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Research Article

Environmental DNA-based detection of *Fascioloides magna* and *Galba truncatula*: a non-invasive approach for monitoring invasive parasites

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Abstract

Invasive alien species, particularly microbial pathogens and parasites, pose significant threats to biodiversity, ecosystem stability, economies, food security, wildlife conservation, and public health. Their introduction can occur through human-mediated translocations or environmental factors such as weather patterns and extreme events. To predict, prevent, and manage emerging infectious diseases caused by invasive parasites, a multidisciplinary approach is essential. Invasion biology focuses on predicting potential invasive parasites before introduction, while wildlife veterinary medicine emphasizes early detection for effective prevention and management. Both approaches rely on continuous monitoring of invasive species and their hosts. The giant liver fluke (*Fascioloides magna*) has proven to be a highly successful invasive parasite, expanding its range through coevolution with native hosts, natural migration, human-facilitated transport, and environmental dispersal mechanisms like flooding and waterborne transmission. Effective monitoring strategies are needed to detect its presence and that of species involved in its life cycle as early as possible. In this study, a three-month environmental DNA (eDNA) screening was conducted in La Mandria Regional Park (Italy) to detect *F. magna* and its lymnaeid snail intermediate host, *Galba truncatula*, using water and soil samples. By integrating surface water dynamics for site selection, collecting multiple environmental matrices, and utilizing highly sensitive molecular methods (ddPCR), the study successfully identified both species without relying on prior biological distribution data, highlighting the effectiveness of eDNA-based surveillance for invasive parasites.

Key words: eDNA, invasive alien species, runoff tracking

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Introduction

Invasions by alien species increasingly threaten biodiversity resources, ecosystem services, regional economies, food security, wildlife conservation and public health. The risk of alien species introduction is growing rapidly worldwide due to expanding transportation corridors, technological breakthroughs, geopolitical dynamics, land use and climate change, (Galil et al. 2015; Muirhead et al. 2015; Seebens et al. 2015; Early et al. 2016), (Fisher et al. 2012; Martel et al. 2014). Alien species are carried along with their symbiome (symbiotic macro- and microbiome) including viruses, bacteria and other eukaryotes among them metazoan parasites (Foster et al. 2021) that may be virulent or pathogenic to native hosts (Lymbery et al. 2014; Blackburn and Ewen 2017).

The emergence of invasive alien pathogens and the challenge to detect their presence are ranked among the top issues in a horizon scan of how future invasion processes and dynamics will be understood and managed (Ricciardi et al. 2017).

Invasive alien pathogens, especially parasites, can cause emerging infectious diseases in wildlife through human intervention (via host or parasite translocations) or natural environmental events. Parasites may be introduced independently or alongside invasive hosts. These invasive alien parasites can infect native wildlife or domestic animals, triggering new diseases (alien-to-native spillover) and potentially endangering native species. (Crowl et al. 2008), (Daszak et al. 2000; Taraschewski 2006). Invasive parasites must adapt to native hosts by overcoming key invasion barriers: introduction (initial contact with native hosts), establishment (successful persistence in native hosts), and spread (expansion across hosts or geographic regions), similar to free-living invasive species (Lymbery et al. 2014). Parasite–host dynamics during invasion stages depend on environmental factors like pollutants (Bojko et al. 2020), temperature changes (Laverty et al. 2017), biological invasions (Dunn et al. 2012), and biodiversity shift (Frainer et al. 2018). Global environmental change, marked by deviations from baseline conditions and extreme weather (Laverty et al. 2017), may enhance the establishment of invasive parasites by creating conditions similar to their native habitat (Hulme 2009). Drastic environmental events such as flooding can also lead to biogeographical expansion of invasive parasites by translocating parasite and species involved in its life cycle beyond their establishment range (Marinković et al. 2013).

Fascioloides magna is an invasive liver fluke in Europe, originally native to North America, which infects wild and domestic ungulates. Its lifecycle involves wild ruminants as definitive hosts and pond snails (specifically freshwater snails with right-handed (dextral) shell coiling, such as the lymnaeid snails *Galba truncatula* and *Radix peregra*) as intermediate hosts. While infection in cattle is usually subclinical, it can be fatal in sheep (Howell and Williams 2020). The parasite was first described in 1865 near Turin, Italy, in imported wapitis (Bassi 1875), and it remains established there, now infecting a broader range of hosts, including cattle, horses, and wild boar (Balbo et al. 1989). Other stable European foci include areas in the Czech Republic, Poland, and the Danube floodplain forests across Austria, Slovakia, Hungary, and Croatia (Králová-Hromadová et al. 2011; Filip-Hutsch et al. 2022).

Traditionally, definitive hosts include red deer, fallow deer, and white-tailed deer, which shed eggs in feces. Wild boar are considered dead-end hosts, and roe deer were previously thought to be aberrant hosts that do not support parasite maturation (Pybus 2001). However, recent findings suggest that roe deer can now act as definitive hosts, and sika deer have also been confirmed as such, indicating host range expansion (Konjević et al. 2021; Rehbein and Visser 2021).

F. magna causes liver damage, reducing host health. Its lifecycle includes egg hatching into miracidia in moist, warm conditions (24–28 °C), which then infect snails. Inside the snail, the parasite develops into cercariae, which emerge at night, encyst on aquatic plants as metacercariae, and are ingested by grazing animals to complete the cycle (Csivincsik et al. 2023). *F. magna* has been demonstrated to be a very successful invader since its expansion relies on a combination of intrinsic factors such as coevolution with native hosts and extrinsic factors such as host-parasite encounter rate (natural migration or by transportation through humans), passive translocations of intermediate host or free-stages of parasite (eggs, miracidia, cercariae and metacercariae), by water, strong wind (rarely), flooding, adhesion in hairs of mammals and feathers of birds as well as transportation of soil and plants for commercial reasons (Sattmann et al. 2014; Rehbein and Visser 2021).

To effectively predict, prevent, and manage emerging epidemiological threats - such as the spread of invasive alien parasites like *F. magna* - a multidisciplinary approach is essential. Invasion biology contributes by identifying parasite traits (morphological, physiological, phenological, and behavioral) that influence success at various invasion stages: entrainment (the process affecting the probability that a parasite is deliberately or accidentally selected for transport from the potential source pool of parasites still in their native range (Pyšek et al. 2020)), transport, introduction, establishment, spread, and impact. Special attention is given to stages before introduction, as early detection is key to prevention (Violle et al. 2007), (Barwell et al. 2023). Meanwhile, wildlife veterinary medicine focuses on the early detection of pathogens as the most important measure of prevention and management from the early introduction stage. The core to both approaches is the monitoring for the presence/absence of known or candidate (predicted) species and other species involved in its lifecycle.

To achieve the early detection aim of a monitoring program within wildlife populations, an integrated surveillance approach where data from pathogen detection merges with host community surveillance is required (Cardoso et al. 2022). Environment with its biotic and abiotic components could serve as a unifying framework for such integrated monitoring programs and eDNA methods are able to potentially capture data from pathogens (e.g. invasive parasites) and host community (i.e. hosts involved in the invasive parasite's lifecycle) at earliest possible (ENETWILD-consortium et al. 2023). Environmental DNA/RNA (eNA)-based methods providing researchers with information on species presence without the need for capture or direct observation are increasingly used for detection, investigation, and surveillance of pathogens (Bass et al. 2023). Nonetheless, application of eNA methods in the context of wildlife surveillance should improve towards a greater standardization of sampling and processing methods. In this context, eNA studies should move from ad-hoc sampling site selection based on species distribution data (e.g. presence/absence of pond snails in studies aiming at trematode detection) forward towards methods with lower independence to species distribution data and aiming at collection of samples containing eNA which represent wider biodiversity allowing increased downstream detection probability (Carraro et al. 2021). Due to dynamic hydrological processes, eDNA samples from running waters and underlying soil are assumed to represent a broader biodiversity signal from contributing areas upstream. Such an approach, particularly in riverine systems, has proven valuable but also presents challenges, as prior hydrological knowledge is essential for meaningful biological interpretation (Carraro et al. 2020, 2021; URycki et al. 2024) Sampling point selection based on surface water dynamics and elevation maps is a promising approach as it showed itself capable of landscape mammalian fauna detection, even for most species present at low density (Lyet et al. 2021). eNA methods are intrinsically non-invasive and low cumbersome relative to direct sampling methods including avoidance of sampling that is stressful and destructive to hosts. However, they can be furthermore simplified at sample collection (e.g. sampling lower volume of water) when coupled with high sensitivity molecular detection approaches such as droplet digital PCR. ddPCR has been demonstrated to outperform qPCR in terms of sensitivity particularly analyzing eDNA samples in various biomonitoring settings (Mauvisseau et al. 2019) enhancing detection rates by reducing false negative.

Successful invasion occurring by *F. magna* requires a monitoring effort which will be able to detect the presence of the invasive parasite and/or species involved in its life cycle continuously and at earliest possible. eDNA methods are promising in this regard and for this reason we performed a three month screening of environmental samples (water and soil) for presence of *F. magna* and *G. truncatula* in its Italian foci of La Mandria Regional Park (LMRP). For this study we 1. Hypothesized sampling site selection based on surface water dynamics would reduce reliance on species (host or intermediate host) distribution data and ad-hoc sampling designs, 2. Collecting water and soil samples continuously would guaranty continuity of sampling through time, 3. Utility of highly sensitive molecular detection methods (e.g. ddPCR) would help decreasing sampling effort by collecting lower sample amounts. 4. Selection of sampling points based on surface water dynamics coupled with simultaneous water and soil sample collection would increase the eDNA capture representing more biodiversity thus increasing the possibility of detecting the targets of interest.

Materials and methods

Study area

"La Mandria" Park is a regional protected area since 1978, spanning approximately 6,557 ha in the northeast of the metropolitan city of Turin in Piedmont Region (Italy). The park features an internal core area of about 3,125 ha, surrounded by a 30 km-long wall from the external pre-park area. Park's internal fenced-off section is home to four ungulate species: wild boar, red deer, roe deer, and fallow deer. This area is registered as an Alpine site of community importance and a Special Area of Conservation (SAC) preserving the most significant example of lowland forest in Piedmont in which measures of conservation for natural and semi-natural habitats and wildlife are applied to achieve the aims of Natura 2000 network in biodiversity safeguarding in Europe (Battisti et al. 2019). The mean annual rainfall is 938 mm and the mean annual temperature is 14.8 °C. It is recognized as a primary hotspot of *F. magna* in Europe. LMRP encompasses diverse freshwater habitats, including artificial lakes, streams, and canals. The park's entire water network contributes to the Ceronda River in its southern region, which flows through the internal area and merges with the Stura di Lanzo River downstream before joining the Po River.

Sampling site selection

Water basins and stream segments were identified using the watershed function of the GRASS plugin in QGIS (3.34.11) on an elevation map of the study area (Fig. 1) (GRASS Development Team 2024). Beginning, endings and confluence junctions among multiple stream-segments in relation to the identified basin were initially identified throughout the study area and subsequently eight sampling points were selected, three of which (points 1, 2, and 8) were located outside the fenced-off area, while the remaining five (points 3, 4, 5, 6, and 7) were inside (Fig. 1). Sampling points were chosen based on their location at the beginning of a stream segment (e.g., Point 1) or at the confluence of multiple stream segments (e.g., Point 4). The identified stream segments included both permanent and temporary lotic water bodies, runoff flows, and areas of land containing lentic water bodies such as temporary waterholes.

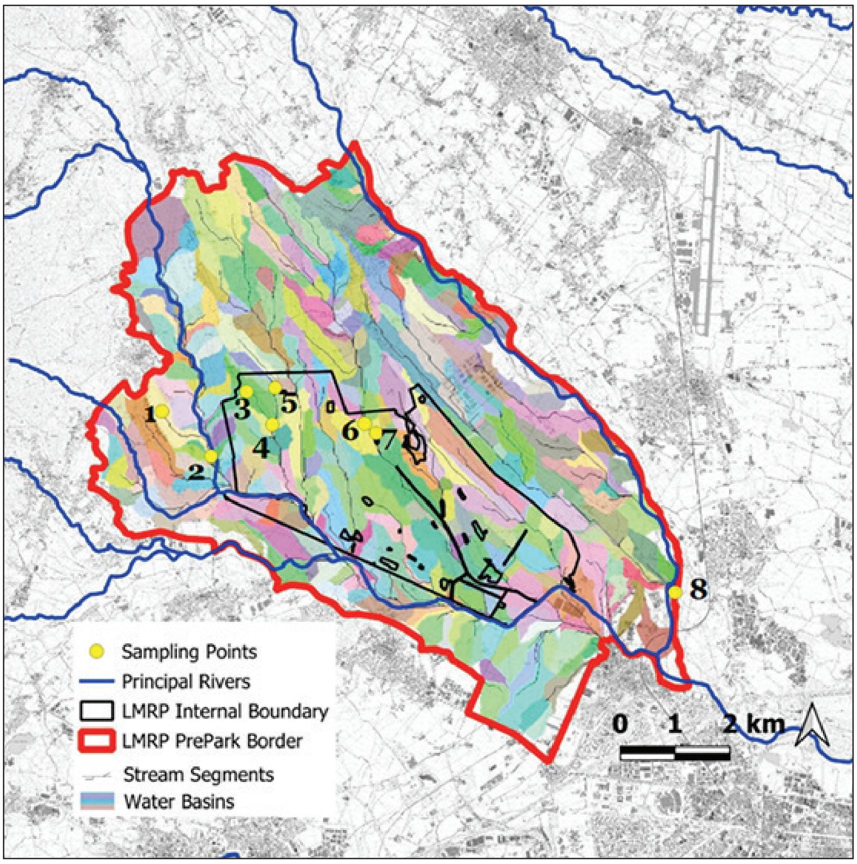

Figure 1. Study area and selected sampling points.

At each sampling site, a visual survey of the landscape was conducted to identify the presence of surface water runoff or traces of past runoff (dried paths). The starting point of a runoff path was visually identified, and the entire path was examined to determine whether it led to a lotic water body (e.g., a stream or river) or a lentic water body (e.g., a pond). This sampling approach was primarily based on the hypothesis that surface water runoff serves as the main vehicle for environmental eDNA (whether cellular or extracellular) transporting genetic material through the landscape and depositing it into downstream water bodies. The selected starting point of the runoff was marked with a wooden stick, and half of the soil sample (25 mL) was collected from the surface soil by removing the top 0.5 mm layer within a 25 cm diameter around the marker. The sample was then transferred to a clean, nuclease-free 50 mL falcon tube. The remaining half of the soil sample was collected at two additional locations along the runoff path, at variable distances from each other, extending toward either a water body (if present) or the point where the runoff path disappeared. Collection of the second half of soil samples was conducted at different points on the identified runoff path between each sampling event depending on the visible runoff path which varied every month. If a water body was present at the end of the runoff path, 200 mL of water was collected in four separate nuclease-free 50 mL falcon tubes (Fig. 2). All collected samples were transported in cool bags (containing ice bags) to the laboratory. Upon arrival to the laboratory, water samples were filtered using syringe filters with two different pore sizes (0.45 μm and 0.22 μm; Merck KGaA, Darmstadt, Germany), each with

Figure 2. Water and soil samples were collected at four distinct points by tracing runoff paths at each sampling site. Yellow markers indicate soil sampling locations along visually identified runoff paths. The first point (No. 1) represents a fixed soil sampling location used consistently throughout the sampling period. Points 2 and 3 denote variable soil sampling sites, selected based on the visible runoff path at each site and sampling event. The blue marker indicates a potential downstream water sampling location at the end of the identified runoff path. Arrows demonstrate our movement direction.

100 mL of collected water. The syringe filters were stored at -20 °C until DNA extraction. Soil samples were kept at 4 °C overnight, and the soil eDNA was extracted the following morning.

DNA extraction

Soil

Soil samples were first homogenized and subsampled to obtain a reduced but representative portion of the original sample, since the downstream DNA extraction requires at most 250 mg of soil. This step was not intended to enrich any specific target organism or parasite stage, but solely to obtain the required sample amount. For each soil sample (initially in a 50 mL falcon tube), the soil was divided into two equal parts: half of the soil from the original tube was transferred to a new 50 mL nuclease-free falcon tube, and the same was done for the remaining half. Molecular-grade nuclease-free water was then added to each tube to bring the total volume to 50 mL. Each tube was vortexed vigorously for 10 s, then allowed to stand briefly (5 s) so that larger particles could settle. After this settling period, 25 mL of the supernatant from each tube was carefully aspirated and transferred into a fresh 50 mL nuclease-free Falcon tube. All samples underwent centrifugation for 80 minutes at 4000 × g. This pre-treatment—comprising the addition of water, supernatant collection, and extended centrifugation—was adapted with modifications from the DNA extraction protocol described by Douchet et al. 2022. Following centrifugation, the supernatant was discarded, and DNA was extracted from approximately 250 mg of soil sediment using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Water

eDNA extraction from water samples was adapted with modifications from a previously validated protocol involving syringe filters, originally used to collect water from tanks containing *F. magna's* miracidia at different time points (Varzandi et al. 2024). In the original protocol, lysis buffer was used to wash and backwash the syringe filters, and DNA was extracted from the resulting liquid. In our modified approach, the lysis buffer provided with the extraction kit was used for this step. Syringe filters (0.22 µm or 0.45 µm) were placed on the top of 1.7 mL microcentrifuge tubes, and 200 µL of PW1 buffer from the DNeasy PowerWater Kit (Qiagen, Hilden, Germany) was added to the filter membrane via pipetting. After a 5-minute incubation, the liquid was aspirated from the opposite side of the filter using a pipette. This membrane-washing step was repeated once more without the incubation period. Additionally, two rounds of backwashing were performed by reversing the liquid input direction, again without the incubation period. Throughout these steps, the PW1 buffer was maintained at 55 °C. DNA was then extracted from the resulting liquid (containing the PW1 buffer) collected from the syringe filters using the DNeasy PowerWater Kit, following the manufacturer's instructions while omitting the initial vortex step intended for paper membrane disruption. Since the LMRP is known to contain both target organisms, every sample could potentially yield a positive result. Considering that we only collected samples in the field and processed them in the laboratory, we included a negative control to monitor for potential false positives. This control consisted of 100 mL of laboratory tap water, filtered through a 0.22 µm syringe filter and extracted using the same protocol as the other samples.

qPCR and ddPCR

qPCR reactions were performed in duplex using primers and probes targeting fragments of the ITS2 region of ribosomal DNA, yielding amplicons of 69 base pairs for *F. magna* and 82 base pairs for *G. truncatula*. The reaction mix and protocol followed those previously described by Varzandi et al., using 30 ng of sample DNA as input. The same primers and probes were used for ddPCR, with the modification of substituting the fluorophore dye in the *G. truncatula* assay with HEX to ensure compatibility with ddPCR instrumentation. ddPCR reaction mix were prepared in a final volume of 22 µL, including 11 µL of ddPCR Supermix for Probes (No dUTP) (Biorad Laboratories, CA, USA), 0.75 µM of each primer, 0.25 µM of each probe and 6.5 µL of sample containing 30 ng of input DNA (quantified using Nanodrop). For the generation of droplets 20 µL of reaction was considered and used for thermocycling program involving a 10-min hold at 95 °C, 40 cycles of 94 °C for 30 s and 60 °C for 1 min, followed by 10-min hold at 98 °C for enzyme deactivation and a final 30-min hold at 4 °C.

Purified cloned vectors containing *F. magna* and *G. truncatula* amplicon inserts, at concentrations ranging from 3×10^5 to 0.06 copies/µL, were prepared for both assays using the QIAGEN PCR Cloning Kit (Hilden, Germany). The following formula was used to calculate the mass of the vector-insert construct, which was then multiplied by the desired copy number to generate the standard curve.

$$m = n \times 1.096 \times 10^{-21}\ g/bp$$

where: n = plasmid size (bp) and $\boldsymbol{m}$ = mass .

In a previous study, we established a limit of quantification (LOQ) of 0.6 copies/µL of DNA for the qPCR assays, based on six 10-fold serial dilutions of positive samples and multiple replicates used for standard curve development (Varzandi et al. 2024). In the present study, we tested positive samples containing 6, 0.6, 0.3, 0.15, and 0.06 target copies/µL of plasmid DNA to compare direct quantification results obtained via ddPCR with qPCR cycle threshold (Ct) values. Additionally, we used these samples to define an amplitude threshold for the inclusion of positive droplets (Suppl. material 1). This threshold determination was performed using a single replicate. The absolute quantity of target genes per µL of sample was calculated using the formula below.

$$Absolute\ \ Quantity = \frac{ddPCR\ \ copy\ \ number \times Reaction\ \ Volume}{Sample\ \ Volume}$$

Where $ddPCR$ copy number reports the identified copy numbers in µL of reaction, reaction final volume was 20 µL and sample volume consisted 6.5 µL of reaction's final volume.

Results

Seven samples were excluded from the analysis: six water samples from sampling point three, where water bodies were consistently absent throughout the sampling period, and one soil sample from sampling point seven, which was inaccessible in August due to its location on private property. The latter was replaced with its actual location in the subsequent months. In total, 65 samples were extracted and analyzed using ddPCR and qPCR, comprising 23 soil samples and 42 water samples. The qPCR analysis detected two positive samples out of 65 (one positive for each target assay). However, quantification was not possible because the cycle threshold (Ct) values for *F. magna* (Ct 40 in a water sample filtered with 0.22 syringe filter) and *G. truncatula* (Ct 36 in a soil sample) were below the previously established limit of quantification (LOQ) of 0.6 copies/µL of plasmid DNA (Varzandi et al. 2024). In contrast, positive control results demonstrated that ddPCR could directly quantify samples with copy numbers below the qPCR LOQ of approximately 0.6 copies/µL. ddPCR successfully quantified positive samples estimated at 0.15 copies/µL, although the actual quantified copy number was higher than the estimate; for example, a sample estimated at 0.15 copies/µL was measured as 0.5 copies/µL by ddPCR. Nonetheless, the LOQ for qPCR remained at 0.6 copies/µL of plasmid DNA (Suppl. material 1: table S1).

Subsequently, all samples were tested using the duplex ddPCR assay, which identified 12 and 17 positive samples for *F. magna* and *G. truncatula*, respectively, using the same primers and probes (Fig. 3A, B). Direct quantification of samples containing 30 ng of input eDNA showed that *G. truncatula* copy numbers ranged from 1.7 to 2.14 in soil samples and from 1.8 to 19.8 in water samples (including both filter types). Similarly, the *F. magna* assay detected copy numbers ranging from 1.7 to 2 in soil samples and from 1.8 to 19.8 in water samples (including both filter types).Although not statistically significant, an overall decreasing trend in detected copy numbers was observed over time for both assays when all sample types were considered (Fig. 4A, B). However, this trend was not evident when soil samples were analyzed separately (Fig. 4A, B). The highest copy numbers were detected exclusively in water samples, while the range of detected copy numbers remained low and relatively stable throughout the sampling period.

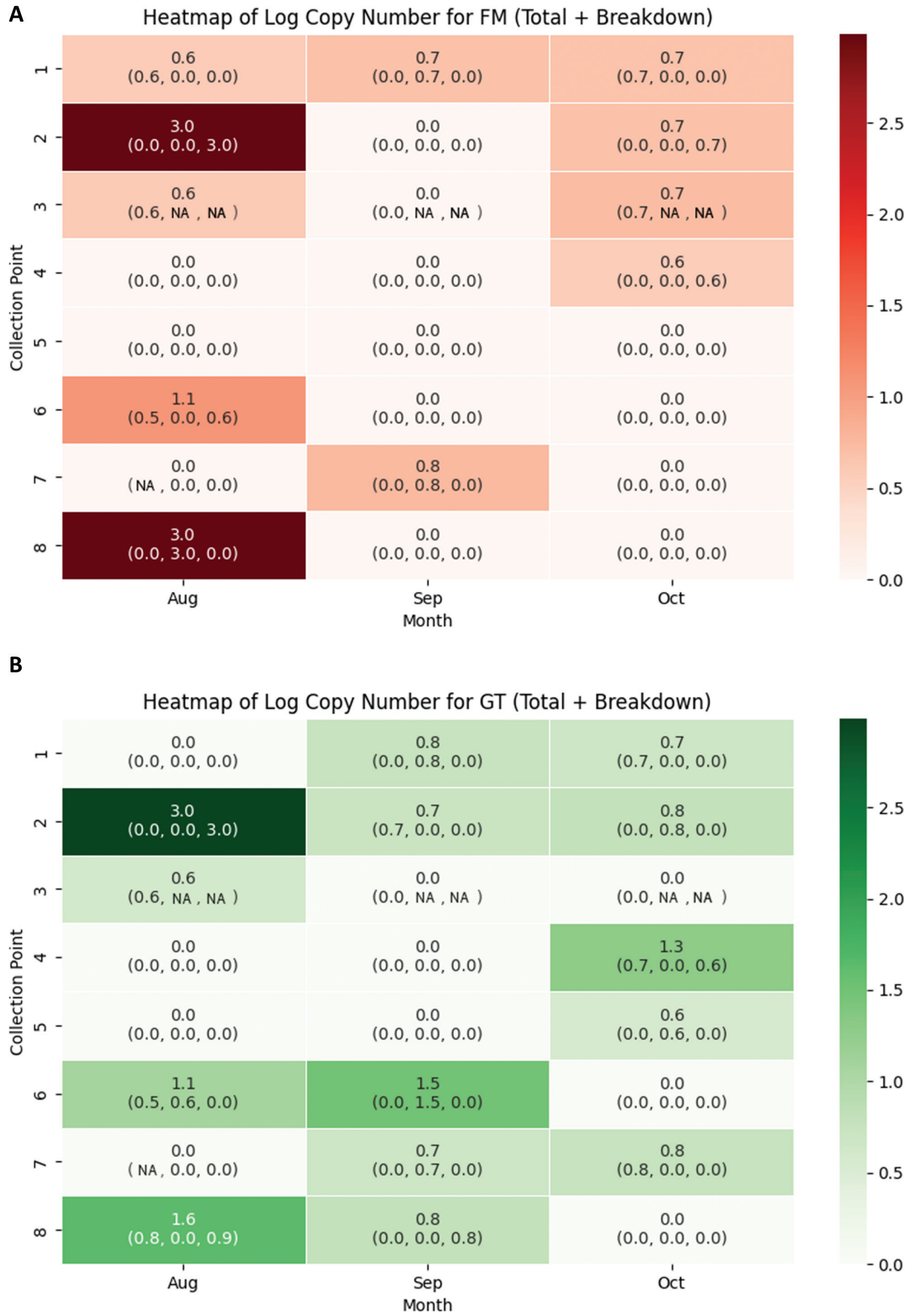

Figure 3. Detected copy numbers (in log scale for **A.** *F. magna*; **B.** *G. truncatula*) at every sampling point during the three months for all sample types (the upper number) and for soil, water 0.22 and water 0.45 filter samples (respectively in parentheses). NAs indicate excluded samples.

Our results demonstrated the presence of *F. magna* and *G. truncatula* eDNA at nearly every sampling point during at least one sampling event, except for point 5, which remained consistently negative for *F. magna*. Notably, sampling points 1 and 2, located outside the fenced-off area, were consistently positive for *F. magna* and *G. truncatula*, respectively. We detected *G. truncatula* in both sample types (water and soil) at three different sampling points (points 4, 6, and 8) across two different months

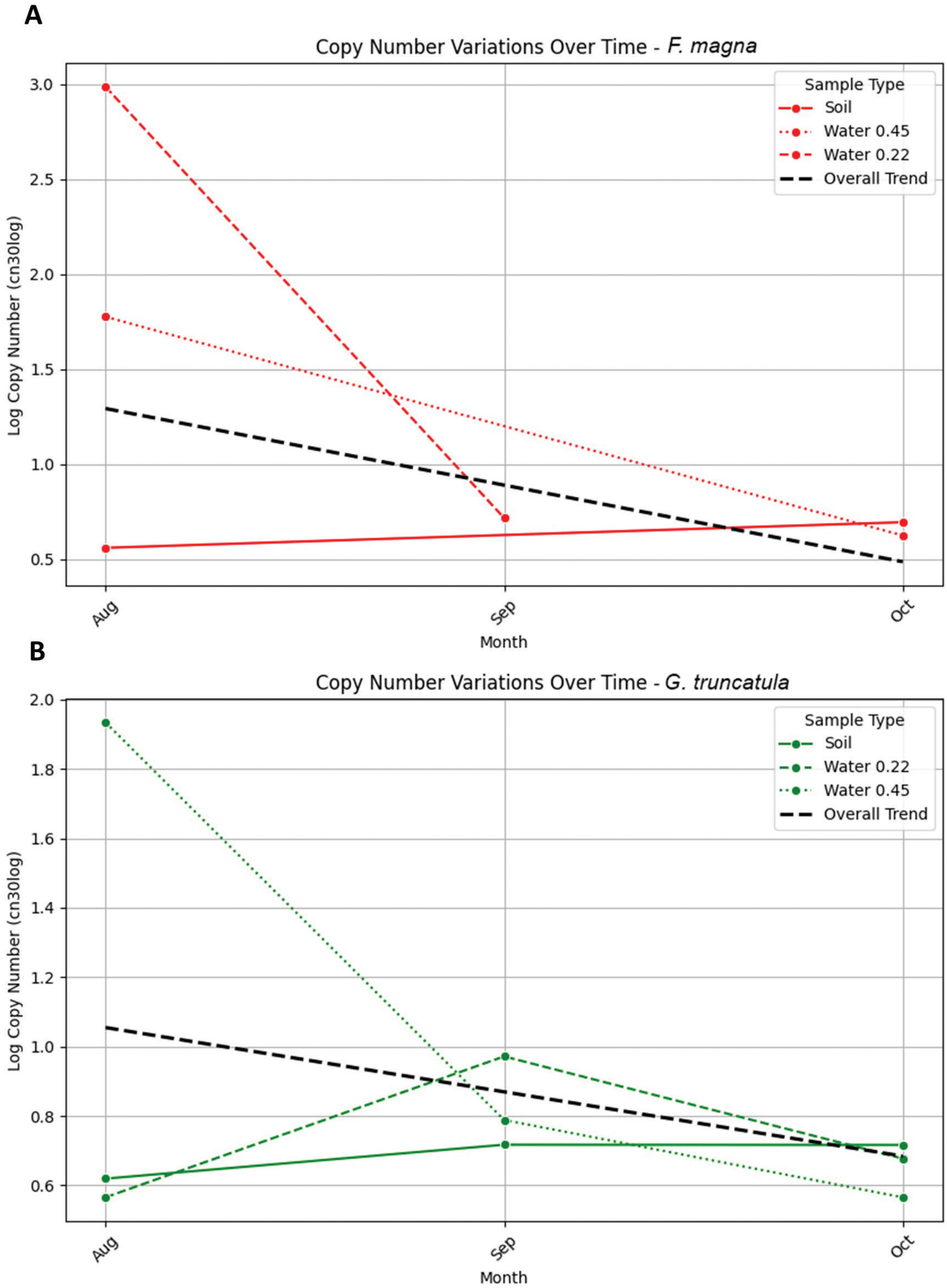

Figure 4. ddPCR positive results for **A.** *F. magna*; **B.** *G. truncatula* assays. lines represent the overall trend in average copy number change (in logarithmic scale per 30 ng of sample tested) during three months period. Kruskal Wallis test showed no significant difference across months, neither separately for each sample type nor for all sample types.

(August and October). Additionally, point 6 was the only site where *F. magna* was detected in both sample types, with positive results recorded in August (Fig. 3A, B).

Discussion

In this study, we investigated eDNA samples collected from different environmental matrices and at various sampling points inside and outside the LMRP fenced-off area to detect the invasive alien parasite *F. magna* and its intermediate host

G. truncatula. We detected DNA from both targets in several collected samples across different sampling points throughout the study period (Aug–Oct 2023).

Our sampling point selection strategy was based solely on hydrological factors, such as surface water dynamics, without relying on biological data, such as species distribution. The sample collection procedure required only minimal water or soil collection, making the non-invasive approach low-effort and practical. Additionally, the use of ddPCR allowed direct and absolute quantification of both targets in low-quantity eDNA samples extracted from a minimal volume of filtered water, an advantage not achieved with qPCR tests. In our previous study, we analyzed 16 water samples from eight sampling points using two different capsule filters (0.45 and 0.1 micron membrane pore sizes), filtering between seven and 20 liters of water per filter, successfully detecting both targets. This sampling strategy required specialized equipment such as pumps and tubing and was time-consuming, as we could only sample two points per day. Battery recharging caused further delays, resulting in a two-week period to collect samples from all eight points. Additionally, site selection involved a prior multi-day survey of water channels to identify candidate points based on the presence or absence of dextral lymnaeid snails, potential intermediate hosts of *F. magna*. In contrast, the current approach involved collecting much smaller sample volumes, required less time (all eight sampling points were visited in a single day), and did not depend on prior biological surveys for host presence. Our results (Figs 3, 4) highlight the effectiveness of integrating multiple factors at different stages (sampling point selection, sample collection, and molecular testing) to improve detection rates while optimizing study effort.

Our findings demonstrated the presence of *F. magna* and *G. truncatula* eDNA in both soil (50 mL) and water samples filtered with syringe filters (100 mL per filter) across different sampling points and months. Notably, these results were obtained independently of prior biological data, making our approach well-suited for monitoring invasive parasites and species, particularly for early detection. This is crucial, as *F. magna*'s definitive hosts include free-ranging wild ungulates, which are difficult to monitor using conventional methods such as fecal examinations (Malcicka 2015). Screening snails also presents challenges, both at the sampling stage (requiring precise morphological identification) and due to the typically low prevalence of *F. magna* infections within snail populations (Hörweg et al. 2011; Haider et al. 2012). Our sampling approach was based solely on hydrological data, assuming that selecting points at the beginning, end, or junctions of different flow segments - combined with diverse sample types (water and soil) - would increase the probability of capturing eDNA representing a broader biodiversity thus increasing the possibility to meet target species of interest (Lyet et al. 2021). This approach is particularly relevant for monitoring species expanding their biogeographic range due to human translocation or environmental events such as flooding. A key example is the Danube floodplains, which have contributed to the introduction of *F. magna* into Europe from the northeastern USA (Sindičić et al. 2023). Such habitats and flood events have played a major role in the spread of fascioloidosis to Germany, Austria, Slovakia, Hungary, Croatia, Serbia, and more recently, Romania (Špakulová et al. 2003; Králová-Hromadová et al. 2011; Malcicka 2015; Popovici et al. 2024).

Our findings indicated an overall decreasing trend in the detected copy numbers of both targets over the three-month sampling period (August to October), though this trend was not statistically significant. This may be attributed, at least in part, to the limited number of samples or replicates. Nevertheless, the observed decline, particularly in water samples, aligns with the seasonal variation and population dynamics of both *F. magna* and *G. truncatula*. The reproductive cycle of *G. truncatula* exhibits bimodal characteristics, with population peaks occurring in spring and autumn. Research suggests that *F. magna* infections in *G. truncatula* are more frequently observed in July and August, indicating a seasonal pattern corresponding to environmental conditions that favor both the snail's activity and the fluke's development (Hörweg et al. 2011; Sattmann et al. 2014).

Detection of both targets in soil samples at consistently low levels throughout the sampling period may be explained by the continuous reproduction of *F. magna* in infected animals. The parasite is excreted onto the soil surface and can be detected due to animal movement and surface water transport. Our sampling included soil collection from areas near different water bodies within the accessible range of snail habitats. However, at sampling point three, water (in the form of a stream or pond) was consistently absent throughout the study period. Interestingly, we still detected a positive soil sample at this point in August (Fig. 4B), which may be linked to the population peak of *G. truncatula* during this month.

Furthermore, our findings confirmed the presence of *F. magna* and *G. truncatula* in both soil and water samples collected outside and upstream of the LMRP's fenced-off area. These results support our previous study, which detected *F. magna* DNA in water samples from a river before it entered the park's fenced-off area (Varzandi et al. 2024). Our current findings consistently detected both targets in multiple sample types, providing further evidence of the presence and potential expansion of this invasive species beyond the protected zone. The fenced-off area is enclosed by a 30 km-long wall; however, it does not cover river entry points, and animals such as roe deer can still jump over the barrier, allowing movement in and out of the area.

Conclusions

Our approach, which integrates sampling site selection based on surface water dynamics, the simultaneous collection of diverse environmental matrices (water and soil), and the use of highly sensitive molecular methods, enabled us to successfully detect the presence of *F. magna* and its intermediate host *G. truncatula* in eDNA samples. This was achieved without relying on prior biological data, such as the presence or absence of snails or the distribution of other hosts within the study area.

This approach enhances the spatiotemporal feasibility of eDNA-based methods across various regions by applying a standardized procedure. Implementing such a standardized approach could further improve the utility of eDNA methods for harmonized monitoring programs and continuous surveillance efforts, facilitating the early detection of invasive species and pathogens.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

Funding

No funding was reported.

Author contributions

Conceptualization: AV. Data curation: AV. Formal analysis: AV. Funding acquisition: SZ, EF. Investigation: AV, ER. Methodology: AV, AT. Supervision: EF. Visualization: AV. Writing - original draft: AV. Writing - review and editing: EF, AV.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Supplementary information

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