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Development of a qPCR Duplex Assay for simultaneous detection of *Fascioloides magna* and *Galba truncatula* in eDNA samples: Monitoring beyond boundaries

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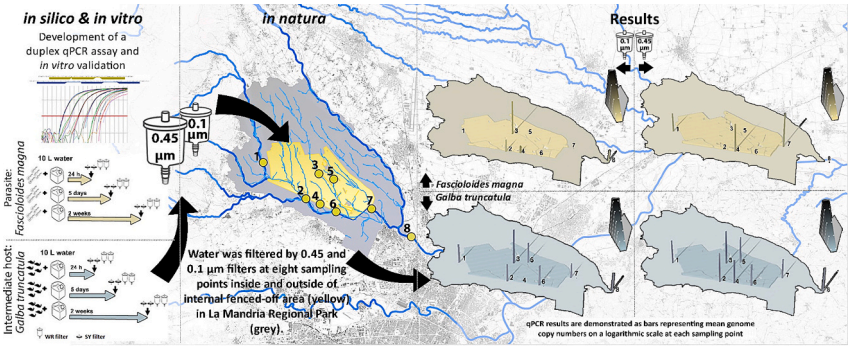
HIGHLIGHTS

- An eDNA method was developed to detect *Fascioloides magna* and its intermediate host *Galba truncatula*.
- Developed duplex qPCR assay was validated *in vitro* achieving LOD of less than 0.6 genome copy number/µ.
- Assays' *in natura* validation resulted in detecting *F. magna*'s eDNA outside its enclosed focus in LMRP for the first time.
- Analysis of stream water flow is a suitable eDNA sampling approach for disease hotspot identification.

GRAPHICAL ABSTRACT



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ABSTRACT

Parasites constitute a significant economic burden and highly impact environmental, public, and animal health. The emergence of many parasitic diseases is environmentally mediated and they share the same biogeography with humans and both domestic and wild animals. American liver fluke, *Fascioloides magna* – a trematode parasite of domestic and wild ungulates – is an example of the anthropogenic introduction of an "invasive alien species" in Italy and Europe. Multiple introductions to Europe have led to the biogeographical expansion of the parasite across the Danube region mainly provided by the presence of suitable habitats for all hosts involved in the parasite's life cycle, human-assisted transport, and drastic environmental events such as flooding. In Italy, it was introduced and established in La Mandria Regional Park (LMRP) near Turin in 1865 along with imported wapitis (*Cervus elaphus canadensis*) from North America (Bassi, 1875), but with no reported expansion to the surrounding areas. LMRP isolated *F. magna* focus, poses an important threat of possible expansion since the enclosed area is vulnerable to occasional bidirectional passage of roe deer. Additionally, tributary rivers to the Po river system, traversing the enclosed area, could further bolster the possibility of such spread. In this study, we developed a duplex qPCR assay for *F. magna* and its principal intermediate host *Galba truncatula* optimized for testing eDNA samples to meet the needs for surveillance of the parasite. Moreover, we validated the developed assay *in natura* by testing samples derived from filtered water and sediments collected inside and outside LMRP's

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fenced-off area. Our findings for the first time demonstrate the presence of *F. magna*'s eDNA outside the park's internal fenced-off area.

1. Introduction

Parasites are among the infectious agents with the highest impact on the global economy, and on environmental, public, and animal health. Parasitic diseases affect local and global (g-local) food security with direct involvement in the loss of livestock production (Rashid et al., 2019). According to Perry et al. (2002), four out of the top ten pathogens most prevalent in livestock and notably impacting impoverished regions are parasites (Perry et al., 2002). Parasites thrive in the same dynamic environment as humans and both domestic or wild animals, and are capable of infecting multiple hosts posing a risk of major outbreaks in all species within the shared biogeography (Gortázar et al., 2021). Environmental monitoring is critical to the management of infectious diseases prevention and control since 75 % of the 150 most burdensome parasites and pathogens tracked by the World Health Organization's Global Burden of Disease show environmental persistence with the ability to remain alive for more than one day outside the human or vertebrate hosts (Hopkins et al., 2022).

Directly or indirectly human-driven activities, like animals' translocation or climate change have profoundly altered the geographic distribution of parasitic diseases. This has led to biogeographical expansion into new areas whether due to the introduction of alien parasites by translocated animals into the new environment (spillover) or the spillback of native parasites to newly introduced hosts (Poulin, 2017; Artois et al., 2009).American liver fluke, *Fascioloides magna* - a trematode parasite of domestic and wild ungulates - is an example of anthropogenic introduction of an "invasive alien species" in Italy and Europe. Originally native to North America, it is an allochthonous species currently invasive to eastern Europe exhibiting a remarkable ability of reproduction and colonization in the new environments (Malcicka, 2015).

In Italy, it was introduced and established in "La Mandria" Regional Park (LMRP) Northwestern Italy in 1865 but with no reported expansion to the surrounding areas. *F. magna*'s prevalence depends on the presence of wild ungulate which act as definitive hosts and upon the presence of dextral lymnaeid (DL) snails[1] as intermediate hosts. White-tailed deer (*Odocoileus virginianus*), Wapiti (*Cervus elaphus canadensis*) and Caribou (*Rangifer tarandus*) serve as primary definitive hosts in the native range of *F. magna*. The unintended anthropogenic introduction of *F. magna* into Europe has resulted in infestation of new definitive (red deer, *Cervus elaphus*, fallow deer, *Dama dama*), dead-end (wild boar, *Sus scrofa*) and aberrant (roe deer, *Capreolus capreolus*, mouflon, *Ovis musimon*) hosts (Sindičić et al., 2023). *Galba truncatula* and *Radix peregra* are its principal intermediate hosts in Europe with *R. peregra* being more widespread but showing much lower infection rates with *F. magna* compared to *G. truncatula* (Malcicka, 2015).Recently, a few cases of *F. magna* infected roe deer have been reported, demonstrating the ability of the parasite to develop liver pseudocysts containing sexually mature flukes. The formation of pseudocysts features characteristics of infestation in definitive hosts such as sexually maturation of flukes and minimal damage to liver suggesting a potential adaptation trend in roe deer (Konjević et al., 2021; Demiaszkiewicz et al., 2018).

LMRP encompasses a fenced-off area that is currently host to four ungulate species, including wild boar, fallow, red and roe deer and a great variety of DL snails, including *G. truncatula* and *R. peregra.* This park is recognized as the first European focus of *F. magna*, following transportation of infected wapitis from North America for hunting reasons in 1865 and consequent rise of mortality within roe deer population (Bassi, 1875). Subsequent introductions to Europe have led to the biogeographical expansion of the parasite across Danube region provided by presence of suitable habitats for both intermediate and definitive hosts, human-assisted transport and drastic environmental events such as flooding. The latter has been particularly effective in the biogeographical expansion of *F. magna* to Serbia through Danube riverine system (Špakulová et al., 2003a; Špakulová et al., 2003b; Marinkovic et al., 2008).

LMRP isolated *F. magna* focus poses an important threat for the potential expansion outside the enclosed area is vulnerable to the movements (towards inside or outside) of roe deer in particular and other domestic and wild ungulates which naturally inhabit the Alpine and plain areas bordering LMRP (Špakulová et al., 2003b; Foreyt and Todd, 1976; Pybus, 2001; Erhardová-Kotrlá and Blazek, 1970). Additionally, tributary rivers to the Po riverine system, traversing the enclosed area, could further bolster the possibility of such spread. Until today, a few small-scale studies have demonstrated the presence of *F. magna* in definitive and intermediate hosts within the LMRP's internal fenced-off area either morphologically in fallow, red and roe deer's liver or through PCR test in *G. truncatula* (Coraglia, 2015; Costanzi, 2010). However, active surveillance of 153 wild ruminants (mouflon, roe deer and chamois) and 226 lymnaeid snails for the presence of *F. magna* has been unsuccessful to detect this parasite outside the park's fenced-off area. Conventional surveillance approaches are inherently invasive and cumbersome. Therefore, environmental monitoring of this emerging epidemiological situation is crucial for wildlife conservation, livestock health protection, and the management of public health at the local level (Artois et al., 2009).

Various methods have been employed for epidemiological surveillance, including fecal examination and PCR testing on samples directly collected from hosts. However, more recently, environmental DNA/RNA (eDNA/eRNA) and to a broader extent environmental Nucleic Acid (eNA) detection methods have demonstrated advantageous over conventional methods (Fediajevaite et al., 2021). eNA based methods are being increasingly employed since they augment sensitivity, specificity, efficiency, spatiotemporal scope and cost effectiveness of a surveillance program. Additionally, eNA methods eliminate the need for invasive or lethal sampling of individual hosts and reduce cumbersome sampling efforts such as collecting feces or conducting snail surveys. These features make eNA based methods suitable for wildlife genomic informed surveillance programs where direct sampling of host is challenging or impossible to be achieved (Bass et al., 2023; Zemanova, 2021). In order to meet the needs for surveillance of *F. magna*; in this study, we developed a duplex qPCR assay for *F. magna* and its principal intermediate host *G. truncatula* optimized for testing eDNA samples and validated it *in natura* by testing samples derived from filtered water and sediments inside and outside of LMRP's fenced-off area.

2. Materials and methods

2.1. qPCR assay development and in vitro validation

2.1.1. Filter types

The yield and richness of obtained eDNA are strongly influenced by filter features, including pore size, membrane type, surface area, and capacity of filtration (Djurhuus et al., 2017; Majaneva et al., 2018a). To optimize eDNA capture, it is recommended to use 0.2 μm filtration or a combination of larger pore size and water volume (Turner et al., 2014). Consequently four distinct filters meeting different recommended criteria were chosen for this study: Millex Mixed Cellulose Syring filters

[1] Dextral lymnaeid snails: freshwater snails with right-handed shell coiling direction such as G. truncatula and R. peregra.

(SY) with 0.22 and 0.45 μm pore-sizes (Merck KGaA, Darmstadt, Germany) and Waterra (WR) polyurethane large surface 0.1 and 0.45 μm pore-sizes eDNA filters (Argaly, Sainte-Helene Du Lac, France). Although laboratory experiments and *in vitro* validation steps were performed using all filter types, *in natura* samplings were carried exclusively out using WR filters which provide a long-surface (600 cm^2) polyethersulfone membrane with a capacity of storing approximately 50 mL of liquid inside the filter capsule (Douchet et al., 2022).

2.1.2. G. truncatula and F. magna collection

DL snails were collected from previously identified suitable habitats in LMRP and were transferred to laboratories of the Dept. of Veterinary Sciences (Grugliasco, TO). These snails were individually maintained in cell culture flasks and reared under laboratory conditions with regular water replacement and a lettuce based diet (Moazeni et al., 2018). After 48 h from water replacement, 20 mL of water hosting each snail was filtered by SY 0.45 μm filters. DNA extraction from filters was performed directly with TRI Reagent® (Chomczynski and Sacchi, 1987).Utilizing the lytic properties of TRI Reagent®, SY filter membranes underwent thorough washing and backwashing by pipetting 100 μL of this solution for five times. Each pipetting step followed by aspiration with pipette after 2 min and subsequently eDNA was extracted according to TRI Regent® protocol from the obtained liquid, totaling about 500 μL.

To confirm snail species, end-point PCR was conducted using primer pairs (F: 5′-GTGAGCTCTCACGCTGCTC-3′ and R: 5′-TAGAGCCCCTTGTTCTCCA-3′), amplifying a 288-bps fragment of *G. truncatula*'s DNA. The thermal cycling program consisted of an initial denaturation (2 min at 95 °C) followed by 40 cycles of denaturation (30 s at 95 °C), annealing (30 s at 61 °C) and extension (60s at 72 °C) with a final extension of 10 min at 72 °C (Jones et al., 2018). The infection status of *F. magna* in snails was assessed using end-point PCR using primer pairs FM_ITS2_SPEC_F (5′-ACCAGTTATCGTTGTGTTG-3′) and FM_ITS2_SPEC_R (5′-CCGTCTTTAAACAACAG-3′), amplifying a 152-bps fragment of *F. magna*'s DNA. The thermal cycling program consisted of an initial denaturation (5 min at 94 °C) followed by 30 cycles of denaturation (1 min at 94 °C), annealing (1 min at 55 °C) and extension (2 min at 72 °C) with a final extension of 5 min at 72 °C (Bazsalovicsová et al., 2010). Snails identified as *G. truncatula* and found to be negative for *F. magna* infestation were included in subsequent water tank analysis (Supplementary materials A Fig. 1).

F. magna eggs were obtained from the biliary system of infected red deer culled in LMRP. Red deer were culled within regular hunting activity carried inside the LMRP. Individual eggs were isolated using a pipette under a microscope, placed in 2 mL tubes with 20 μL of distilled water and stored at −20 °C. *F. magna* eggs were also collected and placed into 250 mL cell culture flasks and were incubated according to Campbell. (1961) until the development of miracidia (Campbell, 1961) (Supplementary materials A Fig. 2).

2.1.3. Mesocosm (water tank) experiments

Experiments were carried out in separate water tanks, each containing 10 L of laboratory tap water. Five confirmed *G. truncatula* snails or five miracidia were placed in each of three separate tanks (3 tanks for snails and 3 tanks for miracidia) and maintained at room temperature. Sampling events occurred at 24 h, 5 days, and 2 weeks after introducing snails or miracidia into the water tanks. Each event involved filtering approximately 4 L of water with each WR filter and 400 mL of water with each SY filter, utilizing four different filters for each sampling event in each water tank (Fig. 4). A separate control water tank (containing tap water from the same source of water used for other tanks) with no miracidia or snail was included, which was filtered at week 2 using all four different filter types. After each filtration event, 40–50 mL of Longmire buffer (LB) was added to each WR filter capsule and they were preserved at room temperature. SY filters were sealed and stored at −20 °C (Williams et al., 2016). Filtration with both filter types (WR and SY) was carried out using a diaphragm pump connected to a 12 V battery. After each filtration event, a closed circuit of 1 L bleach solution (20 %) was run to prevent cross contamination and all disposable parts (tubes and fittings) were replaced after each filtration (Supplementary materials A Fig. 3).

2.1.4. DNA extraction

SY filters, kept on ice, were washed and back washed with 2 mL of LB using 2 mL sterile syringes. Approximately 50 mL and 1.7 mL of the buffer content derived from WR and SY filters were separately transferred to 50 mL and 2 mL centrifuge tubes respectively. SY and WR sample tubes were centrifuged for 20 min at 16000*g* and 80 min at 4000 g, respectively, according to the preparation procedure described by Douchet et al. (2022) for WR filters using LB (Douchet et al., 2022). Sediment (where available) or a maximum of 800 μL of the sample were transferred to Powerbeads tubes, and DNA was extracted according to DNeasy Powersoil DNA Extraction Kit (Qiagen GmbH, Hilden, Germany) protocol. During the water tank experiments, all samples from filters were extracted using this kit since the principal aim of this study was to validate the assays on eDNA isolated from water filtered at sediment/water surface. DNA from single replicates of *F. magna* eggs or single miracidium was extracted using Chelex according to Dolnik et al. (2009). (Dolnik et al., 2009)

2.1.5. Duplex qPCR assay development

Considering higher PCR amplification success of degraded ribosomal DNA and higher differentiating power of ribosomal marker genes, primers and probes for TaqMan qPCR assay were designed on the ITS2 region of ribosomal DNA (Foran, 2006; Holland and Parsons, 1999; Toju et al., 2012). Primers were designed using Geneious® on alignments of closely related organisms where DNA sequences visually exhibited the highest diversity among species. *in silico* specificity tests were performed using NCBI's BLAST and eDNAssay, a machine learning tool for predicting qPCR cross-amplification that also considers probe sequences (Kronenberger et al., 2022). The results from eDNAssay and the degree of dissimilarity to closely related organisms are reported in Table 1 and Fig. 4 of Supplementary materials A. The primers and probes sequences are listed in Table 1.

For both assays, PCR amplicons from positive samples were cloned to pDrive Cloning Vector (QIAGEN PCR Cloning Kit, Hilden, Germany). Subsequently, six 10-fold serial dilutions of purified cloned vectors containing *F. magna* and *G. truncatula* amplicon inserts ranging from 3*10^5 to 3 copies/μL of DNA were prepared. The below formula was used to calculate the mass of vector containing the insert and multiplied by the copy number of interest for development of standard curves.

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

where: n = plasmid size (bp) and m = mass

All qPCR reactions were conducted in duplicate, with a final volume of 25 μL, including 12.5 μL of TaqMan, 0.9 μM of each primer, 0.27 μM of each TaqMan probe and 5 μL of DNA. The real-time thermocycling program involved a 10-min hold at 95 °C, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. All the reactions were performed in duplex

Table 1
Primers and Probes sequence.

Assay	Primer	Amplicon length	Sequence (5′-3′)
F. magna	Forward Reverse Probe	69 bps	TTTATCGTCGGTTTGATGCTA GAAGGATACCGTCTTTAAACAAC FAM – GGCTTGGTCATGTATCTGATGC – BHQ1
G. truncatula	Forward Reverse Probe	82 bps	ACTTTATTTATTATCGTGGCGTTC CGTCTTAGAGCCCCTTGT CY5 – TCCATGGCATCGCAGCTC – BHQ3

since standard curve cycle thresholds (Ct) for singlex and duplex assays showed almost identical values (Supplementary materials B sheet 1).

2.1.6. Specificity and sensitivity tests

To evaluate the specificity of the both assays, qPCR reactions containing 40 ng of genomic DNA extracted from adult *F. magna*, *Dicrocelium dendriticum* and *Fasciola hepatica* were analyzed. Furthermore, to evaluate the sensitivity, qPCR reactions were performed on dilution series of DNA extracted from *F. magna* single egg and 15 mL of water harboring single *G. truncatula* collected after 24 h.

2.2. In natura validation

2.2.1. Study area

"La Mandria" Regional Park (LMRP) is a protected area covering approximately 6500 ha in the northeastern Turin. The park is divided into an internal area (approximately 3200 ha) separated by a 30 km-long wall from the external pre-park area. LMRP boasts a rich variety of freshwater habitats, including artificial lakes, streams and canals. The entire water network in the park contributes to the Ceronda River situated in the southern part of the park, which traverses through the internal area and flows into the Stura di Lanzo River downstream the park before joining the Po River (Fig. 1).

2.2.2. In natura sample collection

Based on the results obtained from water tank experiments, *in natura* filtration of water was carried out at water/sediment surface using WR filters with different pore-sizes (0.1 and 0.45 μm) and a prefilter with a 300 μm mesh plate (Supplementary materials A Fig. 3). Eight sampling points inside and outside the LMRP's area were identified depending on the noticeable presence or absence of DL snails in the proximate surrounding area (Fig. 1). Sampling points were designated from 1 to 8 with point 1 being in the upstream and point 8 in the downstream of the study area. Moreover, sampling points 1, 7 and 8 were located outside the internal fenced-off area. Each sampling event was performed on a separate day within from September 15th, to September 30th 2022. Equipment was thoroughly cleaned or replaced before every sampling event. For instance, the mesh prefilter was autoclaved before filtration, pump, tubing and shoes were washed using 20 % bleach solution.

Filtration was performed at each collection site, both along the sides and in the center of each water stream, with varying volumes of water filtered based on the pore size of the filters. Additionally, filtration was performed in an area spanning 10 to 15 m alongside beginning downstream of identified area and progressively moving upstream. The filtration volume was measured by graduated tanks filled with output water (Supplementary materials A Fig. 5 and Supplementary materials B sheet 7). Subsequently, filters were filled with LB, immediately agitated and stored at room temperature until extraction.

Furthermore, varying numbers of DL snails were collected at each sampling point and transported to the laboratory. These snails were placed in a clean beaker with 0.5 L of tap water for 24 h. Subsequently, approximately 200 mL of water hosting each snail community was filtered using either SY 0.22 or 0.45 μm filters and eDNA was then extracted with TRI Reagent® following the previously described procedure.

2.2.3. eDNA extraction and qPCR

WR filters were prepared for eDNA extraction following the same procedure and protocol employed in water tank experiments. With a slight modification, the liquid obtained from filters was divided into two extraction replicates with equal volumes. eDNA was extracted from precipitated soil content of liquid obtained from WR filters. For each filter type a negative sample was considered, wherein 10 L of extraction laboratory tap water was filtered and filter's capsules were filled with

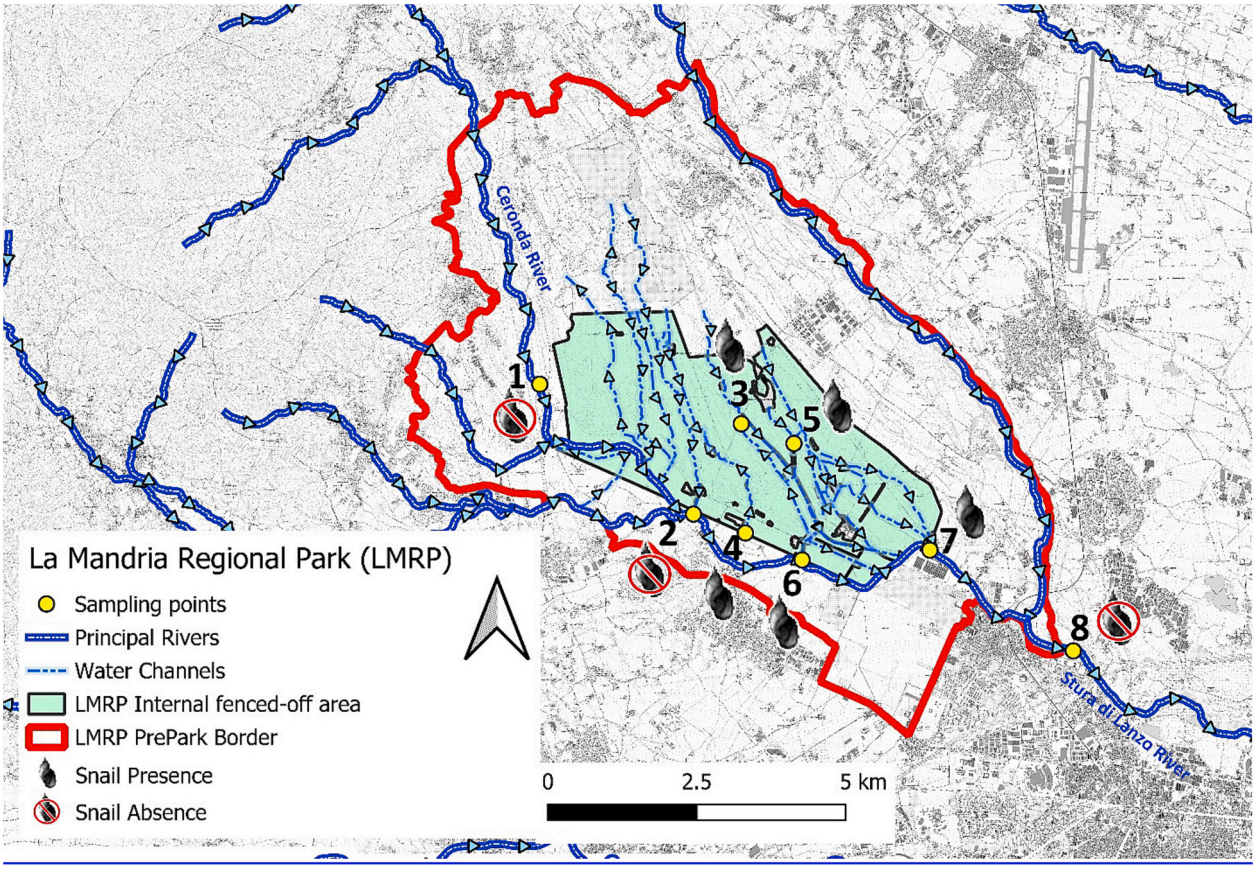

Fig. 1. Study area map. La Mandria Regional Park – sampling points are named from 1 to 8. No DL snail was visually seen at points 1, 2 and 8.

LB. Negative samples underwent the same procedure of preparation and extraction used for *in natura* samples. Throughout laboratory procedures, all the required measures to avoid cross-contamination were applied; samples were prepared, extracted and tested in separate laboratories. Water tank experiments were carried out in a separate facility room different from eDNA extraction and qPCR laboratories. eDNA samples were analyzed by duplex qPCR assay using the previously mentioned protocol.

3. Results

3.1. Duplex qPCR assay

The limit of quantification (LOQ) and detection (LOD) achieved for both assays was 0.6 genome copies/μL, and lower than 0.6 genome copies/μL respectively. The reported LOQ and LOD values correspond to the cycle threshold at which the most diluted standard (3 copies/reaction) was quantifiable. To calculate LOD and LOQ, Ct values of 10 qPCR technical replicates (from four different qPCR runs) were analyzed according to a curve-fitting modeling approach proposed by Klymus et al. (2020) for eDNA qPCR assays (Supplementary materials B sheet 8) (Klymus et al., 2020). Considering that all reactions were performed in duplex and also considering the reported LOQ, samples with one replicate quantified above 0.6 genome copies/μL of input eDNA were considered positive. The qPCR assay showed high specificity for *F. magna* since specificity tests on closely related parasites (*F. hepatica* and *D. dendriticum*) yielded copy numbers significantly below LOQ with cycle thresholds above 42 (more than 10-fold difference with same amount of input genomic DNA) (Fig. 2 and Supplementary materials B sheet 2)

As demonstrated in Fig. 3, the sensitivity of our test achieved a biological limit of detection of 40 pg and 400 pg of input DNA for *F. magna* and *G. truncatula*, respectively. The represented values were obtained by analyzing two replicates of eggs' and snails' DNA and eDNA, respectively, in duplicate qPCR reactions (Supplementary materials B sheet 3).

3.2. Water tank experiments

The results from water tank experiment revealed a higher amount of target eDNA detected by both WR filters, which correlated with higher filtration volumes. The amount of target eDNA detected by all filter types and particularly in water tanks containing miracidia, interestingly demonstrated an increase over time (Fig. 4). To better assess the efficiency of filters (considering total yield of eDNA from each sample and total volume of filtered water) the average detected copy number of both assays per mL of sample's filtered water volume was calculated. The results demonstrated a gradual decrease in average detected copy number, with the following order: WR 0.1, SY 0.22, WR 0.45 and SY 0.45 with 2.56, 1.53, 1.47 and 0.64 genome copies, respectively (Supplementary materials B sheet 4).

3.3. In natura validation

G. truncatula's eDNA was detected in all sampling points, irrespective of its visual presence/absence. Detected genome copy numbers for each assay are represented separately for WR 0.01 and WR 0.45 filters in Figs. 5(a) and 6(a) respectively (Supplementary materials B sheet 5 and 6). Presence of *G. truncatula* was detected in all sampling points by WR 0.45 filters, while only one sample from WR 0.1 series (sampling point 4) resulted negative. Regarding detection of *F. magna*, six sampling points demonstrated positive results (points 2,3 and 4 within the fenced-off area and points 1,7 and 8 outside this area) for the presence of the parasite but the results from the both filters overlapped only at sampling point 4.

It's worth mentioning that *F. magna* was detected upstream of the internal fenced-off area in the Ceronda River (sampling point 1) or downstream the park's boundary in Ceronda and Stura Rivers (points 7 and 8 respectively). The genome copy number density maps represented in Figs. 5(b and c) and 6(b and c) illustrate the mean genome copy numbers detected in every sampling point transformed on logarithmic scale prepared by Rayshader package in R (Morgan-Wall, 2024).

Additionally, qPCR results from eDNA extracted from each snail community collected at every sampling point demonstrated detection of both *F. magna* and *G. truncatula* at all points where snails were visually

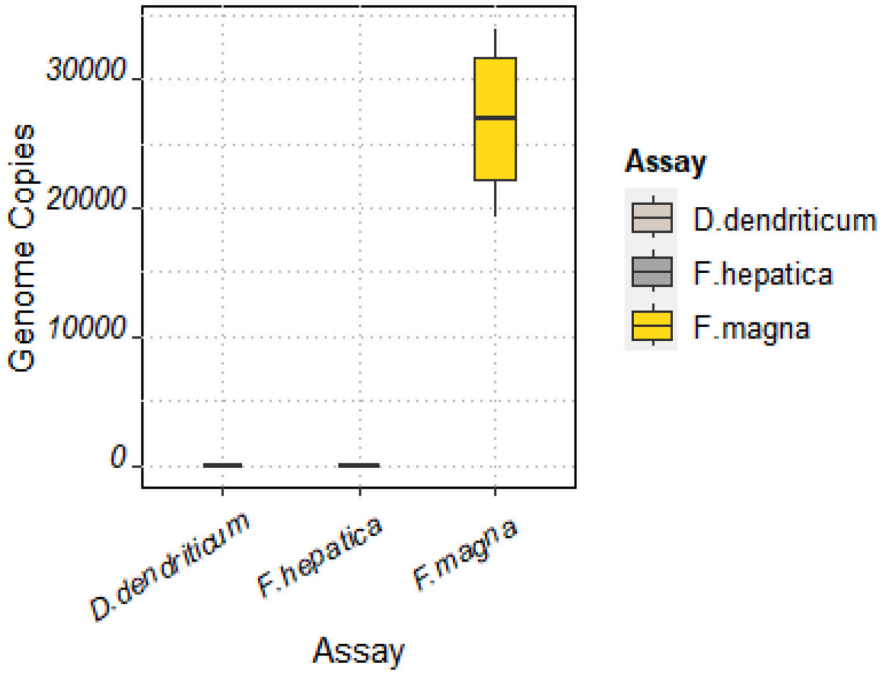

Fig. 2. Specificity tests - qPCR results from 40 ng input DNA comparing closely related parasites (each box and whisker plot represents four values since DNA was extracted in two replicates and qPCRs were carried out in duplicates).

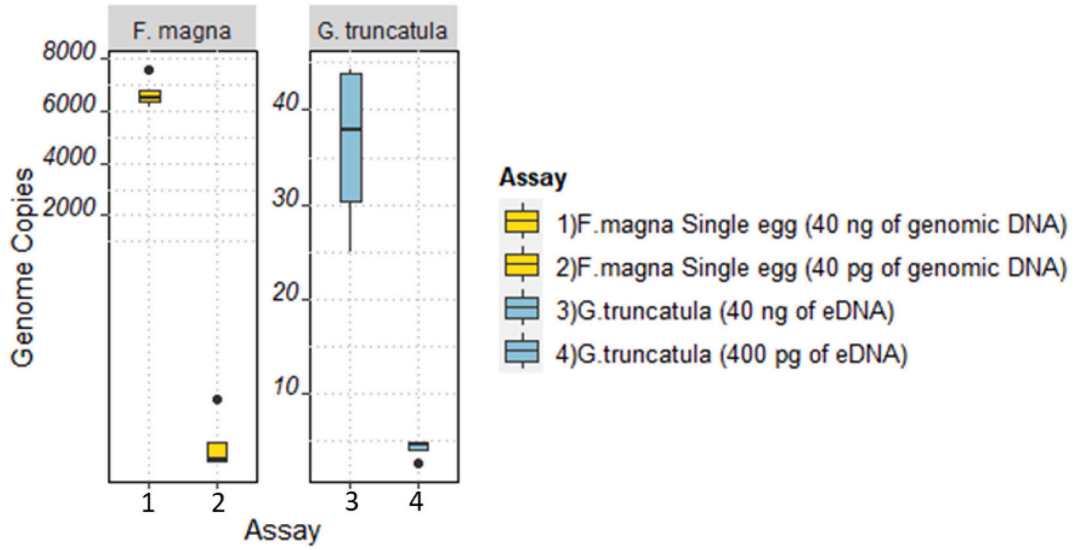

Fig. 3. Sensitivity tests showing the limit of detection for *F. magna* and *G. truncatula* assays.

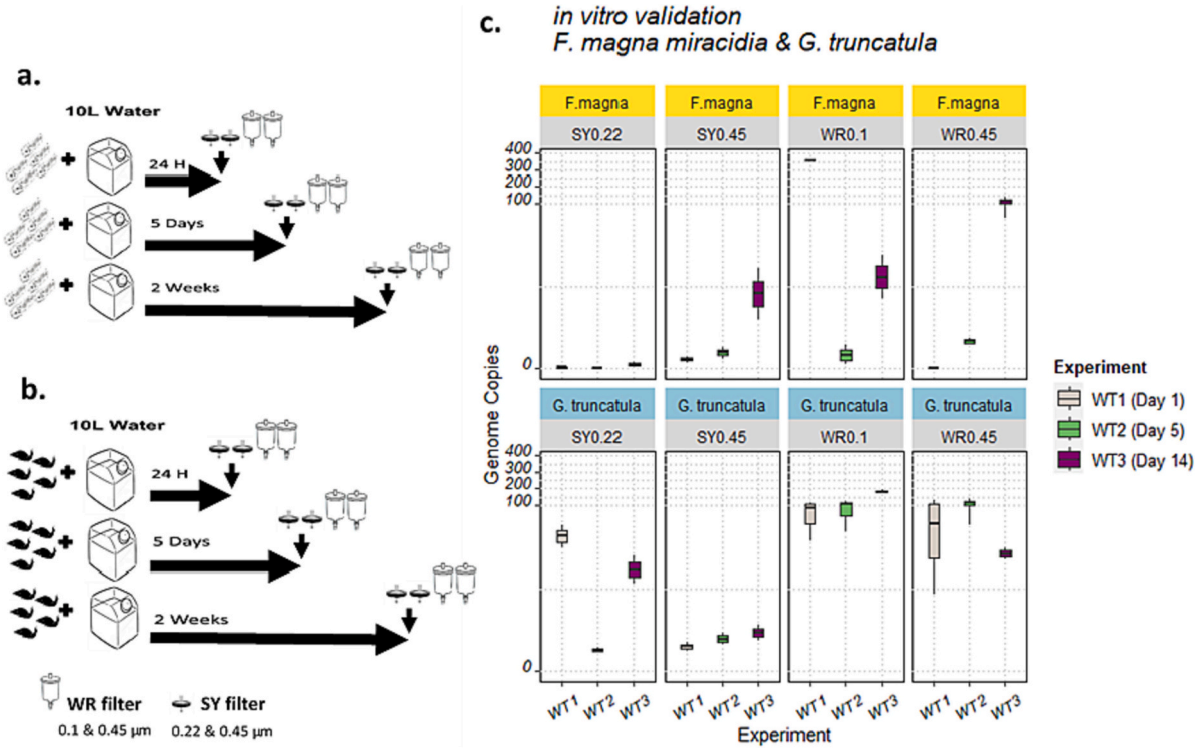

Fig. 4. Water tank experiments for *in vitro* validation of eDNA extraction and qPCR assay. a. Miracidia containing water tank experiments – b. *G. truncatula* containing experiments. C. Water Tank experiments' results. (10 L of water in each water tank was filtered).

observed and collected (points 3,4,5 and 6) except at point 7 where only *F. magna* was detected (Supplementary materials B sheet 9).

4. Discussion

The developed duplex qPCR assay demonstrated a remarkable sensitivity as it successfully detected the target DNA of interest from samples originally containing low copy number of target genome of interest (*i.e.* a single *F. magna* egg in its very early stage of cell division or from water hosting a single snail after 24 h). In addition, the developed assay demonstrated the ability to detect quantities less than one target genome copy/µL of input DNA indicating a high level of LOQ (Fig. 3). Given that we used a consistent amount (40 ng) of input DNA for all qPCR reactions, regardless of whether they were from samples obtained from laboratory experiments or in the field, and considering the specificity test results for *F. magna* (Fig. 2), the developed assay demonstrates a high reliability in terms of specificity when distinguishing closely related or co-occurring parasites such as *F. hepatica* and *D. dendriticum*.

The main objective to conduct water tank experiments was to compare the environmental detection limits achieved by different filter types since, according to the literature, eDNA filter features might influence the eDNA total yield and downstream biodiversity recovery (Djurhuus et al., 2017; Majaneva et al., 2018b). It is noteworthy that the two selected filters in this study have highly distinct features; SY filter

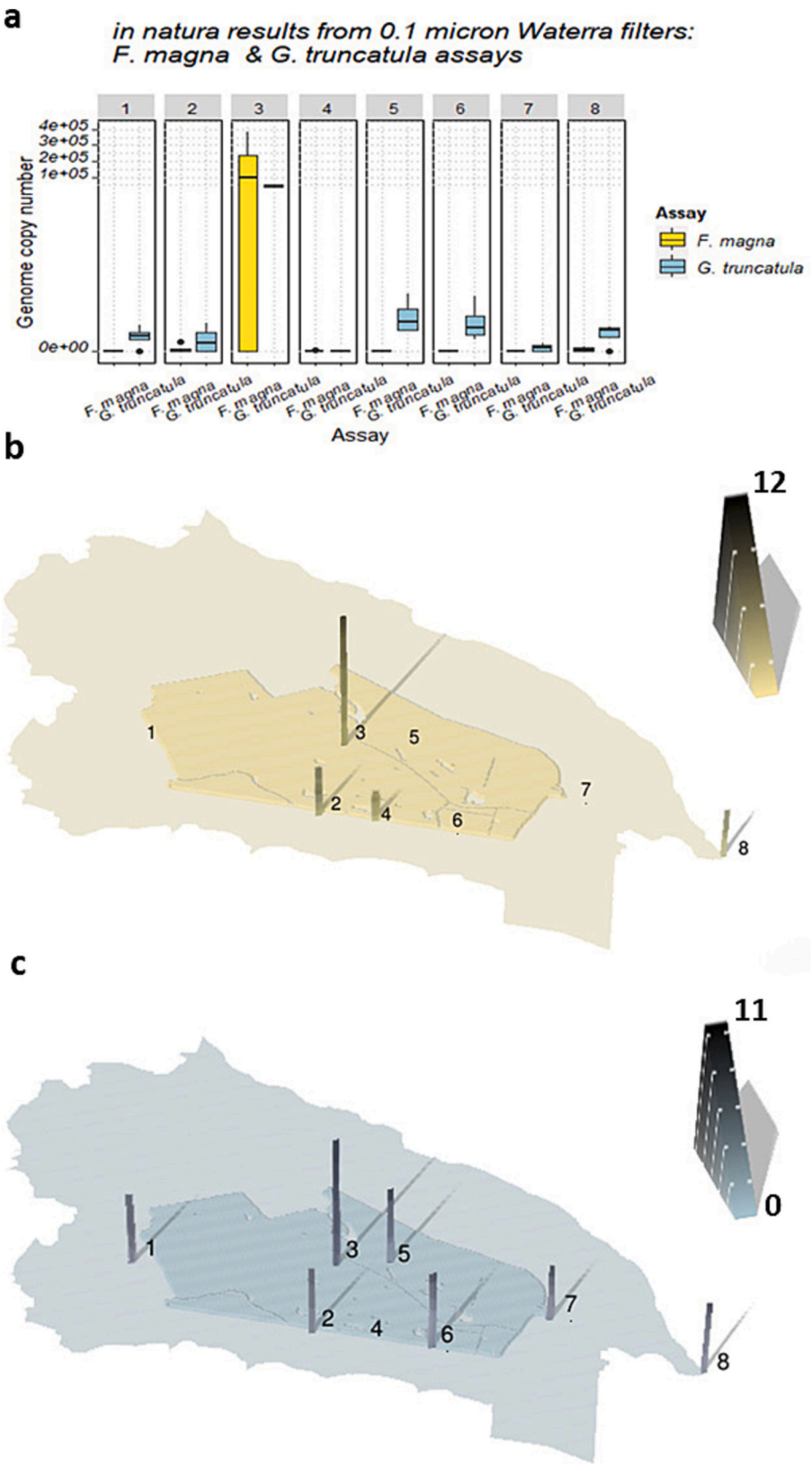

Fig. 5. In natura results for *F. magna* (yellow) and *G. truncatula* (blue) from 0.1 μm Waterra (WR) filters– a. Detected genome copy numbers from four qPCR replicates of each sampling point. b and c. Mean genome copy number results from 0.1 μm WR filters demonstrated on logarithmic scale and exact coordination (dark color on the density maps highlights internal fenced-off area.

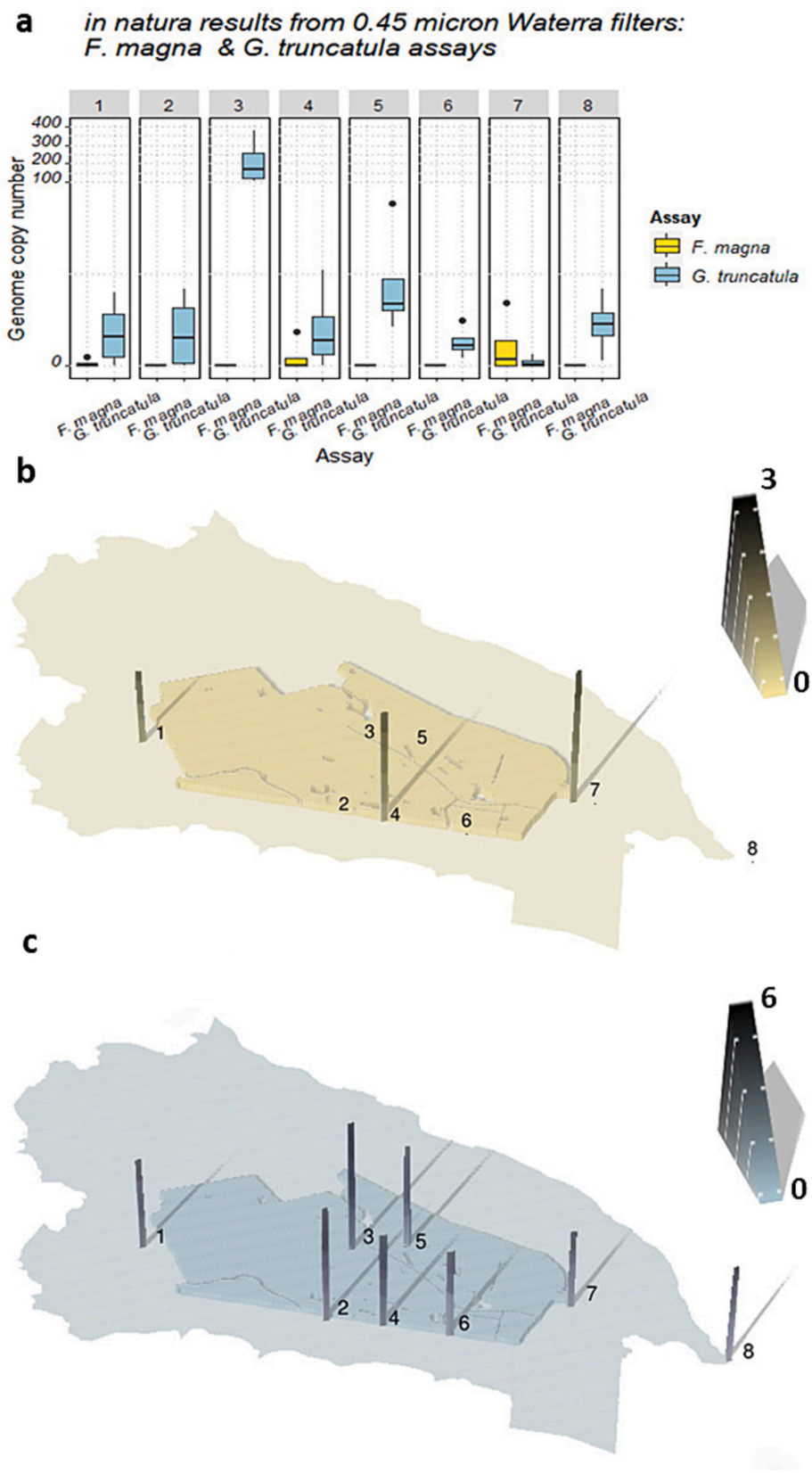

Fig. 6. In natura results for *F. magna* (yellow) and *G. truncatula* (blue) from 0.45 μm Waterra (WR) filters– a. Detected genome copy numbers from four qPCR replicates of each sampling point. b and c. Mean genome copy number results from 0.1 μm WR filters demonstrated on logarithmic scale and exact coordination (dark color on the density maps highlights internal fenced-off area).

membrane has a high affinity towards DNA, but provides a low surface area and filtration volume capacity. Conversely WR filters provide a very large surface area of approximately 600 cm^2 and high filtration volume capacity but have membrane with low DNA affinity. Both filter types were able to capture target eDNA, while the higher amount of target DNA detected in WR-derived samples could be attributed to the filtration capacity of these filters. Considering low amount of water filtered by SY filters, these filters demonstrated promising results. However, based on the sample collection location which is characterized by streams with rapid flow of water, WR filters were selected for field sampling at LMRP.

Results from water tank experiments showed an incremental detection rate over time. Different studies have focused on eDNA degradation and decay of eDNA, reporting a wide range of half-lives, from 0.7 h in multi-species lotic mesocosm at approximately 15 °C (Seymour et al., 2018) to 71.1 h in arctic settings (Cowart et al., 2018). Considering the fixed number of miracidia placed in the water tank and physical degradation and spontaneous decay rate of eDNA (the primary eDNA degradation factor in water tanks), the observed detection rate was mainly influenced by the availability of fragmented extracellular eDNA (true eDNA) and the filter's ability to capture it. This could also explain the significant difference in detected target DNA between WR filters at the first miracidia containing water tank experiment which was performed after 24 h of the placement of newly hatched miracidia in the water tank. Miracidia remain viable and intact for up to 24 after hatching, which could result in capturing of whole miracidia by one filter and leaving the other without at the first experiment since the amount of true eDNA is minimal at this early stage. The higher copy number detected in water tanks containing snails could be due to presence of live snails and continuous eDNA shedding occurring during the experiment period.

The represented box plots in Figs. 5 and 6 report four values as eDNA extraction from every filter was divided in two replicates and qPCR assay was also performed in duplicates. Therefore, the variance observed between the values and results from the same sampling point, primarily arises from the use of several technical and PCR replicates. These results suggest the importance of replicates at different stages of an eDNA study. Therefore, use of at least two filters at each sampling point, consideration of technical replicates at extraction and performing PCRs in duplicate is highly recommended.

Prior studies have indicated a higher prevalence of *G. truncatula* inside the fenced-off area compared to the pre-park zone with this intermediate host dominating over *R. peregra*. These studies have also identified *G. truncatula* positive for *F. magna* infection inside the fenced-off area, but were unsuccessful in detecting any positive snail or roe deer outside of this designated area (Coraglia, 2015). This might partially explain our challenges in visually locating DL snail communities beyond the park's internal fenced-off area and our inability to detect *G. truncatula*'s eDNA in the snail community collected at point 7, immediately downstream of the park's internal area. Nevertheless, our findings indicate the presence of both *F. magna* and its main intermediate host, *G. truncatula*'s eDNA, both within and outside the fenced-off area.

Considering that our selected sampling points, mainly were situated along lotic water bodies, the detection of *F. magna* eDNA at each sampling point could stem from both an established infection hotspot and the capturing of transported eDNA from an upstream established infection zone. This is plausible given that eDNA travel downstream within a range of a few meters to tens of kilometers in riverine systems (Jo and Yamanaka, 2022). Moreover, simultaneous detection of the parasite and its intermediate host's eDNA upstream of the fenced-off area (point 1) suggests, for the first time, a potential presence of the parasite, whether as a result of a contamination or an established infection expansion event. This finding could be primarily attributed to the permeability of the fence to roe deer movements and holds significance in light of reports indicating potential adaptation trends towards definitive host by the formation of *F. magna* pseudocysts in roe deer's liver (Konjević et al., 2021; Halász et al., 2023)

G. truncatula demonstrates a seasonal pattern of distribution as reported by Haider et al. (2012) in a study conducted in three distinct locations in the Danube wetlands east of Vienna, Austria (Haider et al., 2012). In their study the highest and lowest number of snails were found in August and April, respectively, which aligns with the trend of snail population found in fenced-off area of LMRP, particularly in locations with continuous flow of water throughout the year similar to our sampling locations (Costanzi, 2010). However, the same study reports a different seasonality pattern for snail infection with several Digeneans, including *F. magna*, showing an infection peak at July and a sharp decrease in the following months. Additionally, the reported prevalence of infection with Digeneans, including *F. magna*, in this study was 2.41 % (determined by both microscopy and molecular biology methods in parallel), while previous study in LMRP reported a prevalence of 3.7 % (detected by PCR). Considering the sampling period in our study, results demonstrate a high sensitivity in the detection of *F. magna* and also a high consistency with distribution of intermediate host population. These findings furthermore emphasize the potential and capability of eDNA methods in monitoring occurrence of parasitic diseases where different hosts and multiple environmental conditions are involved.

Finally, the sample collection method used in our study, is adapted to collect samples from high volumes of filtered water from different type of water bodies such as water with high or low levels of turbidity at water/sediment surface or from streams and riverine systems with powerful flow rate. This adaptation would make sampling feasible at every point of a riverine system with different conditions such as those after drastic environmental events like overflowing or flood.

5. Conclusion

The developed duplex qPCR assay demonstrated high sensitivity and specificity towards both target organisms of interest, making it highly suitable for analyzing environmental DNA samples. When combined with sampling methods adapted for collecting samples from large volumes of water of varying levels of turbidity, this assay becomes a valuable tool facilitating environmental surveillance of *F. magna* simultaneously with *G. truncatula* in areas without prior information. Detection of *F. magna* in eDNA samples collected outside the LMRP's internal fenced-off area asserts applicability of this sampling method and assay for disease hotspot identification beyond the boundaries of LMRP, encompassing other riverine systems like the Po River. This comprehensive approach further enhances the monitoring of wildlife populations as well as domestic animals for parasitic diseases in regions near such rivers, enabling rapid detection and effective prevention of an emerging epidemiological situation.

Ethics and consent

No approval of research ethics committees was required to accomplish the goals of this study.

CRediT authorship contribution statement

Amir reza Varzandi: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. **Stefania Zanet:** Conceptualization, Supervision, Writing – review & editing. **Elisa Rubele:** Formal analysis, Investigation. **Flavia Occhibove:** Writing – review & editing. **Rachele Vada:** Investigation. **Francesco Benatti:** Investigation. **Ezio Ferroglio:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors have no competing interests to declare that are relevant to the content of this article.

Data availability

Datasets analyzed during the current study are available to readers and could be accessed as supplementary information.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170338.

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