previously defined invasion front. (C) A comprehensive screening of metapopulation 1 resulted in the identification of one breeding site that is likely to serve as the source for this satellite metapopulation.

only one breeding site was identified near the previously identified invasion front, while the other sampled water bodies did not contain any bullfrog eDNA (n = 10) or were refuge sites (n = 11).

Four metapopulations (metapopulation 0, 1, 2, and 8) were identified that were highly spatially isolated compared to the other metapopulations. The conducted eDNA survey confirmed the successful eradication of satellite metapopulation 0 in the valley of the river Mark after intensive management interventions in the years before sampling (Adriaens et al. 2013). Two satellite metapopulations were identified in the valley of the river Wamp, and one in the valley of the Dyle. No breeding sites were identified in metapopulations 2 and 8. Only one breeding site was found in metapopulation 1, while 11 water bodies contained very low bullfrog eDNA concentrations (Figure 3c). No bullfrog eDNA was detected in the 3 sampled water bodies located along the Wamp downstream of the breeding site.

Environmental characteristics of breeding sites

There was no single logistic regression model based on the quantified habitat variables that could discriminate bullfrog-free sites from bullfrog refuge sites (Tables 2, 3). The best fit model included percentage tree cover as only explanatory variable (AIC_c = 83.79, Pseudo R^2 = 0.078; Table 3), which was the most important variable throughout all models (Table 4).

Table 2. Comparison of the best GLM's ($\Delta AIC_c < 2$) predicting the occurrence of either bullfrog refuge sites (bullfrog colonisation status 0 and 1 were binary coded) or bullfrog breeding sites (bullfrog colonisation status 0+1 and 2 were binary coded) based on habitat variables.

Variables[1]	Log(L)[2]	AIC_c[3]	Δ_i[4]	Weight[5]
Bullfrog refuge sites				
TREE	−39.79	83.79	0.00	0.03
TREE + SUBM VEG	−38.77	83.96	0.18	0.03
SHRUB	−40.25	84.71	0.93	0.02
SHRUB + SUBM VEG	−39.36	85.15	1.36	0.02
1	−41.59	85.25	1.46	0.02
EMER VEG + TREE + SUBM VEG	−38.28	85.28	1.50	0.01
TREE + PER	−39.46	85.35	1.56	0.01
TREE + SHRUB	−39.54	85.52	1.73	0.01
SUBM VEG	−40.68	85.58	1.79	0.01
TREE + FLOAT VEG	−39.60	85.62	1.83	0.01
TREE + PER + SUBM VEG	−38.50	85.73	1.94	0.01
Bullfrog breeding sites				
EMER VEG + TREE + PER	−11.67	31.82	0.00	0.08
EMER VEG + TREE + PER + COND	−11.05	32.82	1.00	0.05
EMER VEG + TREE	−13.27	32.82	1.01	0.05
EMER VEG + TREE + COND	−12.57	33.61	1.79	0.03
EMER VEG + TREE + PER + SHRUB	−11.46	33.63	1.81	0.03
EMER VEG + TREE + PER + DO	−11.51	33.74	1.93	0.03

[1]Variable codes for the quantified habitat variables, as defined in Table 1.
[2]The log-likelihood value of a model is a measure of the goodness of fit, with higher values indicating a better fit.
[3]Akaike's information criterion (AIC) corrected for small sample size.
[4]Difference in AIC_c between the best model and the given model.
[5]Akaike weight, representing the probability that the current model is the best-approximating model among those considered.

Table 3. Variable estimates for the best-supported logistic regression model predicting bullfrog refuge sites (bullfrog colonisation status 0 and 1 were binary coded) and bullfrog breeding sites (bullfrog colonisation status 0+1 and 2 were binary coded) based on habitat variables. Variables for which the associated z-values are indicated in bold are significant predictors.

Variable	Sign[1]	$\lvert\beta\rvert$[2]	SE[3]	|z-value|[4]	Significance[5]
Bullfrog refuge sites					
(Intercept)	+	0.8438	0.8290	1.018	
TREE	–	0.3508	0.1912	1.835	
Bullfrog breeding sites					
(Intercept)	–	0.1415	3.5981	0.039	
EMER VEG	+	21.968	8.0335	**2.735**	**
TREE	–	1.9655	0.7750	**2.545**	*
PER	–	1.0866	0.6872	1.581	

[1]Indicates whether the variable estimate was positive or negative.
[2]Absolute value of the multiple logistic regression coefficient.
[3]Standard error.
[4]Test statistic of the multiple logistic regression coefficient.
[5]Significance codes: * < 0.05; ** < 0.01; *** < 0.001.

Alternatively, bullfrog breeding sites were found to be significantly differing from all other sampled water bodies (Tables 2, 3). The best fit model for predicting bullfrog breeding sites contained percentage emergent vegetation, percentage tree cover, and the perimeter to area ratio as explanatory variables (AIC_c = 31.82; Pseudo R^2 = 0.88; Table 3), which were

Table 4. Model averaged coefficients with either bullfrog refuge sites (bullfrog colonisation status 0 and 1 were binary coded) and bullfrog breeding sites (bullfrog colonisation status 0+1 and 2 were binary coded) as response variables based on 512 (2^9) GLMs per response variable. Variance represents the adjusted standard error of each predictor and significant predictors are indicated in bold.

Variable	*Bullfrog refuge sites*			*Bullfrog breeding sites*		
	Relative Importance	Parameter estimate	Variance	Relative Importance	Parameter estimate	Variance
(Intercept)	–	0.63	1.91	–	−1.30	5.95
EMER VEG	0.28	−0.25	0.63	**1.00****	**19.92**	**7.35**
TREE	0.55	−0.19	0.24	**1.00***	**−1.89**	**0.82**
SUBM VEG	0.46	0.14	0.20	0.27	−0.15	0.30
FLOAT VEG	0.27	−0.030	0.79	0.25	0.045	0.48
SHRUB	0.39	−0.099	0.17	0.27	0.26	0.48
PER	0.27	0.047	0.095	0.58	−1.08	0.75
DO	0.26	−0.00054	0.0026	0.28	−0.011	0.017
ACID	0.26	0.016	0.11	0.26	0.26	0.78
COND	0.24	0.000021	0.00037	0.38	−0.0046	0.0043

Significance codes: * < 0.05; ** < 0.01; *** < 0.001.

the most important across all possible GLMs (Table 4). Holding all other predictor variables constant, the odds of a site being suited for breeding increased by 659% (95% CI [3.16, 18.26]), decreased by 71% (95% CI [0.51, 0.83]), and decreased by 7% (95% CI [-0.28, 0.33]) for a one-unit increase in emerging vegetation, tree cover along the shoreline, and perimeter to area ratio, respectively. Water bodies with an increasing percentage emergent vegetation (which in many cases was common reed *Phragmites australis* [(Cav.) Trin. ex Steud.] or soft rush *Juncus effusus* [L.]) and a decreasing percentage tree cover along the shoreline are significantly more likely to be favourable sites for bullfrog breeding (Figure 4). All bullfrog breeding sites included in this study contained a percentage of emergent vegetation of at least 6–25% and a tree cover of maximum 6–25%.

Discussion

Spatial distribution

The large-scale eDNA survey conducted in this study revealed a fragmented distribution pattern of bullfrogs in Flanders spanning a total area of 364.76 km². Eight metapopulations located in five different river valleys were identified, which suggests the occurrence of multiple anthropogenically-mediated introductions of bullfrogs in Belgium (Figure 2). The trade in live bullfrogs at a local pet market in the early 2000s can be expected to have contributed to the observed distribution pattern. A non-continuous spatial distribution of invasive bullfrogs linked to secondary translocations after initial introduction has also been reported in France and Italy (Ficetola et al. 2007a). Considering that the first introductions in Flanders occurred only 20 to 30 years ago and that invasive spread has continued unabated despite intensive management programmes (Adriaens et al. 2013), the extent of the occupied area is alarming. Moreover, the area that is presently invaded only constitutes a fraction of the potential spatial distribution of invasive bullfrogs in Belgium according to the climate and land-use models of Ficetola et al. (2007b). If no effective and sustained management interventions are implemented,

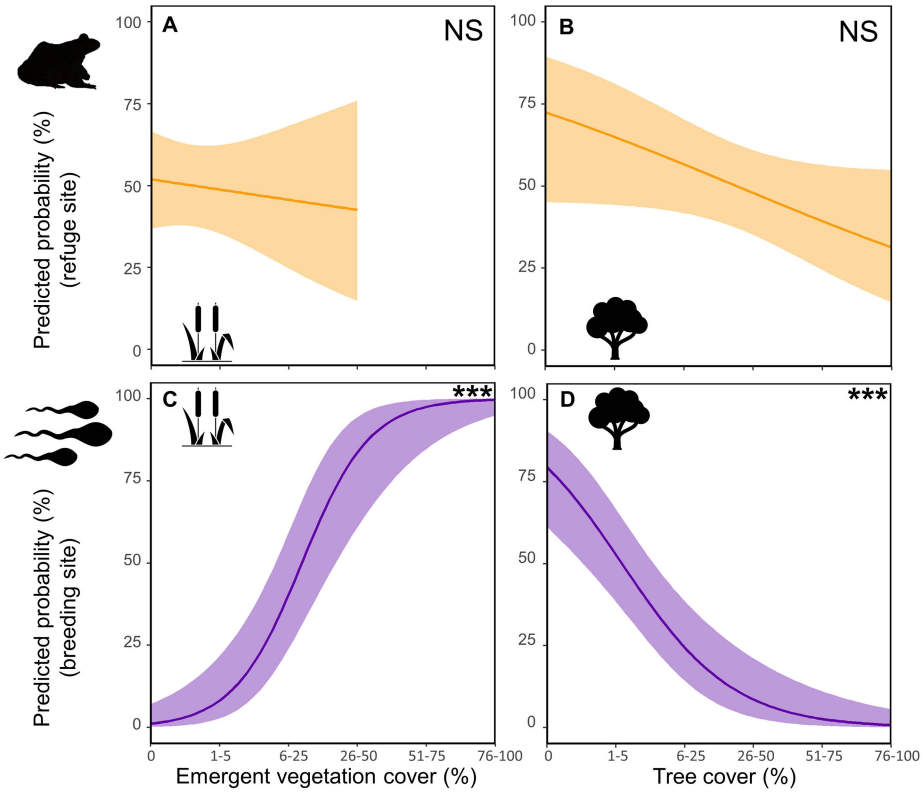

Figure 4. Logistic regression model output predicting the occurrence of either (A, B) bullfrog refuge sites (colonisation status 0 and 1 were binary coded) or (C, D) bullfrog breeding sites (colonisation status 0+1 and 2 were binary coded). Only the models including percentage emergent vegetation (A, C) and tree cover (B, D) as predictors were shown, as these were found to be strong predictors of bullfrog breeding sites ($p < 0.001$), while no measured variable appeared to be related to bullfrog refuge sites. This suggests that bullfrog breeding, opposed to its general presence, is highly dependent on specific habitat types, which can be used in an integrated management approach. Asterisks represent significant relationships. NS stands for non-significant.

the situation here can be expected to progressively evolve into one that can currently be found in France and Italy, where bullfrogs have occupied approximately 2000 km² and 5000 km² since their first introduction in 1968 and the 1930s, respectively (Ficetola et al. 2007a). These enormous occupied areas have also been reported for other invasive amphibian species, such as the African clawed frog in France, which has occupied 2055 km² since its single introduction in the 1980s (Measey et al. 2012; Vimercati et al. 2019). Nonetheless, the confirmation of the successful eradication of metapopulation 0 (Figure 2) supports the belief that intensive management interventions coordinated by eDNA-based analyses can effectively reduce and even locally eradicate invasive bullfrogs (Kamoroff et al. 2020).

The valley of the Grote Nete comprised the largest part of the occupied area, where invaded water bodies were located along 72% of the length of the river, and primarily downstream from the first recorded bullfrog observation (Figure 2). Similarly, invasive bullfrogs in the Yellowstone River floodplain in the USA have colonised a considerable part of the valley and have spread mainly in a downstream direction since their introduction in 1999 (Sepulveda et al. 2015). Hence, bullfrog dispersal seems to be facilitated by

a river system, either through passive downstream dispersal, or through active movement along the rural floodplain. Nonetheless, our results indicate the presence of three clearly distinct metapopulations in the valley of the Grote Nete. Considering that no bullfrog eDNA was detected in a large number of water bodies in between these metapopulations, and that the buffer sizes on which the constructed AOO's were based had a radius that slightly exceeded the maximal annual bullfrog displacement distance observed, it is likely that multiple anthropogenically-mediated introductions have occurred even within this valley (Ficetola et al. 2007a). In other words, our results seem to contradict the common belief that invasive bullfrogs have spread in a linear fashion all the way from the region where they were first introduced, resulting in one large, interconnected metapopulation. Instead, it is more likely that an interplay between dispersal along the river floodplain and multiple anthropogenically-mediated introductions has resulted in the present distribution of bullfrogs in Flanders. Genetic analyses can provide valuable insights into the observed distribution patterns. Such analyses allowed Kamath et al. (2016), for example, to demonstrate that invasive bullfrogs have spread naturally and mainly in a downstream direction along the Yellowstone river without secondary human-mediated introductions. If the identified metapopulations in Flanders are truly geographically isolated, it is key from a management point of view to prevent them from coalescing (With 2002).

Invasion fronts

A screening of nearly all permanent water bodies located at the periphery of the main distribution area for bullfrog eDNA has clearly delineated the upstream boundary of metapopulation 4 (Figure 3a). The location of this invasion front was only 6.5 km upstream of the first recorded bullfrog observation in Flanders (Figure 2), confirming earlier findings that upstream dispersion along a river is considerably slower than downstream dispersal (Sepulveda et al. 2015). Notably, this area was not known to be invaded in 2013 (Adriaens et al. 2013), which implies that either bullfrogs made use of a culvert that channels the Grote Nete under a man-made canal in an upstream direction (Figure 3a), or that bullfrogs were already introduced at that time but remained unnoticed by conservation managers. In any case, bullfrogs have increased in abundance since then, and preventing this invasion front from expanding further upstream is crucial to preserve the ecological integrity of the multitude of water bodies in the upstream nature reserve (Adriaens et al. 2013).

The conducted eDNA survey also detected a range expansion in a region where no active management efforts have yet been undertaken (Figure 3b). Here, the expansion occurred independent of any river system, supporting the importance of overland dispersal for invasive bullfrog spread (Peterson

et al. 2013). The low ratio of positive to negative ddPCR replicates indicates that this range expansion was carried out by a limited number of bullfrogs that had not (yet) reproduced in the sampled water bodies and thus that it was recent (Everts et al. 2022). This suggests that complete elimination of this early invasion stage from this region is feasible if measures are rapidly undertaken (Tingley et al. 2017; Greenlees et al. 2018). Apart from providing the opportunity to intercept newly arriving colonisers, accurately tracing the leading edge of expanding invasions has the additional advantage that this knowledge can inform the installation of barriers to contain the invaded area. This was clearly demonstrated by Bylemans et al. (2016), who used eDNA-based analyses to delineate the invasion front of redfin perch (*Perca fluviatilis* [Linnaeus, 1758]) in Australia for the construction of an exclusion barrier aimed at preserving an upstream population of the endangered Southern pygmy perch (*Nannoperca australis* [Günther, 1861]). Interestingly, when determined by eDNA-based analyses, this invasion front was located 2.8 km more upstream than when determined by conventional surveillance techniques, which underlines the value of this technique to track advancing invasions (Bylemans et al. 2016).

Refuge versus breeding sites

Considerable parts of the occupied area may consist of sink habitat that is not suited for reproduction but rather serve as refuge sites that cannot persist without a continuous influx of immigrating individuals (Vander Zanden and Olden 2008; Gahl et al. 2009; Sepulveda 2018). Conversely, breeding sites function as continuous sources of propagules driving invasive spread (Gahl et al. 2009; Mizumoto et al. 2022). The ability of quantitative eDNA analyses to discriminate between refuge and breeding sites (Everts et al. 2022) was applied to provide an overview of the spatial configuration of source (i.e. breeding sites) and sink (i.e. refuge sites) populations within the occupied area (Figure 3). Overall, bullfrog metapopulations were found to consist of numerous refuge sites and only a handful of breeding sites. For example, one bullfrog breeding site was detected in satellite metapopulation 1 that was surrounded by a large number of refuge sites (Figure 3c). The identified breeding site was only 2 km away from the border with the Netherlands, where bullfrogs have been successfully eradicated (Adriaens et al. 2013), necessitating the prioritisation of this area for intensive management interventions. In general, breeding sites that are located in the immediate vicinity of the outer distribution limits should be targeted to halt invasive spread and minimize recolonisation while pre-emptively monitoring water bodies just outside the delineated distribution range so that early colonisers can be detected and removed before new populations can establish (Florance et al. 2011; Tingley et al. 2017; Greenlees et al. 2018).

Our results further showed that bullfrog breeding sites, unlike refuge sites, were strongly associated with specific environmental conditions. The percentage of emergent vegetation as well as tree cover along the shoreline of permanent water bodies proved to be strong predictors of bullfrog breeding sites (Tables 3, 4). Permanent water bodies with shorelines abundantly covered with emergent vegetation and with hardly any trees casting shade on the riparian area appeared to offer conditions that are suitable for breeding (Figure 4). Similar breeding habitat requirements were found for invasive bullfrogs in Chinese wetlands (Liu et al. 2016), along the anthropogenically altered Trinity River in California (Fuller et al. 2010), and along the more natural Yellowstone river (Sepulveda et al. 2015). Water bodies with abundant emergent vegetation can provide important microhabitat for bullfrog oviposition (Liu et al. 2016) and, by supplying shelter, reduce the predation of eggs, tadpoles, juveniles and adults (Fuller et al. 2010; Chuang et al. 2019). Permanent water bodies under open canopies receive more sunlight and hence support a better growth of algae, which is the primary food source of bullfrog tadpoles (Bury and Whelan 1984). Additionally, these water bodies favour basking and accelerate egg and tadpole development by reaching higher water temperatures (Skelly et al. 2002), which is particularly important for this warm-adapted species to thrive in temperate maritime climates (Ficetola et al. 2007b). However, sunny water bodies with abundant emergent vegetation are also highly suited for a myriad of indigenous European amphibian species as well (Ficetola and De Bernardi 2004; Vági et al. 2013). This overlap in breeding habitat preferences is worrying as invasive bullfrogs have been linked to declines in the abundance and diversity of co-occurring indigenous amphibian species in North America (Johnson et al. 2011), South America (Gobel et al. 2019), and Asia (Yiming et al. 2011).

Implications for management

The interface between quantitative eDNA results and management interventions is still largely lacking and impedes their implementation by conservation managers (Sepulveda et al. 2020; Keller et al. 2022). This study highlighted five complementary aspects of how the spatial configuration of eDNA concentrations in the landscape can provide important insights for a more effective management of widespread AIS. First, metapopulations can be identified and delineated in a cost-efficient way (Figure 2), which facilitates the division of an extensive spatial distribution area in functional management units and the guidance of targeted control efforts (King et al. 2022). Second, distribution boundaries can be accurately delineated (Figure 3a), which can be extremely useful when designing spatial management strategies that minimize recolonisation of previously managed areas (Tingley et al. 2017; Greenhalgh et al. 2022). The invaded area can be

expected to be effectively contained or even reduced over time by initially removing individuals from the outer region and gradually proceeding to the inner region (Epanchin-Niell and Wilen 2012; Simberloff 2020). Third, the unparalleled sensitivity of eDNA-based analyses enables the detection of expanding invasions in an early stage and inference of direction (Figure 3b), so that measures can be taken to prevent the establishment of newly arriving colonisers and halt further spread (Bylemans et al. 2016; Keller et al. 2022). Fourth, breeding sites serving as local invasion hubs can be located in order to prioritise the allocation of resources to such source populations (Figure 3c). Depleting and isolating these breeding sites can slow down or halt further range expansions (Florance et al. 2011; Mizumoto et al. 2022) and suppress local population sizes (Green and Grosholz 2021), the effectiveness of which can be evaluated on the basis of eDNA concentrations (Everts et al. 2022). Fifth, knowledge of the type of habitat associated with these invaders (Figure 4) not only facilitates the prioritisation of surveillance and management efforts, but can also limit secondary spread by preventing access to nearby suitable habitats that are still uncolonised but at high risk of invasion (With 2002; Sepulveda 2018; Vimercati et al. 2019; Araya-Donoso et al. 2022). Altogether, this study shows the potentially invaluable role eDNA-based analyses can fulfil in risk-assessment and the science-based management of widely established invasive species.

Acknowledgements

The authors would like to thank Nico De Regge, Vincent Van Obbergen, Sander Devisscher, David Halfmaerten, Jan Vercammen, Daan Van Eenaeme, Io Deflem, Caroline Mouton, Marc Schouppe, Thomas Impens, and Els Luyckfasseel for their help in collecting samples, as well as two reviewers for their valuable comments on a previous version of the manuscript.

Funding declaration

Funding was provided by Research Foundation Flanders (grant numbers 1S01822N [T.E.] and 1S23822N [C.V.D.]), the 3n-Bullfrog project (LIFE18 NAT/BE/001016 [R.B]), the Provinces of Antwerp and Limburg, the Sigma plan and the Research Institute for Nature and Forest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contribution

T.E. and R.B. conceived the study design, T.E., R.B. and C.V.D. conducted the field work, and S.N. performed the molecular analyses. T.E. analysed and interpreted the data and led the writing of the manuscript with significant input from H.J. and R.B. All authors contributed critically to previous drafts and provided final approval for publication. T.E., R.B., and C.V.D received funding for this work.

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