Environmental DNA

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First European Interlaboratory Ring Test Study to Detect DNA of Crayfish and the Crayfish Plague Pathogen From Water Samples

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ABSTRACT

In recent years, European countries have intensified efforts to control or limit the spread of invasive freshwater crayfish and the crayfish plague pathogen *Aphanomyces astaci*, while working to conserve native species such as the noble crayfish (*Astacus astacus*). Although crayfish shed relatively low amounts of DNA into their environment, environmental DNA (eDNA) approaches have proven effective for detecting their presence. A range of protocols and equipment is currently used in eDNA-based monitoring of freshwater crayfish. To evaluate how methodological variation influences detection accuracy, we conducted the first European interlaboratory ring test using eDNA to detect *A. astacus*, the invasive signal crayfish *Pacifastacus leniusculus*, a chronic carrier of *A. astaci*, and the pathogen itself. The aim is to harmonize monitoring methods for crayfish and disease surveillance across laboratories. Eleven teams from thirteen European countries participated, each using its own equipment and protocols to collect and filter water from indoor tanks and outdoor ponds where the presence of *A. astacus* and *P. leniusculus* had been experimentally manipulated, as well as from a natural lake containing a *P. leniusculus* population. The resulting samples

†Deceased.

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were analyzed in each team's laboratory. Despite methodological differences, all teams successfully detected DNA from both crayfish species in indoor tanks (3–10 crayfish/m^3). However, detection accuracy declined in outdoor ponds where crayfish density was an order of magnitude lower (0.32 crayfish/m^3). Detection was most variable for *A. astaci*, likely due to its very low prevalence in the host stock. Our study demonstrates the challenges of achieving consistent eDNA results across laboratories and highlights the importance of interlaboratory comparisons. It also underscores the need to identify sources of variability and error, an essential step toward developing robust and standardized protocols. This multinational intercalibration and exchange of knowledge improved methodology and enhanced reliability in crayfish detection.

1 | Introduction

Since the first publications on using *environmental DNA* (eDNA) to detect and monitor macro-organisms almost two decades ago (Ficetola et al. 2008; Haile et al. 2009), the number of eDNA studies has increased drastically (Schenekar 2023). Compared to many other available methods of biodiversity surveys, eDNA screening is a fast, efficient, sensitive, and noninvasive methodology (Takahashi et al. 2023). Analysis of eDNA can be used to assess whole community composition or screen for specific target species, which may include those with a cryptic lifestyle as well as various threatened and protected taxa (Thomsen et al. 2012), species with bioindicator value, as well as taxa that are harmful to the environment or socioeconomy. These include invasive alien species (Dejean et al. 2012; Jerde et al. 2011), various pathogens (Bass et al. 2015, 2023; Sieber et al. 2024), or combinations of all of them (e.g., Rusch et al. 2020; Strand et al. 2019). Consequently, eDNA-based methods are increasingly being used to monitor freshwater species distribution and ecology.

Crayfish are large, long-lived freshwater invertebrates found across diverse habitats, from cold, oligotrophic headwaters to warmer, eutrophic downstream systems (Souty-Grosset et al. 2006). Their omnivorous benthic lifestyle and broad niche width (Ercoli et al. 2015) make them an important link in aquatic food webs where they influence organic matter breakdown, vegetation structure, and invertebrate communities (Albertson and Daniels 2018; Creed Jr. and Reed 2004; Dorn and Wojdak 2004; Reynolds et al. 2013). Some crayfish species are culturally and economically important and were introduced beyond their native ranges in the distant or recent past (Gouin et al. 2001; Swahn 2004). Also, some species are targeted by fisheries and aquaculture (Holdich 1993; Gherardi 2011), while others are threatened, formally protected, and serve as umbrella species for the conservation of aquatic ecosystems (Reynold and Souty-Grosset 2012). Still others are invasive, causing serious environmental impacts in the habitats they invade (Lodge et al. 2000; Gherardi 2007; Dragičević et al. 2020).

In Europe, several native crayfish species of the genera *Astacus*, *Pontastacus*, and *Austropotamobius* have historically been exploited by fisheries (Jussila et al. 2021). However, crayfish populations have collapsed across much of Europe, mainly due to the introduction of crayfish plague, a disease caused by the oomycete *Aphanomyces astaci*. This occurred after the mid-19th century (Alderman 1996), when the crayfish plague pathogen was likely introduced along with its native carriers from North America. Following the collapse of native crayfish stocks, North American crayfish species were introduced to replace them (Bohman et al. 2006). These species were generally considered resistant to the disease, as they are natural carriers of the pathogen (Jussila et al. 2021; Martínez-Ríos et al. 2023), and their introduction led to further declines in native crayfish populations.

The most widespread native crayfish in central and northern Europe is *A. astacus*, which remains exploited in Fennoscandia thanks to both economic and cultural importance (Edsman and Smietana 2004; Gren et al. 2009) but is considered vulnerable (Edsman et al. 2010) and is under some level of legal protection in most European countries (Souty-Grosset et al. 2006). Among the introduced species, the signal crayfish *Pacifastacus leniusculus* and the red swamp crayfish (*Procambarus clarkii*) are of particular concern, as they are important for fisheries and aquaculture, highly invasive (Holdich et al. 2009), and carriers of the *A. astaci* pathogen (Persson and Söderhäll 1983; Diéguez-Uribeondo and Söderhäll 1993). In addition, several other invasive crayfish, often with origin from the aquarium trade, have established populations in European freshwater environments (e.g., Aluma et al. 2023; Jussila et al. 2021; Kouba et al. 2014; Laffitte et al. 2023; Weiperth et al. 2020), many of which are potential or proven carriers of *A. astaci* (Mrugała et al. 2014; Panteleit et al. 2017; Laffitte et al. 2023; Svoboda et al. 2017).

In different regions, different invasive species of North American crayfish contribute to the spread of crayfish plague, which has resulted in the involvement of different *A. astaci* genotypes in the disease outbreaks (Ungureanu et al. 2020). *Pacifastacus leniusculus* is the most widespread invasive crayfish on the European continent, particularly common in western and northern Europe (Kouba et al. 2014). This species and five other invasive crayfish species have been listed as "Invasive Alien Species of Union Concern" in the European Union (European Union 2014) due to their documented or potential impact on invaded ecosystems, and on native crayfish populations in particular. Similarly, due to its devastating impact on European crayfish, *A. astaci* has been listed among the "100 world's worst invasive alien species" (Lowe et al. 2000), and crayfish plague is classified as a listed disease by the World Organization for Animal Health (Yu et al. 2022). Consequently, there is an urgent need to monitor the distribution of both hosts and the pathogen, not only to lead conservation efforts (James et al. 2017) but also to inform fisheries management.

Various methods have been used to assess crayfish presence and abundance, including manual search in suitable shelters, night surveys, use of lift-nets, traps, electrofishing, and scuba diving (Kozák and Kuklina 2015; Parkyn 2015). The success of these methods varies in different environments, depends on the skill and experience of the person conducting the survey, and is strongly influenced by the size structure of the sampled population.

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The substantial impact of invasive crayfish makes it essential to continue developing improved methods for assessing their distribution. Environmental DNA provides a promising and standardizable tool for monitoring both native and invasive crayfish species (reviewed in Baudry et al. 2024), as well as *A. astaci* (e.g., Rusch et al. 2020; Wittwer et al. 2019), offering clear advantages for conservation given the severe effects of the pathogen on European populations. Unlike conventional methods, which require handling live animals and often detect the pathogen only at advanced stages of infection, eDNA enables simultaneous detection of both crayfish and *A. astaci*. As a result, several qPCR assays have been developed for crayfish detection (Baudry et al. 2024; see Table S1). The pathogen can be detected in filtered water samples because it colonizes host exoskeletons and releases short-lived motile zoospores that disperse in the water column (Söderhäll and Cerenius 1999).

Several studies have simultaneously targeted the eDNA detection of multiple aquatic pathogens (including *A. astaci*), or both the pathogen and their crayfish hosts (e.g., Robinson et al. 2018; Rusch et al. 2020; Strand et al. 2011, 2014, 2019; Sieber et al. 2024; Wittwer et al. 2018, 2019), successfully applying and validating this approach on field samples. However, despite these advances, crustaceans, and by extension crayfish, are expected to shed lower amounts of eDNA than fish (see for instance Allan et al. 2021 and Rounds et al. 2024), making them harder to detect and underscoring the need for standardized, calibrated protocols. Indeed, even though a general European standard for eDNA sampling, capture, and preservation from water has been recently proposed (CEN 2023), multiple independent research groups developed their own assays and protocols that differ in, for example, sample collection systems, filter type and pore size, filtered water volume, eDNA sample preservation and storage, DNA isolation, and qPCR assay of choice. This may lead to variability in final outcomes, making comparisons difficult and slowing the implementation of crayfish eDNA screening as a tool broadly recognized by stakeholders.

Here, we perform an interlaboratory comparison as a ring test to compare established detection methods and success rates (including false positives and false negatives) among several European research groups that use eDNA for monitoring crayfish and crayfish plague. Our goal was to evaluate the success rate (i.e., the proportion of eDNA analyses that led to the expected outcome) of various eDNA methods targeting three key species: *A. astacus* native to Europe, invasive *P. leniusculus*, and the crayfish plague pathogen *A. astaci*. This evaluation aimed to improve the accuracy of detecting freshwater crayfish and *A. astaci* across Europe.

2 | Materials and Methods

This present study was initiated during a 2-day workshop on eDNA methodology in February 2020, at the Institute of Freshwater Research, Swedish University of Agricultural Sciences (SLU), Stockholm, Sweden. The workshop had 28 participants from nine countries (Sweden, Croatia, Denmark, Estonia, Finland, Germany, Norway, Spain, and Switzerland). The ring test itself was conducted on May 24, 2023, at the Institute of Freshwater Research, SLU. The experiment involved 11 research teams comprising 26 participants from 13 European countries (Sweden, Croatia, Czechia, Estonia, Finland, France, Germany, Ireland, Norway, Switzerland, Slovenia, Spain, and the UK). Each team brought and operated their own eDNA sampling equipment (including filtration pumps, tubes, and filters) and followed their own protocols from eDNA isolation to qPCR amplification and analysis. Each team used their own laboratory, and Switzerland used two different laboratories. Full details of the materials and procedures employed by each team are provided in Table S3. The participants were affiliated with a range of European universities and governmental institutions representing varying levels of expertise in the eDNA-based monitoring of crayfish and associated pathogens. For clarity and consistency, all protocols have been categorized into three distinct phases: presampling, sampling, and postsampling (Figure 1).

2.1 | Presampling

2.1.1 | Indoor Sampling Locations

Six indoor tanks were prepared 16 days before the experiment by filling each with approximately 1 m^3 of water from Lake Mälaren. To minimize background contamination, including *A. astaci* spores, the water was passed through a sand filtration system. This method has proven effective in eliminating the spores, as *A. astacus* have been kept in the same facility using sand-filtered lake water for several years without developing crayfish plague symptoms (Edsman, L. and Bohman, P., unpublished data). Also, one of the teams used this water as blank control samples and detected no traces of *A. astaci* DNA. After filling, all tanks were sealed from incoming water to prevent water exchange and external contamination. Crayfish were then introduced to allow for acclimation and natural eDNA shedding. Four of the tanks (T1–T2, T4–T5) were stocked with either *A. astacus* or *P. leniusculus* at low (3 crayfish/m^3) or high (10 crayfish/m^3) densities, with each species kept separately. A fifth tank (T6) of 1 m^3 of water contained a mixture of *A. astacus* ($n=3$) and *P. leniusculus* ($n=7$) to assess the ability to detect both species simultaneously and to evaluate potential transmission of *A. astaci* from the *P. leniusculus*, which were sourced from a population known to harbor the pathogen (Bohman et al. 2014). The sixth tank (T3) contained only filtered lake water and served as a negative control to detect any background eDNA or cross-contamination. Detailed information on crayfish numbers and source populations is provided in Table S3.

2.1.2 | Outdoor Sampling Locations

Sixteen days prior to the experiment, *A. astacus* and *P. leniusculus* were also placed in two separate outdoor ponds to allow for eDNA accumulation under seminatural conditions (Figure 2). Each pond measured approximately 25 m in length, 10 m in width, and 1 m in depth, with an estimated volume of 250 m^3. The ponds were continuously supplied with slowly circulating water from Lake Mälaren, which was first passed through slot filters (Model: RS-500/0.05–0.8 mm, SPA products/FilterteknikBW, Sweden) to remove fine sediment and reduce the risk of introducing *A. astaci* spores. Into each pond, 80 crayfish individuals

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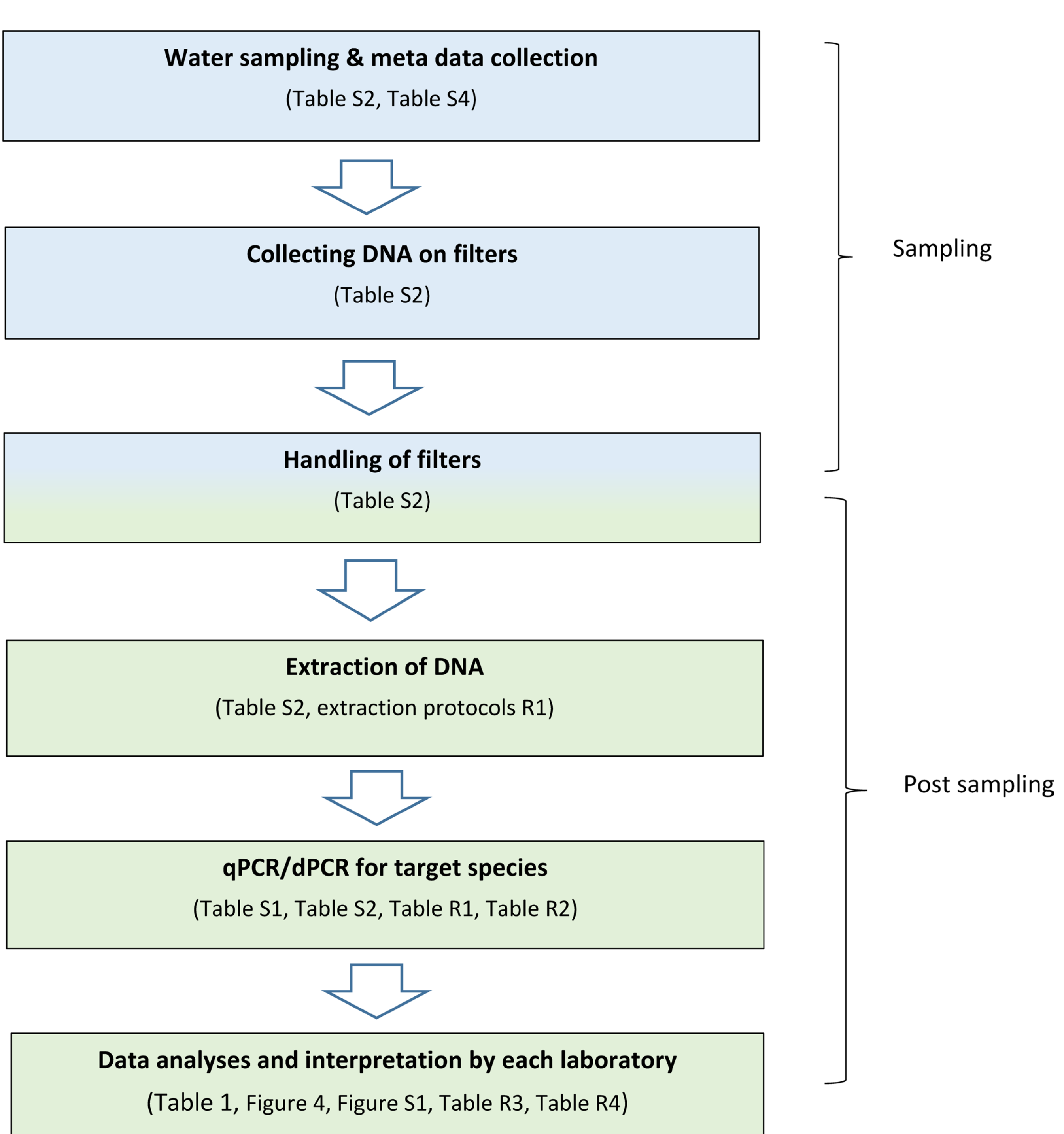

FIGURE 1 | Flow chart of general methodological steps in the eDNA analysis process, from water sampling to interpretation. Tables provide more details. The abbreviation "S" refers to Figure S1 and Tables S1–S4 and "R" refers to repository protocols and tables (Bohman 2025a).

were inserted in eight cylindrical cages with no escape openings (length: 400 mm, diameter: 250 mm, mesh size: 14 mm), with 10 crayfish per cage (0.32 crayfish/m^3). The cages were evenly arranged along the bottom to ensure uniform crayfish distribution and eDNA release. A third outdoor pond was designated as a negative control and contained no crayfish, serving to detect

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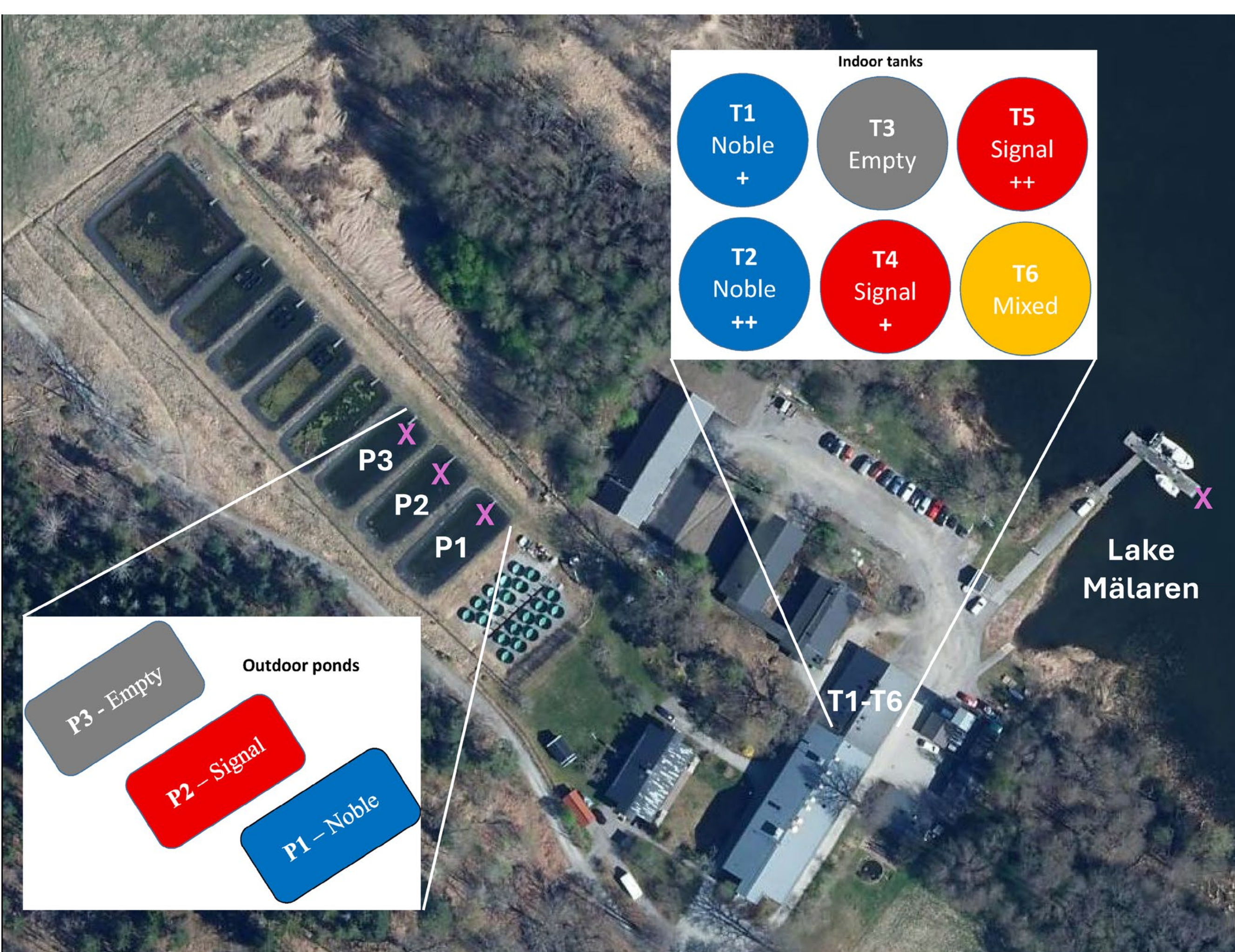

FIGURE 2 | Aerial overview of the eDNA sampling locations (indoor tanks, ponds and lake) used in the ring test experiment at the Institute of Freshwater Research, SLU, Stockholm. The indoor tanks (T1–T6), located within a dedicated facility, contained *P. leniusculus* and *A. astacus* at varying densities (mixed tank contains both species). Negative controls ("Empty") were included both indoors and outdoors. Outdoor sampling sites are indicated by purple crosses in the ponds and at the lake jetty. Abbreviations used in the figure include "T" for tank and "P" for pond, "Signal" for *P. leniusculus* and "Noble" for *A. astacus*, "+" indicate low densities and "++" indicate high densities. Photo: Lantmäteriet (Eniro).

potential environmental contamination. Detailed information on crayfish origin and allocation is provided in Table S3.

The last outdoor sampling site was at the jetty in Lake Mälaren, which harbors a *P. leniusculus* population. While its abundance in the area around the jetty is unknown, based on previous surveys and local ecological knowledge (Bohman 2025b) we can estimate at least 100 individuals over an area of 500 m outside the jetty.

2.1.3 | Preparation of Subsamples and Reference DNA Samples

To evaluate the efficiency of laboratory procedures of each lab (including DNA extraction and PCR), standardized filters were prepared so that every team could analyze equivalent samples. In total, 11 replicates, one for each team, were sampled from three tanks (high density of *A. astacus*, high density of *P. leniusculus*, and a mixed tank with both species but with a higher density of *P. leniusculus*). The sampling was performed by one person on a single day, 7 days before the experiment. For each sample, 5 L of water was filtered with a peristaltic pump (Cole-Parmer Masterflex E/S portable sampler, USA), using an open filter holder with hydrophilic glass fiber filters with binder resin (pore size: 2.0 μm, AP2504700, Merck Millipore, Germany). The tubes and filter holders were disinfected (when changing tanks) with a 10% solution of commercial bleach for several minutes, then by a 5% solution of sodium thiosulfate, and finally rinsed with tap water. Each eDNA-containing filter was then stored in a sterile plastic zip bag marked with ID code and filled with 35 g of silica gel (Real Marine AS, Norway) to dry out, according to Majaneva et al. (2018) and Wilcox et al. (2016). The filters were stored in the dark at room temperature until they were handed over to each participating team at the end of the sampling day.

In addition, total DNA extracts of the three target species were prepared using muscle tissue (claws) from *A. astacus* (6.9 ng/μL) and *P. leniusculus* (152 ng/μL), and from *A. astacus* naturally infected by *A. astaci* (20.7 ng/μL, mixed-genome sample). The DNA concentrations in these extracts were measured on a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, USA) using the Qubit

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1× dsDNA High Sensitivity (HS) kit (Q33230; Thermo Fisher Scientific, Waltham, USA). The extracts were used to generate standardized dilution series for qPCR calibration across participating teams. These reference samples were kept in the freezer (−20°C) and handed out at the end of the sampling day.

2.2 | Sampling

The sampling, filtration and stabilization of filtrate was conducted during a full day on May 24, 2023, from 09:00 to 18:00. All 11 participating teams began sampling at different designated sources (indoor tanks, outdoor ponds, and Lake Mälaren), as outlined in Figure 2. Each team operated independently, using their own field equipment and following their respective protocols for filtration and sample handling (Figure 1; Table S3).

2.2.1 | Sampling Design and Procedure

Water samples were collected by all teams from standardized locations and depths, although sampling times varied between teams. For the outdoor ponds, sampling was performed near the water outlet, while for Lake Mälaren, samples were collected from the jetty. In the case of indoor tanks, a fixed siphon was installed at each tank to avoid cross-contamination during water collection. Each team collected two to seven replicate filters per sampling source, resulting in a total of 20 to 70 filters per team across all ten sites: six indoor tanks, three outdoor ponds, and the lake.

2.2.2 | Disinfection Routines

To minimize the risk of cross-contamination between sampling sources, strict disinfection protocols were followed for all reusable equipment. After sampling at each site, equipment, including filtration units, tubes, and sampling containers, was disinfected using a three-step process. First, items were submerged in a 10% bleach for several minutes to ensure effective decontamination. For internal surfaces such as tubes, bleach was circulated using pumps used by the respective teams. Following this, all equipment was immersed in a 5% sodium thiosulfate solution to neutralize residual chlorine, preventing downstream DNA degradation. Finally, the equipment was thoroughly rinsed with tap water to remove any remaining chemical residues. As an alternative to this procedure, several teams employed disposable or predisinfected materials to minimize contamination risk.

2.2.3 | Metadata

The following metadata was recorded during the sampling day to aid interpretation of the eDNA results. Water temperature was measured once for each indoor tank and twice for the outdoor ponds and Lake Mälaren (once in the morning and again in the afternoon) to capture potential diurnal variation. In parallel, additional water samples were collected, stored in cool and dark conditions, and sent to the Department of Aquatic Sciences and Assessment, SLU, for analysis of physical and chemical parameters. These included turbidity (FNU), total organic carbon (TOC), absorbance, and pH. These variables provide insight into water quality and may influence eDNA degradation, transport, inhibition, and therefore detectability. A comprehensive summary of all metadata measurements and analytical results is presented in Table S4.

2.3 | Post Sampling

Following the field sampling activities, each participating team transported their collected eDNA filtrates to their respective home laboratories for molecular analysis. Transport conditions and timelines varied slightly among teams, but all samples were handled in accordance with the teams' standard practices to maintain sample integrity during field surveys. Further methodological details are provided in Table S3.

2.3.1 | DNA Extraction

DNA extraction (including inhibition control) was performed independently by each team using their preferred laboratory protocols (repository Protocols R1; Bohman 2025a). The majority ($n = 8$) utilized commercial extraction kits, such as Qiagen DNeasy Blood and Tissue or Qiagen DNeasy PowerWater kits (Qiagen, Hilden, Germany), either according to manufacturer instructions or with minor laboratory-specific modifications. Other teams ($n = 3$) employed in-house extraction protocols tailored to their filter types, including CTAB-chloroform-based methods and high-salt extraction techniques, particularly for samples collected using Sterivex filters (Merck Millipore, Darmstadt, Germany). Details of the protocols are provided in the repository (Protocols R1; Bohman 2025a).

2.3.2 | qPCR Analyses

Most participating laboratories conducted molecular detection of eDNA using quantitative PCR (qPCR; $n = 7$), three laboratories used a combination of qPCR and droplet digital PCR (ddPCR), and one laboratory used digital PCR (dPCR), following their in-house protocols (Table S3). To enable cross-comparison, all teams included a standardized calibration curve derived from extracts of DNA of the target species that had been distributed in advance of the experiment (Figure S1). A variety of thermocycler platforms and manufacturers were used across laboratories, reflecting current diversity in available instrumentation. The number of technical replicates per sample ranged from one to seven, depending on each laboratory's standard practice and resource availability. PCR amplification protocols also varied, particularly in primer and probe selection (Table S3), which were adapted to the target species being analyzed. For detection of *A. astacus*, most laboratories used previously validated assays described by Rusch et al. (2020) or Agersnap et al. (2017), while a few employed protocols developed by King et al. (2022) or Knudsen et al. (2019). Assays targeting *P. leniusculus* were more heterogeneous, using protocols from Agersnap et al. (2017), Chucholl and Chucholl (2021), King et al. (2022), Knudsen et al. (2019) and Rusch et al. (2020). Most laboratories used singleplex assays, but a few ($n = 2$) used multiplexing/duplexing assays. Also, most laboratories ($n = 7$) used blind samples in their

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pipelines. For detection of *A. astaci*, most teams relied on the qPCR assay developed by Vrålstad et al. (2009), though minor variations in primers and probes were observed. In some cases, teams used in-house designed probe-based assays tailored to their own laboratory conditions. A full list of eDNA assays and their corresponding references is presented in Table S1.

2.3.3 | Control for *A. astaci* Prevalence

To assess the presence of *A. astaci* among the experimental animals, targeted diagnostic testing was conducted on a subset of individuals (Table S3). All crayfish from the mixed-species tank (three *A. astacus* and seven *P. leniusculus*) were examined, along with ten randomly selected *P. leniusculus* from the outdoor pond. Sampling procedures and molecular diagnostics for pathogen detection were developed and carried out by the Swedish Veterinary Agency (SVA), Uppsala, Sweden, using established PCR-based protocols for *A. astaci* detection (Strand et al. 2023).

2.3.4 | Data Collection and Presentation

To ensure consistency and traceability throughout the study, all participating teams were instructed to record metadata, sampling details, and molecular analysis results using preformatted Excel "results sheets", produced by SVA. These standardized templates facilitated uniform data entry across laboratories. A summary of the methodological details and recorded data can be found in Table S3.

2.3.5 | Analytical Approach: Methodological Diversity and Detection Success

To assess how methodological differences influenced detection success, Sankey diagrams were constructed for *A. astacus* and *P. leniusculus* detection (but not for *A. astaci* due to ambiguous results). These diagrams effectively visualize complex workflows involving key variables such as filter type, preservation method, DNA extraction protocol, and PCR assay. By tracing the flow of eDNA from sampling to detection, the diagrams reveal how specific methodological choices correlate with successful detections, defined as correct identification in at least 85% of samples. The width of each flow represents the proportion of laboratories using a given method. To enhance clarity, only a subset of critical variables was included (filter pore size, water volume filtered, filter type, preservative, eDNA extraction protocol, and primer assay), selected by expert judgment based on their likely impact on detection outcomes. For full methodological details, see Table S3.

2.3.6 | Analytical Approach: Standard Dilution Series of Total Genomic DNA and LOD

To evaluate the analytical sensitivity of the qPCR assays used across laboratories, each team performed a 10 point 10-fold standard dilution series, using preextracted total genomic DNA from *A. astacus*, *P. leniusculus*, and *A. astaci* (Figure S1). The dilution series enabled determination of the limit of detection (LOD), defined here as the lowest standard concentration of template DNA that produced at least 95% positive replicate, that is, the "threshold method" according to Klymus et al. (2020). By isolating this step from upstream variables (e.g., sampling, filtration, DNA extraction), these standard curves served as a control to assess the intrinsic performance of each PCR assay and platform, offering a benchmark for interlaboratory comparison.

2.3.7 | Analytical Approach: Qualitative Ratings of qPCR Results

To support management decisions and to allow laboratories to reflect on the reliability of their results, each team assigned a qualitative score to their final qPCR outcomes using a five-point scale (Table 1). This scale provided a nuanced assessment of detection confidence based on the overall performance of technical replicates and Cq values and was preferred over a simpler three-point scale (e.g., "positive", "negative", or "uncertain"). A five-point scale may also enable better communication of uncertainty to stakeholders and policy makers (Darling and Mahon 2011). The scoring was defined as 1 = Fully negative (no amplification in any replicate), 2 = Predominantly negative (majority of replicates negative, but with minor inconsistencies), 3 = Uncertain (ambiguous results), 4 = Predominantly positive (most replicates amplified, but with some uncertainty), 5 = Fully positive (consistent amplification in all replicates). It should be noted that the application of this scale was not entirely uniform: some laboratories applied more stringent criteria than others. For example, some considered any signal with a Cq value above 35 as negative, even when amplification occurred consistently in all replicates. Additionally, the number of technical replicates varied between laboratories (ranging from one to six), potentially affecting detection outcomes. A secondary layer of analysis, comparing successful and failed eDNA detections across teams, is also presented within the same matrix tables. This illustrates the concordance between each laboratory's species identification results and the verified presence or absence of the target species at each sampling site (Figure 2). This correspondence is represented using colors (Table 1). The application of this color-coded classification facilitates visual identification of key diagnostic errors, such as false negatives and false positives. "Detection accuracy" in Table 1 is used to define how well the method classifies presence and absence correctly, that is, accuracy = (true positives + true negatives)/total number of samples x 100. This combines both sensitivity (true positive rate) and specificity (true negative rate) and is a good measure of overall performance, especially when both false positives and false negatives matter.

3 | Results

3.1 | Workflows and Success Rate for Species Detection

Both diagrams in Figure 3 share a similar structure, reflecting parallel analytical pipelines across teams, but differ in the distribution of yellow (above average) and purple (below average detection success), highlighting species-specific variation

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TABLE 1 | Matrix tables showing results for eDNA detection of *A. astacus* (top), *P. leniusculus* (middle), and *A. astaci* (bottom) assays across multiple teams in the ring test experiment.

Results Noble crayfish

Sample	Expected	Cze	Cro	Est	Fin	Fra	Ger	Irl	Nor	Swe	Swi	Swi2	Acc
T Noble +	Pos	5	5	5	5	5	5	4	5	5	5	5	100%
T Noble ++	Pos	5	5	5	4	5	5	5	5	5	5	5	100%
T Signal +	Neg	1	2	1	1	1	1	1	1	1	1	1	100%
T Signal ++	Neg	1	1	1	1	1	1	1	1	1	1	1	100%
T Mixed	Pos	5	3	5	1	5	1	4	5	5	1	5	70%*
T Empty	Neg	1	2	1	2	1	1	1	2	1	1	1	100%
P Noble	Pos	4	2	5	2	4	1	1	4	1	1	1	36%
P Signal	Neg	1	1	1	2	1	1	1	1	1	1	1	100%
P Empty	Neg	2	1	1	1	1	1	1	1	1	1	1	100%
Lake	Neg	1	1	1	1	4	1	1	1	1	1	1	91%
Accuracy		100%	89%*	100%	80%	90%	80%	90%	100%	90%	80%	90%	90%

Results Signal crayfish

Sample	Expected	Cze	Cro	Est	Fin	Fra	Ger	Irl	Nor	Swe	Swi	Swi2	Acc
T Noble +	Neg	1	3	5	1	1	1	1	1	1	1	1	90%*
T Noble ++	Neg	2	1	1	4	1	1	1	1	1	2	1	91%
T Signal +	Pos	5	5	5	5	5	4	4	5	5	5	4	100%
T Signal ++	Pos	5	5	5	5	5	5	5	5	5	5	5	100%
T Mixed	Pos	5	3	5	5	5	5	5	5	5	5	5	100%*
T Empty	Neg	1	1	1	4	1	1	1	4	1	3	1	70%*
P Noble	Neg	2	1	1	1	3	1	1	4	1	1	1	90%*
P Signal	Pos	5	2	1	1	5	5	1	5	5	5	3	60%*
P Empty	Neg	5	1	1	1	1	1	1	1	1	1	1	91%
Lake	Pos*	1	1	1	1	4	1	1	4	3	2	1	20%*
Accuracy		80%	75%*	70%	60%	100%*	90%	80%	80%	100%*	89%*	89%*	83%

Results crayfish plague pathogen (A. astaci)

Sample	Expected	Cze	Cro	Est	Fin	Fra	Ger	Irl	Nor	Swe	Swi	Swi2	Acc
T Noble +	Neg	1	5	1	4	4	1	1	1	1	1	1	73%
T Noble ++	Neg	1	5	1	5	1	1	1	1	1	1	1	82%
T Signal +	?	1	2	1	2	1	4	1	1	1	1	1	
T Signal ++	?	1	1	1	4	4	1	1	1	1	1	1	
T Mixed	?	2	3	1	2	4	1	5	1	1	5	3	
T Empty	Neg	1	5	1	5	4	1	1	1	1	1	1	73%
P Noble	Neg	1	1	1	1	4	2	1	1	1	1	1	91%
P Signal	Pos	1	3	1	1	4	1	1	1	1	1	1	10%*
P Empty	Neg	1	1	1	1	4	1	1	1	1	1	1	91%
Lake	Pos	2	3	1	1	4	2	1	1	1	1	1	10%*
Accuracy		71%*	40%*	71%*	29%*	43%*	71%*	71%*	71%*	71%*	71%*	71%*	62%

Note: Teams (by country) scored their PCR results from 1 to 2 (negative) to 4–5 (positive). Score 3 (uncertain) is excluded from accuracy calculation (Acc*). Colors indicate consistency with expected species: dark and light green = consistent, light blue = uncertain, light and dark beige = inconsistent. Expected species are shown for each sample ("?" shows dubious expectations). "T" for tank and "P" for pond, "Signal" for *P. leniusculus* and "Noble" for *A. astacus*, "+" indicates low densities and "++" indicates high densities.
Abbreviations: Cro, Croatia; Cze, Czechia; Est, Estonia; Fin, Finland; Fra, France; Ger, Germany; Irl, Ireland; Nor, Norway; Swe, Sweden; Swi, Switzerland lab 1; SWI2, Switzerland lab 2.

in eDNA detection success. This variation is influenced by combinations of filter pore size and material, volume of water filtered, preservation medium, extraction method, and PCR assay. Since teams were allowed to use their own equipment and methods, this led to variation in filtration systems and the volume of water processed (Table S3). Most teams ($n = 6$) used glass fiber filters, which typically have rather large pore sizes (1.0–2.0 μm), allowing them to process larger water volumes (3.5–5 L per sample). Other teams used Sterivex cartridge filters of 0.45 μm pore size ($n = 2$), while the remaining groups

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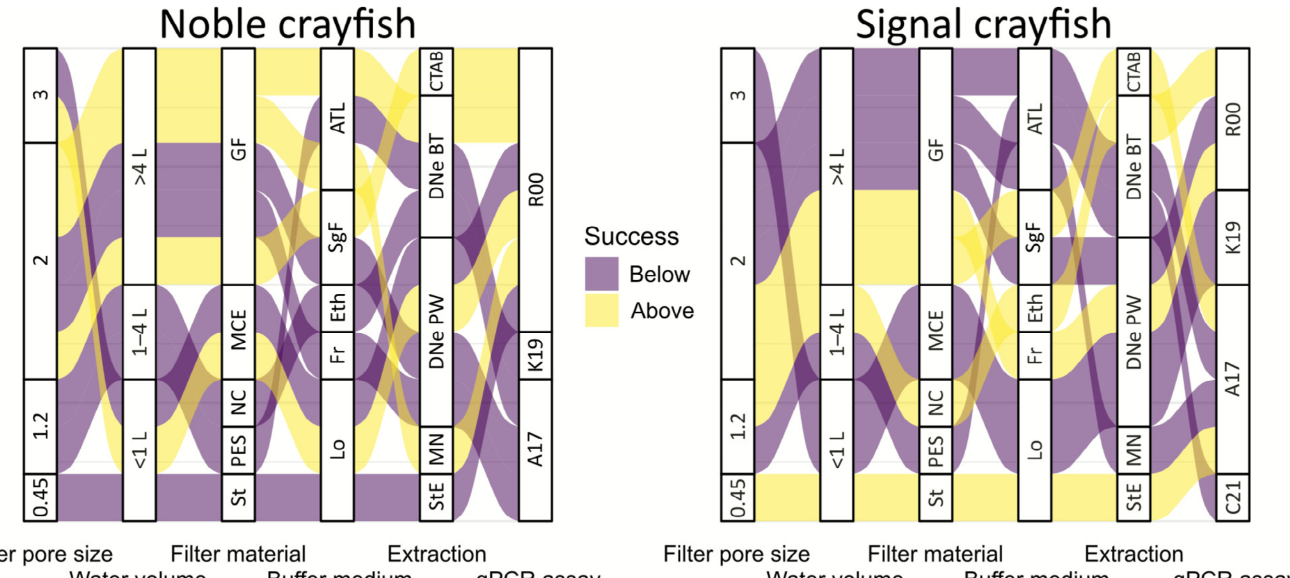

FIGURE 3 | Sankey diagrams illustrating the methodological workflows and detection outcomes for *A. astacus* (left) and *P. leniusculus* (right) across multiple laboratories in the ring test experiment. Each diagram visualizes the flow of samples through successive methodological steps: Filter pore size (μm), water volume (L), filter material (GF, glass fiber; MCE, mixed cellulose ester; NC, nitrocellulose; PES, polyethersulfone; St, sterivex capsule), buffer medium (Eth, ethanol; Fr, frozen; Lo, longmire; Sgf, silica gel and frozen, ATL), DNA extraction method (CTAB, CTABchloroform-based extraction; DNeBT, DNeasy Blood and Tissue; DNePW, DNeasy PowerWater kit; MN, modified nucleospin; StE, Sterivex high salt extraction), and qPCR assay used (R00 = Rusch et al. 2020; K19 = Knudsen et al. 2019; A17 = Agersnap et al. 2017; C21 = Chucholl et al. 2021). Yellow streams indicate highly successful detections of the target species (above the median success rate of 85%), while purple streams represent less successful detections. Variability in detection success is associated with differences in filtration materials, buffer types, extraction protocols, and qPCR assays.

applied a variety of cellulose and polyethersulfone (PES) filters, each with differing pore sizes and filtration capacities. Also, a variety of preservation strategies were employed across teams. In accordance with the European standard of eDNA sampling (CEN 2023) most teams ($n = 6$) used preservation buffers such as Longmire's solution, ATL buffer, or ethanol to stabilize the DNA on the filters. A smaller number of teams ($n = 2$) used silica gel desiccants for drying filters, which were subsequently kept frozen for long-term storage, while others ($n = 3$) immediately froze their samples upon collection.

3.2 | Qualitative Ratings of PCR

Table 1 provides a comparative overview of each laboratory's species-specific detection performance across different environmental samples. Overall, detection accuracy of crayfish was notably higher for samples derived from indoor tanks than from outdoor ponds or the lake. Interestingly, in the mixed indoor tank of 1 m^3, containing three *A. astacus* and seven *P. leniusculus*, the detection accuracy for *A. astacus* was lower than in the single-species tank with low-density *A. astacus* (3 individuals per m^3). In outdoor samples, detection accuracy declined markedly, to 36% for *A. astacus* and 60% for *P. leniusculus* in the ponds (with the density of 0.32 individuals per m^3), highlighting the challenges of eDNA detection in larger, more complex natural environments. Notably, the detection success for *P. leniusculus* in Lake Mälaren, where crayfish spatial distribution is unknown and densities are expected to be low (according to Bohman 2025b), was only 20%.

Aphanomyces astaci was more challenging to detect, and the results showed large variation. Unfortunately, due to low initial prevalence of *A. astaci* in the host crayfish (see below), it is unclear whether *A. astaci* was present in three of the indoor tanks containing *P. leniusculus*. Therefore, these tanks are left without color in Table 1 ("T Signal+", "T Signal ++" and "T Mixed"). In addition, some participating teams reported false-positive results for both species of crayfish ($n = 7$) and *A. astaci* ($n = 10$), which may confuse the overall patterns. For example, in the case of Lake Mälaren, where *A. astaci* would have been expected, the only team reporting its presence also reported false positive detections of this pathogen in tanks and ponds, making the positive results of its detection in the lake doubtful (Table 1).

3.3 | Crayfish Plague Prevalence

Two individuals out of the 10 sampled from the pond with *P. leniusculus* (total $n = 80$) tested positive for *A. astaci* DNA, indicating that the *P. leniusculus* used in the experiment carried the pathogen, but its prevalence was low (12%; 95% confidence interval: 1.4 to 35.9%; Table S3). Analyzed tissues from all individuals in the mixed indoor tank tested negative ($n = 3$ *A. astacus* and $n = 7$ *P. leniusculus*), which reflects the uncertainty regarding the expected species in the tank (indicated by "?" in Table 1). The same applies to the two tanks with *P. leniusculus* ("T Signal+" and "T Signal ++" in Table 1), as no individuals from these tanks were tested.

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3.4 | Limit of Detection (LOD) and Cq Values

Comparison of LOD of various PCR assays and systems (Figure S1) shows that the methods generally are comparable, with most of the teams clustering closely together. For *A. astaci*, two teams represent clear outliers, with one of them (GER) having approximately a factor 1000 lower LOD compared to most other teams. Two teams (CRO and FRA) appear to have the potential to increase the sensitivity of their qPCR systems for *A. astacus* and *P. leniusculus*. For a few teams, the dilution series for the *A. astaci* assay continued to yield Cq values well beyond their defined LOD.

Overall, the Cq values were high, and in most cases well above a Cq value of 30 (Figure 4). In controlled tank conditions, target species were detected with higher sensitivity, and Cq values inversely correlated with crayfish density, reflecting increased eDNA yield with higher biomass. Water samples from outdoor ponds and the lake exhibited higher Cq values and greater variability, with Cq values clustering near the detection threshold (~Cq 40).

4 | Discussion

Here, we present the results of an interlaboratory ring test aimed at evaluating how different eDNA protocols influence the detection of crayfish species and the crayfish plague pathogen. Our main finding is that, while detection of crayfish species was generally successful in indoor tanks, performance declined in outdoor ponds. In contrast, detection of *A. astaci* was highly inconsistent, with false negatives (likely caused by very low concentrations of target eDNA in the source samples) but also false positives. By granting participants full procedural autonomy, from field sampling to qPCR analysis, this study reflects the current diversity of methodologies employed by eDNA practitioners for detecting crayfish and crayfish plague across Europe. A key difference from previous interlaboratory studies, which focused on individual methodological steps such as filtration, DNA extraction, or PCR optimisation (e.g., Burian et al. 2021; Rodriguez et al. 2025), is that our study evaluated the entire eDNA workflow as an integrated process (Figure 1, Table S3). While this holistic focus better reflects real-world practices, it also introduced substantial heterogeneity in both procedures and outcomes in this study, making it difficult to attribute variability to specific steps within the workflow. The observed interlaboratory variation likely stems not only from differences in protocol and equipment but also from unaccounted-for sources, such as stochastic fluctuations in target DNA concentration in environmental samples (especially for *A. astaci*), inadvertent contamination, and inconsistent handling of PCR inhibitors or other technical artifacts (see e.g., Jones et al. 2025). Indeed, a recent study by

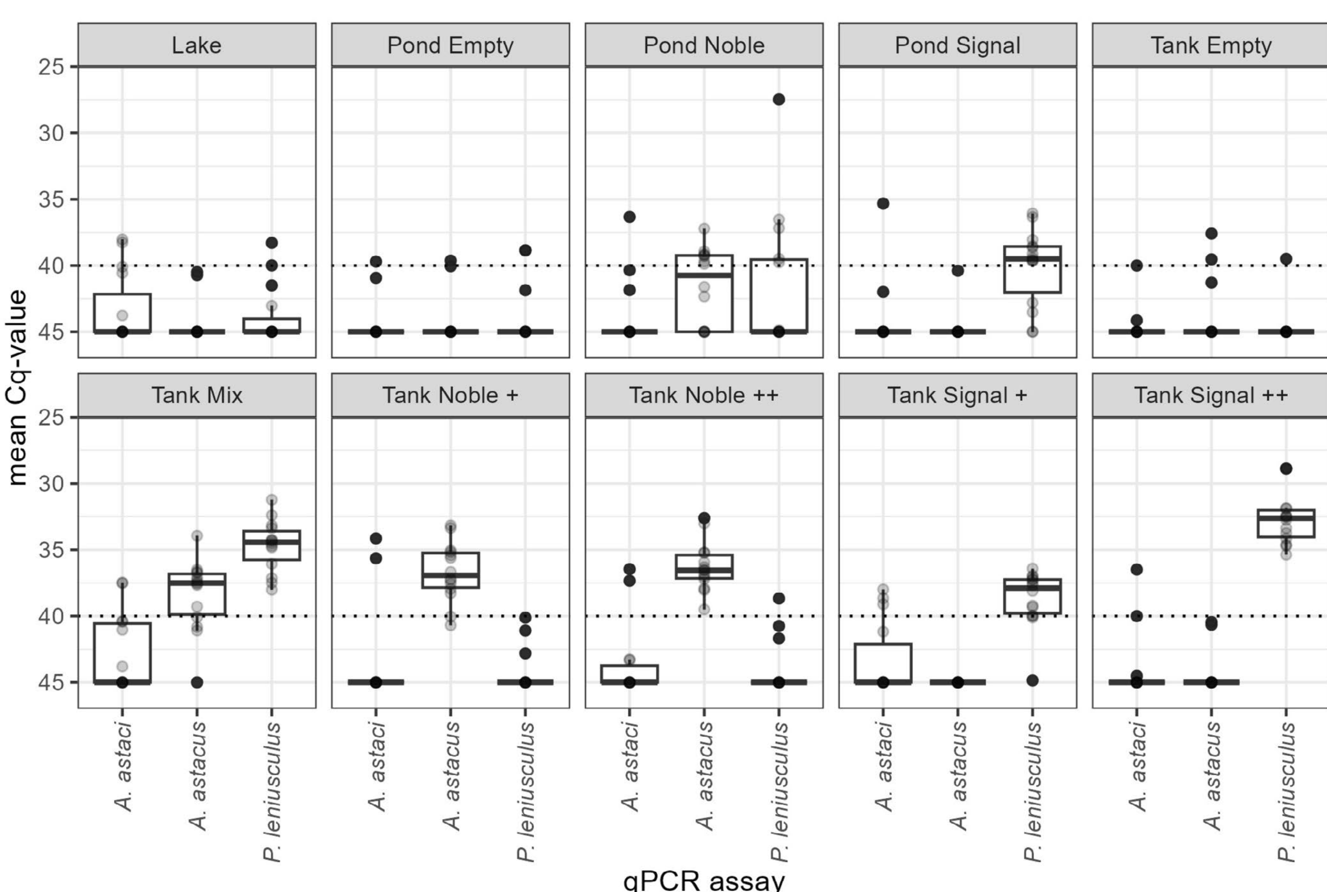

FIGURE 4 | Boxplot with jitter of mean Cq values of positive amplifications from each filter, analyzed with species specific qPCR assays (*A. astaci*, *A. astacus*, or *P. leniusculus*), per tanks ($n = 6$), ponds ($n = 3$), and lake ($n = 1$). The boxplot shows the median (horizontal line), interquartile range (box), and whiskers extending to 1.5× the interquartile range; individual data points (mean Cq values from each lab) are shown as jittered dots. Data from the two teams using dPCR are not included. "No amplification" is set to Cq value 45. Dotted lines indicate a threshold Cq value of 40. Abbreviations used in the figure include "Signal" for *P. leniusculus* and "Noble" for *A. astacus*; "+" indicate low densities and "++" indicate high densities.

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Parsley et al. (2025) indicated that even when seemingly homogeneous sources of eDNA such as mesocosms are sampled, substantial variation is observed among parallel biological replicates. Since the design did not allow for the partitioning of variation across individual methodological steps, these factors remain difficult to quantify directly. We further explore these potential sources of variability and their implications for data interpretation below. Nevertheless, our interlaboratory study has led several participating laboratories to review and refine their own eDNA protocols to enhance the yield of target DNA which aligns with the primary objective of the study: to progressively improve the detection of crayfish and *A. astaci* across Europe through increased collaboration and the adoption of a harmonized and optimized methodological framework.

4.1 | Association Between Workflows and Success Rates in Species Detection

Although the Sankey diagrams in Figure 3 revealed notable methodological differences across laboratories, we found no clear connection between the methodologies used and the success rates of the different labs, indicating that the main source of variation in success rates remains unknown in a study like this. A few methodological factors, such as the use of a larger amount of water (associated with larger pore size of used filters), seem associated with higher success rates, but it is not possible to disentangle their effect fully from other factors. This is due to the large number of methodological steps involved and the limited number of participating teams, which together result in a sample size too small for reliable analysis.

4.2 | Detection of Crayfish and *A. astaci*

Despite the use of diverse equipment and protocols by all 11 participating teams, detection accuracy of crayfish was high in four controlled indoor tanks. These tanks housed either low-density (three individuals per m^3) or high-density (ten individuals per m^3) populations of single crayfish species (Table 1, Table S3). The high detection accuracy in these tanks likely reflects their simplified and stable environmental conditions, that is, clean, closed systems with limited water volume ($1\,m^3$) and constant temperature (15°C), which reduce DNA degradation and enhance eDNA persistence. In contrast, in larger and more complex outdoor environments (ponds and lakes), detection accuracy declined dramatically to 36%–60% in ponds (0.32 individuals per m^3) and 20% in the lake (Table 1). These differences are consistent with the lower eDNA concentrations inferred from higher Cq values observed in these systems (Figure 4).

The reduced detection accuracy in outdoor systems is likely due to several factors. Larger water volumes lead to dilution of eDNA, and spatial and temporal heterogeneity increases stochastic effects (Parsley et al. 2025), which can result in false negatives (Goldberg et al. 2016). Sampling from multiple locations in the ponds and lake (e.g., near the shore) or pooling water samples with pseudo-replicates may reduce the impact of these factors, thereby decreasing the number of false negatives. Additionally, outdoor ponds and lakes introduce greater environmental complexity, such as variable substrate, open water exchange, increased solar radiation, and fluctuating temperatures, which can influence DNA degradation and amplification efficiency (Barnes et al. 2014; Barnes and Turner 2016; Yates et al. 2019). Although metadata from these sites indicated low turbidity, total organic carbon (TOC), and absorbance, water temperatures in the ponds rose from 16.4°C in the morning to 19.4°C by afternoon, potentially affecting DNA stability and increasing enzymatic activity (Table S4). Despite these challenges, previous studies have successfully detected crayfish eDNA in both lotic (Chucholl et al. 2021; King et al. 2022; Rusch et al. 2020; Wittwer et al. 2018) and lentic systems (Dougherty et al. 2016; Johnsen et al. 2020; Larson et al. 2017; Tréguier et al. 2014), even at very low population densities. However, detection rates (defined as "proportion of positive results") are typically lower under natural conditions. For example, Dougherty et al. (2016) and Johnsen et al. (2020) reported detection frequencies as low as 10% in low-density populations, compared to nearly 100% in high-density lakes. Therefore, the low detection accuracy found in this study for ponds (36%–60%) and the lake (20%) align with previous findings when accounting for similar environmental complexity and crayfish density, and limited sampling effort per each site in our ring test. Lastly, although TOC and suspended solids, which may introduce inhibitors to the eDNA samples, were measured at low levels, the presence of other, unmeasured inhibitors (e.g., laboratory-specific chemical compounds) may still have affected PCR performance (Schrader et al. 2012), as supported by previous studies (Eichmiller et al. 2016; Parsley et al. 2024; van Bochove et al. 2020).

In the mixed-species indoor tank ($1\,m^3$), containing seven *P. leniusculus* and three *A. astacus*, detection accuracy varied significantly between species. *P. leniusculus* were consistently detected by all participating laboratories, while *A. astacus* were only detected by 70% of the teams. These false negatives may reflect assay-specific limitations or broader methodological inconsistencies affecting *A. astacus* detection. Although all teams reporting negative results employed previously validated, high-sensitivity probe-based qPCR assays (taken from Agersnap et al. 2017; King et al. 2022; Knudsen et al. 2019; Rusch et al. 2020), variation in upstream methodological steps could have contributed to reduced detection success. As displayed in Table S1, species-specific assays for qPCR/ddPCR/dPCR have already been developed and applied to detect all widespread native crayfish known from Europe, except for locally endemic species or evolutionary significant units of *Austropotamobius torrentium* (Klobučar et al. 2013; Pârvulescu et al. 2019; Lovrenčić et al. 2020), as well as to screen for prominent invasive species of North American origin. In several cases, assays for the same species have been developed multiple times in parallel, for example, for *P. leniusculus*, *A. astacus* or for the parthenogenetic marbled crayfish *Procambarus virginalis* (Baudry et al. 2024; Mauvisseau et al. 2019; Rusch et al. 2020).

Detection of the crayfish plague pathogen *A. astaci* in this study was unexpectedly low, with most teams not confirming its presence in the mixed tank (set up with the intention of inducing crayfish plague by placing the potential carriers, *P. leniusculus*, together with susceptible *A. astacus*), as well as in other systems containing *P. leniusculus*, including tanks, ponds, and the lake. Although *P. leniusculus* acts frequently as a chronic carrier of *A. astaci*, the pathogen prevalence within its populations is highly

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variable, and *A. astaci* can be apparently in some cases entirely absent (Filipová et al. 2013; James et al. 2017; Mojžišová et al. 2022; Vrålstad et al. 2011). Several studies have demonstrated the potential of eDNA monitoring of *A. astaci*, both in localities with North American host populations (Robinson et al. 2018; Rusch et al. 2020; Strand et al. 2011; Wittwer et al. 2019) and during crayfish plague outbreaks (Strand et al. 2019; Casabella-Herrero et al. 2023). Moreover, Strand et al. (2014) showed a positive correlation between eDNA concentration of *A. astaci* and the prevalence of *A. astaci* in *P. leniusculus* populations. The frequent failure to detect *A. astaci* in our study seems primarily due to the very low prevalence and limited sporulation of *A. astaci* in *P. leniusculus* used for the study, rather than poor methodological approaches (Martínez-Ríos et al. 2023). The very low LOD for *A. astaci* reported by one of the participating teams may be due to either a more efficient PCR system (Francesconi et al. 2021) or an unidentified experimental error. This result hence requires further investigation to determine its reproducibility.

A serious matter is the findings of the false-positive detections for *P. leniusculus* ($n=6$) and *A. astaci* ($n=10$) observed in samples from the indoor tanks and outdoor ponds, while only one false positive (from the lake) was recorded for *A. astacus* (Table 1). These false positives are clustered in some labs and not across all teams. Some of the false positives might depend on the implementation of a particular detection assay and its specific parameters used by those teams. In the dilution series, the qPCR *A. astaci* assay by Vrålstad et al. (2009) gave consistent amplification well past LOD for two teams. However, only one of these teams reported false positive results for *A. astaci*. This highlights the need to verify each detection assay to each laboratory-specific reagents and instrument employed. In diagnostic applications, a false-positive rate of $>5\%$ is generally considered unacceptable (Wilcox et al. 2013). However, in the context of eDNA as a screening tool, such a rate may be tolerable if supported by confirmatory testing (Goldberg et al. 2016). The most likely causes of these false positives include contamination during sampling or laboratory processing, particularly since only a few laboratories were affected, suggesting isolated procedural errors rather than systematic flaws. Additionally, despite precautionary measures, residual eDNA contamination from the source water cannot be ruled out, as all experimental water (whether from tanks, ponds, or tap) originated from Lake Mälaren, which hosts established populations of *P. leniusculus* and *A. astaci* (Bohman 2025b). However, the lake water was always treated by filtration, and one team used tap water as blank controls and did not detect any detectable traces of *A. astaci* DNA.

The original plan was to conduct the ring test using a "business-as-usual" approach to assess the potential variability in results, with implications for data interpretation and interlaboratory comparability. However, due to methodological and logistical constraints, it was not possible to standardize DNA input, extraction efficiency, or amplification conditions across participating laboratories. As a result, interlaboratory comparisons depicted in Figure 4 are certainly influenced by variation in sample processing and analytical sensitivity that was not controlled for. Consequently, the data should be interpreted with caution, as observed differences may partially reflect procedural discrepancies rather than true biological or methodological effects. Being aware of such variation, however, is important when interpreting eDNA screening results from various countries and laboratories or when reporting to the stakeholders.

5 | Conclusions

To further investigate how methodological differences can affect detection accuracy in upcoming eDNA studies, interlaboratory comparisons are vital. We recommend the implementation of blind ring tests using known concentrations of target DNA to objectively assess assay performance. Harmonization of primer-probe sets across laboratories is essential to ensure comparability of results, alongside the consistent integration of internal positive controls throughout the entire eDNA workflow (from field sampling to PCR amplification) to monitor for inhibition and procedural errors. Standardization of protocols should be prioritized over individual laboratory preferences to enhance reproducibility, comparability of results, and overall reliability across studies. Additionally, comprehensive training in assay execution is crucial to reduce variability introduced by human error. Finally, each PCR system must be validated in the context of the specific reagents, equipment, and protocols used by individual laboratories to ensure optimal compatibility and performance.

Author Contributions

A.A., A.V., K.A., L.E., P.B. conceived and designed the study. A.K., A.P., A.V., A.Z., B.G., C.D., D.A.S., F.E., F.G., I.M., J.D.-U., J.-Y.G., K.A., K.K., K.T., L.M., L.M.-T., L.O., L.P., M.A., M.B., M.M., M.M.-R., P.B., P.K., S.R.R.P., T.B., T.I.-T., T.P.M., T.J.R. collected the data. A.P., B.R., D.A.S., K.A., T.B. conducted the statistical analyses and created the figures. A.A., A.P., B.R., D.A.S., I.M., K.A., K.T., L.L.B., P.B., T.B. drafted the manuscript, with contributions from all coauthors. All authors approved the final submission.

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06.0121.PZ/0304). Team SP. J.D.-U., M.M.-M., and L.M.-T. were supported by a project of the Spanish Ministry of Science and Innovation I+D+I (grant number PID2020-16115GB-100). All other laboratory work was funded by the teams' own institutions, university departments, or private companies.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in First European ring test to detect crayfish eDNA at https://doi.org/10.6084/m9.figshare.29525897.v1.

References

Agersnap, S., W. Larsen, S. Knudsen, et al. 2017. "Monitoring of Noble, Signal and Narrow-Clawed Crayfish Using Environmental DNA From Freshwater Samples." *PLoS One* 12: e0179261. https://doi.org/10.1371/journal.pone.0179261.

Albertson, L. K., and M. D. Daniels. 2018. "Crayfish Ecosystem Engineering Effects on Riverbed Disturbance and Topography Are Mediated by Size and Behavior." *Freshwater Science* 37, no. 4: 836–844. https://doi.org/10.1086/700884.

Alderman, D. J. 1996. "Geographical Spread of Bacterial and Fungal Diseases Ofcrustaceans." *Revue Scientifique et Technique de l'OIE* 15, no. 2: 603–632. https://doi.org/10.20506/rst.15.2.943.

Allan, E. A., W. G. C. Zhang, A. F. Lavery, and A. Govindarajan. 2021. "Environmental DNA Shedding and Decay Rates From Diverse Animal Forms and Thermal Regimes." *Environmental DNA* 3: 492–514. https://doi.org/10.1002/edn3.141.

Aluma, M. O., L. Pukk, M. Hurt, and K. Kaldre. 2023. "Distribution of Non-Indigenous Crayfish Species in Estonia and Their Impacts on Noble Crayfish (*Astacus astacus L.*) Populations." *Diversity* 15, no. 4: 474. https://doi.org/10.3390/d15040474.

Barnes, M. A., C. R. Turner, C. L. Jerde, M. A. Renshaw, W. L. Chadderton, and D. M. Lodge. 2014. "Environmental Conditions Influence eDNA Persistence in Aquatic Systems." *Environmental Science & Technology* 48, no. 3: 1819–1827. https://doi.org/10.1021/es404734p.

Barnes, M. A., and C. R. Turner. 2016. "The Ecology of Environmental DNA and Implications for Conservation Genetics." *Conservation Genetics* 17: 1–17. https://doi.org/10.1007/s10592-015-0775-4.

Bass, D., K. W. Christison, G. D. Stentiford, L. S. J. Cook, and H. Hartikainen. 2023. "Environmental DNA/RNA for Pathogen and Parasite Detection, Surveillance, and Ecology." *Trends in Parasitology* 39, no. 4: 285–304. https://doi.org/10.1016/j.pt.2022.12.010.

Bass, D., G. D. Stentiford, D. T. J. Littlewood, and H. Hartikainen. 2015. "Diverse Applications of Environmental DNA Methods in Parasitology." *Trends in Parasitology* 31, no. 10: 499–513. https://doi.org/10.1016/j.pt.2015.06.013.

Baudry, T., V. Vasselon, C. Delaunay, M. Sweet, and F. Grandjean. 2024. "When Methodological Innovation Changes the Game: A 10-Year Review of Environmental DNA (eDNA) Applied to Crayfish." *Aquatic Conservation: Marine and Freshwater Ecosystems* 34: e4245. https://doi.org/10.1002/aqc.4245.

Bohman, P. 2025a. *First European Ring Test to Detect Crayfish eDNA*. Figshare Dataset. https://doi.org/10.6084/m9.figshare.29525897.v1.

Bohman, P. 2025b. *Swedish Crayfish Database*. Swedish University of Agricultural Sciences (SLU), Department of Aquatic Resources. http://www.slu.se/kraftdatabasen.

Bohman, P., L. Edsman, and F. Nordwall. 2006. "The Effect of the Large-Scale Introduction of Signal Crayfish on the Spread of Crayfish Plague in Sweden." *Bulletin Français de la Pêche et de la Pisciculture* 380–381: 1291–1302. https://doi.org/10.1051/kmae:2006026.

Bohman, P., L. Edsman, A. Sandström, A. Asp, F. Engdahl, and J. och Dahlberg. 2014. *Report in Swedish: Kompletterande uppgifter till uppföljningsrapport för projektet 'Utveckling av förvaltningen av signalkräfta', Fas 3*, 74. SJVs Dnr. 18-11740/11, inom ramen för Europeiska fiskerifonden.

Burian, A., Q. Mauvisseau, M. Bulling, S. Domisch, S. Qian, and M. Sweet. 2021. "Improving the Reliability of eDNA Data Interpretation." *Molecular Ecology Resources* 21, no. 5: 1422–1433. https://doi.org/10.1111/1755-0998.13367.

Casabella-Herrero, G., M. Higuera-Gamindez, V. A. Azcona, L. Martín-Torrijos, and J. Diéguez-Uribeondo. 2023. "Austropotamobius Pallipes can be Infected by Two Haplotypes of Aphanomyces Astaci: A Key Example from an Outbreak at an Ex-Situ Conservation Facility." *Journal of Invertebrate Pathology* 201: 107989. https://doi.org/10.1016/j.jip.2023.107989.

CEN. 2023. "Water Quality—Sampling, Capture and Preservation of Environmental DNA From Water, Cen En 17805: 2023." https://www.iso.org/standard/87980.html.

Chucholl, F., and C. Chucholl. 2021. "Differences in the Functional Responses of Four Invasive and One Native Crayfish Species Suggest Invader-Specific Ecological Impacts." *Freshwater Biology* 66, no. 11: 2051–2063. https://doi.org/10.1111/fwb.13813.

Chucholl, F., F. Fiolka, G. Segelbacher, and L. S. Epp. 2021. "eDNA Detection of Native and Invasive Crayfish Species Allows for Year-Round Monitoring and Large-Scale Screening of Lotic Systems." *Frontiers in Environmental Science* 9: 639380. https://doi.org/10.3389/fenvs.2021.639380.

Creed, R. P., Jr., and J. M. Reed. 2004. "Ecosystem Engineering by Crayfish in a Headwater Stream Community." *Journal of the North*

26374943, 2026, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70238 by Fachhochschule Nordwestschweiz, Wiley Online Library on [16/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

American Benthological Society 23, no. 2: 224–236. https://doi.org/10.1899/0887-3593(2004)023<>2.0.CO;2.

Darling, J. A., and A. R. Mahon. 2011. "From Molecules to Management: Adopting DNA-Based Methods for Monitoring Biological Invasions in Aquatic Environments." *Environmental Research* 111, no. 7: 978–988. https://doi.org/10.1016/j.envres.2011.02.001.

Dejean, T., A. Valentini, C. Miquel, P. Taberlet, E. Bellemain, and C. Miaud. 2012. "Improved Detection of an Alien Invasive Species Through Environmental DNA Barcoding: The Example of the American Bullfrog *Lithobates catesbeianus*." *Journal of Applied Ecology* 49, no. 4: 953–959. https://doi.org/10.1111/j.1365-2664.2012.02171.x.

Diéguez-Uribeondo, J., and K. Söderhäll. 1993. "*Procambarus clarkii* Girard as a Vector for the Crayfish Plague Fungus, *Aphanomyces astaci* Schikora." *Aquaculture Research* 24: 761–765. https://doi.org/10.1111/j.1365-2109.1993.tb00655.x.

Dorn, N. J., and J. M. Wojdak. 2004. "The Role of Omnivorous Crayfish in Littoral Communities." *Oecologia* 140, no. 1: 150–159. https://doi.org/10.1007/s00442-004-1548-9.

Dougherty, M. M., E. R. Larson, M. A. Renshaw, et al. 2016. "Environmental DNA (eDNA) Detects the Invasive Rusty Crayfish *Orconectes rusticus* at Low Abundances." *Journal of Applied Ecology* 53, no. 3: 722–732. https://doi.org/10.1111/1365-2664.12621.

Dragičević, P., M. Faller, P. Kutleša, and S. Hudina. 2020. "Update on the Signal Crayfish, *Pacifastacus leniusculus* (Dana, 1852) Range Expansion in Croatia: A 10-Year Report." *BioInvasions Records* 9, no. 4: 793–807. https://doi.org/10.3391/bir.2020.9.4.13.

Edsman, L., L. Füreder, F. Gherardi, and C. Souty-Grosset. 2010. *Astacus astacus*, e.T2191A9338388. IUCN Red List of Threatened Species 2010. https://doi.org/10.2305/IUCN.UK.2010-3.RLTS.T2191A9338388.en.

Edsman, L., and P. Smietana. 2004. "Roundtable—Session 2 Exploitation, Conservation and Legislation." *Bulletin Francais de la Peche et de la Pisciculture* 372: 457–464. https://doi.org/10.1051/kmae:2004019.

Eichmiller, J. J., S. E. Best, and P. W. Sorensen. 2016. "Effects of Temperature and Trophic State on Degradation of Environmental DNA in Lake Water." *Environmental Science & Technology* 50, no. 4: 1859–1867. https://doi.org/10.1021/acs.est.5b05672.

Ercoli, F., T. J. Ruokonen, H. Hämäläinen, et al. 2015. "Does the Introduced Signal Crayfish Occupy an Equivalent Trophic Niche to the Lost Native Noble Crayfish in Boreal Lakes?" *Biological Invasions* 16: 2025–2036. https://doi.org/10.1007/s10530-014-0645-x.

European Union. 2014. "Regulation no 1143/2014." https://eur-lex.europa.eu/eli/reg/2014/1143/oj/eng.

Ficetola, G. F., C. Miaud, F. Pompanon, and P. Taberlet. 2008. "Species Detection Using Environmental DNA From Water Samples." *Biology Letters* 4, no. 4: 423–425. https://doi.org/10.1098/rsbl.2008.0118.

Filipová, L., A. Petrusek, K. Matasová, C. Delaunay, and F. Grandjean. 2013. "Prevalence of the Crayfish Plague Pathogen *Aphanomyces astaci* in Populations of the Signal Crayfish *Pacifastacus leniusculus* in France: Evaluating the Threat to Native Crayfish." *PLoS One* 8, no. 7: e70157. https://doi.org/10.1371/journal.pone.0070157.

Francesconi, C., J. Makkonen, A. Schrimpf, J. Jussila, H. Kokko, and K. Theissinger. 2021. "Controlled Infection Experiment With *Aphanomyces astaci* Provides Additional Evidence for Latent Infections and Resistance in Freshwater Crayfish." *Frontiers in Ecology and Evolution* 9: 647037. https://doi.org/10.3389/fevo.2021.647037.

Gherardi, F. 2007. "Biological Invasions in Inland Waters: An Overview." In *Biological Invaders in Inland Waters: Profiles, Distribution, and Threats*, edited by F. Gherardi, vol. 2. Springer. Invading Nature—Springer Series in Invasion Ecology. https://doi.org/10.1007/978-1-4020-6029-8_1.

Gherardi, F. 2011. "Towards a Sustainable Human Use of Freshwater Crayfish (Crustacea, Decapoda, Astacidea)." *Knowledge and Management of Aquatic Ecosystems* 401: 22. https://doi.org/10.1051/kmae/2011038.

Goldberg, C. S., C. R. Turner, K. Deiner, et al. 2016. "Critical Considerations for the Application of Environmental DNA Methods to Detect Aquatic Species." *Methods in Ecology and Evolution* 7, no. 11: 1299–1307. https://doi.org/10.1111/2041-210X.12595.

Gouin, N., F. Grandjean, D. Bouchon, J. D. Reynolds, and C. Souty-Grosset. 2001. "Population Genetic Structure of the Endangered Freshwater Crayfish *Austropotamobius pallipes*, Assessed Using RAPD Markers." *Heredity* 87, no. 1: 80–87. https://doi.org/10.1046/j.1365-2540.2001.00909.x.

Gren, I. M., M. Campos, L. Edsman, and P. Bohman. 2009. "Incomes, Attitudes, and Occurrences of Invasive Species: An Application to Signal Crayfish in Sweden." *Environmental Management* 43: 210–220. https://doi.org/10.1007/s00267-008-9210-7.

Haile, J., D. G. Froese, R. D. E. MacPhee, et al. 2009. "Ancient DNA Reveals Late Survival of Mammoth and Horse in Interior Alaska." *Proceedings of the National Academy of Sciences* 106, no. 52: 22352–22357. https://doi.org/10.1073/pnas.0912510106.

Holdich, D. M. 1993. "A Review of Astaciculture: Freshwater Crayfish Farming." *Aquatic Living Resources* 6, no. 4: 307–317. https://doi.org/10.1051/alr:1993032.

Holdich, D. M., J. D. Reynolds, C. Souty-Grosset, and P. J. Sibley. 2009. "A Review of the Ever Increasing Threat to European Crayfish From Non-Indigenous Crayfish Species." *Knowledge and Management of Aquatic Ecosystems* 394–395: 11. https://doi.org/10.1051/kmae/2009025.

James, J., S. Nutbeam-Tuffs, J. Cable, et al. 2017. "The Prevalence of *Aphanomyces astaci* in Invasive Signal Crayfish From the UK and Implications for Native Crayfish Conservation." *Parasitology* 144, no. 4: 411–418. https://doi.org/10.1017/S0031182016002419.

Jerde, C. L., A. R. Mahon, W. L. Chadderton, and D. M. Lodge. 2011. "'Sight-Unseen' Detection of Rare Aquatic Species Using Environmental DNA." *Conservation Letters* 4, no. 2: 150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x.

Johnsen, S. I., D. A. Strand, J. C. Rusch, and T. Vrålstad. 2020. "Environmental DNA (eDNA) Monitoring of Noble Crayfish *Astacus astacus* in Lentic Environments Offers Reliable Presence-Absence Surveillance—But Fails to Predict Population Density." *Frontiers in Environmental Science* 8: 612253. https://doi.org/10.3389/fenvs.2020.612253.

Jones, J. I., A. Arnold, D. Buchner, et al. 2025. "Sources of Uncertainty in DNA Metabarcoding of Whole Communities: Implications for Its Use in Biomonitoring." *Methods in Ecology and Evolution* 16: 1658–1673. https://doi.org/10.1111/2041-210X.70093.

Jussila, J., L. Edsman, I. Maguire, J. Diéguez-Uribeondo, and K. Theissinger. 2021. "Money Kills Native Ecosystems: European Crayfish as an Example." *Frontiers in Ecology and Evolution* 9: 648495. https://doi.org/10.3389/fevo.2021.648495.

King, A. C., R. Krieg, A. Weston, and A. K. Zenker. 2022. "Using eDNA to Simultaneously Detect the Distribution of Native and Invasive Crayfish Within an Entire Country." *Journal of Environmental Management* 302: 113929. https://doi.org/10.1016/j.jenvman.2021.113929.

Klobučar, G. I. V., M. Podnar, M. Jelić, et al. 2013. "Role of the Dinaric Karst (Western Balkans) in Shaping the Phylogeographic Structure of the Threatened Crayfish *Austropotamobius torrentium*." *Freshwater Biology* 58, no. 6: 1089–1105. https://doi.org/10.1111/fwb.12110.

Klymus, K. E., C. M. Merkes, M. J. Allison, et al. 2020. "Reporting the Limits of Detection and Quantification for Environmental DNA Assays." *Environmental DNA* 2, no. 3: 271–282. https://doi.org/10.1002/edn3.29.

26374943, 2026, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70238 by Fachhochschule Nordwestschweiz, Wiley Online Library on [16/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Knudsen, S. W., S. Agersnap, P. R. Møller, and J. H. Andersen. 2019. *Development of Species-Specific eDNA-Based Test Systems for Monitoring of Freshwater Crayfish*. Norwegian Institute for Water Research. https://niva.brage.unit.no/niva-xmlui/handle/11250/2633765.

Kouba, A., A. Petrusek, and P. Kozák. 2014. "Continental-Wide Distribution of Crayfish Species in Europe: Update and Maps." *Knowledge and Management of Aquatic Ecosystems* 413: 31. https://doi.org/10.1051/kmae/2014007.

Kozák, P., and I. Kuklina. 2015. "Crayfish as Tools of Water Quality Monitoring." In *Freshwater Crayfish, A Global Overview*, edited by T. Kawai, Z. Faulkes, and G. Scholtz, 275–297. CRC Press. https://doi.org/10.1201/b18723-15.

Laffitte, M., T. Baudry, M. Guilmet, et al. 2023. "A New Invader in Freshwater Ecosystems in France: The Rusty Crayfish *Faxonius rusticus*." *BioInvasions Records* 12: 457–468. https://doi.org/10.3391/bir.2023.12.2.10.

Larson, E. R., M. A. Renshaw, C. A. Gantz, et al. 2017. "Environmental DNA (eDNA) Detects the Invasive Crayfishes *Orconectes Rusticus* and *Pacifastacus leniusculus* in Large Lakes of North America." *Hydrobiologia* 800, no. 1: 173–185. https://doi.org/10.1007/s10750-017-3210-7.

Lodge, D. M., C. A. Taylor, D. M. Holdich, and J. Skurdal. 2000. "Nonindigenous Crayfishes Threaten North American Freshwater Biodiversity: Lessons From Europe." *Fisheries* 25: 7–20. https://doi.org/10.1577/1548-8446(2000)025<0007:NCTNAF>2.0.CO;2.

Lovrenčić, L., L. Bonassin, L. L. Boštjančić, et al. 2020. "New Insights Into the Genetic Diversity of the Stone Crayfish: Taxonomic and Conservation Implications." *BMC Evolutionary Biology* 20: 146. https://doi.org/10.1186/s12862-020-01709-1.

Lowe, S. M., M. Browne, S. Boudjelas, and M. De Poorter. 2000. *100 of the World's Worst Invasive Alien Species: A Selection From the Global Invasive Species Database*, 12. Invasive Species Specialist Group (ISSG) a Specialist Group of the Species Survival Commission (SSC) of the World Conservation Union (IUCN). First Published as Special Lift-out in Aliens.

Majaneva, M., O. H. Diserud, S. H. C. Eagle, E. Boström, M. Hajibabaei, and T. Ekrem. 2018. "Environmental DNA Filtration Techniques Affect Recovered Biodiversity." *Scientific Reports* 8, no. 1: 4682. https://doi.org/10.1038/s41598-018-23052-8.

Martínez-Ríos, M., L. Martín-Torrijos, and J. Diéguez-Uribeondo. 2023. "Protocols for Studying the Crayfish Plague Pathogen, *Aphanomyces Astaci*, and Its Host-Pathogen Interactions." *Journal of Invertebrate Pathology* 201: 108018. https://doi.org/10.1016/j.jip.2023.108018.

Mauvisseau, Q., S. Tönges, R. Andriantsoa, F. Lyko, and M. Sweet. 2019. "Early Detection of an Emerging Invasive Species: eDNA Monitoring of a Parthenogenetic Crayfish in Freshwater Systems." *Management of Biological Invasions* 10: 461–472. https://doi.org/10.3391/mbi.2019.10.3.04.

Mojžišová, M., J. Svobodová, E. Kozubíková-Balcarová, et al. 2022. "Long-Term Changes in the Prevalence of the Crayfish Plague Pathogen and Its Genotyping in Invasive Crayfish Species in Czechia." *Neobiota* 74: 105–127. https://doi.org/10.3897/neobiota.74.79087.

Mrugała, A., E. Kozubíková-Balcarová, C. Chucholl, et al. 2014. "Trade of Ornamental Crayfish in Europe as a Possible Introduction Pathway for Important Crustacean Diseases: Crayfish Plague and White Spot Syndrome." *Biological Invasions* 17, no. 5: 1313–1326. https://doi.org/10.1007/s10530-014-0795-x.

Panteleit, J., N. Keller, H. Kokko, et al. 2017. "Investigation of Ornamental Crayfish Reveals New Carrier Species of the Crayfish Plague Pathogen (Aphanomyces Astaci)." *Aquatic Invasions* 12, no. 1: 77–83. https://doi.org/10.3391/ai.2017.12.1.08.

Parkyn, S. 2015. "A Review of Current Techniques for Sampling Freshwater Crayfish." In *Freshwater Crayfish, A Global Overview*, edited by T. Kawai, Z. Faulkes, and G. Scholtz, 205–220. CRC Press. https://doi.org/10.1201/b18723.

Parsley, M. B., J. L. Brunner, E. J. Crespi, T. A. G. Rittenhouse, and C. S. Goldberg. 2025. "Sample-To-Sample Variability Impacts eDNA Quantification, With Implications for Estimating Species Abundance." *Environmental DNA* 7, no. 5: e70195. https://doi.org/10.1002/edn3.70195.

Parsley, M. B., E. J. Crespi, T. A. G. Rittenhouse, J. L. Brunner, and C. S. Goldberg. 2024. "Environmental DNA Concentrations Vary Greatly Across Productive and Degradative Conditions, With Implications for the Precision of Population Estimates." *Scientific Reports* 14, no. 1: 17392. https://doi.org/10.1038/s41598-024-66732-4.

Pârvulescu, L., J. L. Pérez-Moreno, C. Panaiotu, et al. 2019. "A Journey on Plate Tectonics Sheds Light on European Crayfish Phylogeography." *Ecology and Evolution* 9, no. 4: 1957–1971. https://doi.org/10.1002/ece3.4888.

Persson, M., and K. Söderhäll. 1983. "*Pacifastacus leniusculus* (Dana) and Its Resistance to the Parasitic Fungus *Aphanomyces astaci* Schikora." *Freshwater Crayfish* 5, no. 1: 292–298. https://doi.org/10.5869/fc.1983.v5.292.

Reynold, J. S., and C. Souty-Grosset. 2012. *Management of Freshwater Biodiversity: Crayfish as Bioindicators*. Cambridge University Press. https://doi.org/10.1017/CBO9781139031790.

Reynolds, J., C. Souty-Grosset, and A. Richardson. 2013. "Ecological Roles of Crayfish in Freshwater and Terrestrial Habitats." *Freshwater Crayfish* 19: 197–218. https://doi.org/10.5869/fc.2013.v19-2.197.

Robinson, C. V., C. Garcia de Leaniz, J. James, J. Cable, P. Orozco-terWengel, and S. Consuegra. 2018. "Genetic Diversity and Parasite Facilitated Establishment of the Invasive Signal Crayfish (*Pacifastacus leniusculus*) in Great Britain." *Ecology and Evolution* 8, no. 18: 9181–9191. https://doi.org/10.1002/ece3.4235.

Rodriguez, L. K., L. D. Bonis, J. McKee, et al. 2025. "Inter-Laboratory Ring Test for Environmental DNA Extraction Protocols: Implications for Marine Megafauna Detection Using Three Novel qPCR Assays." *Metabarcoding and Metagenomics* 9: e128235. https://doi.org/10.3897/mbmg.9.128235.

Rounds, C. I., T. W. Arnold, C. L. Chun, et al. 2024. "Aquatic Invasive Species Exhibit Contrasting Seasonal Detectability Patterns Based on Environmental DNA: Implications for Monitoring." *Freshwater Biology* 69: 1479–1493. https://doi.org/10.1111/fwb.14320.

Rusch, J. C., M. Mojžišová, D. A. Strand, J. Svobodová, T. Vrålstad, and A. Petrusek. 2020. "Simultaneous Detection of Native and Invasive Crayfish and *Aphanomyces astaci* From Environmental DNA Samples in a Wide Range of Habitats in Central Europe." *NeoBiota* 58: 1–32. https://doi.org/10.3897/neobiota.58.49358.

Schenekar, T. 2023. "The Current State of eDNA Research in Freshwater Ecosystems: Are We Shifting From the Developmental Phase to Standard Application in Biomonitoring?" *Hydrobiologia* 850, no. 6: 1263–1282. https://doi.org/10.1007/s10750-022-04891-z.

Schrader, C., A. Schielke, L. Ellerbroek, and R. Johne. 2012. "PCR Inhibitors—Occurrence, Properties and Removal." *Journal of Applied Microbiology* 113, no. 5: 1014–1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x.

Sieber, N., A. King, R. Krieg, A. Zenker, C. Vorburger, and H. Hartikainen. 2024. "Large-Scale eDNA Monitoring of Multiple Aquatic Pathogens as a Tool to Provide Risk Maps for Wildlife Diseases." *Environmental DNA* 6, no. 1: e427. https://doi.org/10.1002/edn3.427.

Söderhäll, K., and L. Cerenius. 1999. "The Crayfish Plague Fungus: History and Recent Advances." *Freshwater Crayfish* 12, no. 1: 11–35.

Souty-Grosset, C., D. M. Holdich, P. Y. Noël, J. D. Reynolds, and P. Haffner. 2006. *Atlas of Crayfish in Europ*. Vol. 64, 187. Scientific

26374943, 2026, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70238 by Fachhochschule Nordwestschweiz, Wiley Online Library on [16/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Publications of the Muséum National D'histoire Naturelle, Paris. Collection Patrimoines Naturels.

Strand, D. A., A. Holst-Jensen, H. Viljugrein, et al. 2011. "Detection and Quantification of the Crayfish Plague Agent in Natural Waters: Direct Monitoring Approach for Aquatic Environments." *Diseases of Aquatic Organisms* 95, no. 1: 9–17. https://doi.org/10.3354/dao02334.

Strand, D. A., T. Jinnerot, A. Aspán, et al. 2023. "Molecular Detection of *Aphanomyces astaci* – An Improved Species Specific qPCR Assay." *Journal of Invertebrate Pathology* 201: 108008. https://doi.org/10.1016/j.jip.2023.108008.

Strand, D. A., S. I. Johnsen, J. C. Rusch, et al. 2019. "Monitoring a Norwegian Freshwater Crayfish Tragedy: eDNA Snapshots of Invasion, Infection and Extinction." *Journal of Applied Ecology* 56, no. 7: 1661–1673. https://doi.org/10.1111/1365-2664.13404.

Strand, D. A., J. Jussila, S. I. Johnsen, et al. 2014. "Detection of Crayfish Plague Spores in Large Freshwater Systems." *Journal of Applied Ecology* 51, no. 2: 544–553. https://doi.org/10.1111/1365-2664.12218.

Svoboda, J., A. Mrugała, E. Kozubíková-Balcarová, and A. Petrusek. 2017. "Hosts and Transmission of the Crayfish Plague Pathogen *Aphanomyces astaci*: A Review." *Journal of Fish Diseases* 40, no. 1: 127–140. https://doi.org/10.1111/jfd.12472.

Swahn, J.-O. 2004. "The Cultural History of Crayfish." *Bulletin Français de la Pêche et de la Pisciculture* 372–373: 243–251.

Takahashi, M., M. Saccò, J. H. Kestel, et al. 2023. "Aquatic Environmental DNA: A Review of the Macro-Organismal Biomonitoring Revolution." *Science of the Total Environment* 873: 162322. https://doi.org/10.1016/j.scitotenv.2023.162322.

Thomsen, P. F., J. Kielgast, L. L. Iversen, et al. 2012. "Monitoring Endangered Freshwater Biodiversity Using Environmental DNA." *Molecular Ecology* 21, no. 11: 2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x.

Tréguier, A., J.-M. Paillisson, T. Dejean, A. Valentini, M. Schlaepfer, and J.-M. Roussel. 2014. "Environmental DNA Surveillance for Invertebrate Species: Advantages and Technical Limitations to Detect Invasive Crayfish *Procambarus clarkii* in Freshwater Ponds." *Journal of Applied Ecology* 51: 871–879. https://doi.org/10.1111/1365-2664.12262.

Ungureanu, E., M. Mojžišová, M. Tangerman, M. C. Ion, L. Pârvulescu, and A. Petrusek. 2020. "The Spatial Distribution of *Aphanomyces astaci* Genotypes Across Europe: Introducing the First Data From Ukraine." *Freshwater Crayfish* 25: 77–87. https://doi.org/10.5869/fc.2020.v25-1.077.

van Bochove, K., F. T. Bakker, K. K. Beentjes, L. Hemerik, R. A. Vos, and B. Gravendeel. 2020. "Organic Matter Reduces the Amount of Detectable Environmental DNA in Freshwater." *Ecology and Evolution* 10, no. 8: 3647–3654. https://doi.org/10.1002/ece3.6123.

Vrålstad, T., S. I. Johnsen, R. F. Fristad, L. Edsman, and D. Strand. 2011. "Potent Infection Reservoir of Crayfish Plague Now Permanently Established in Norway." *Diseases of Aquatic Organisms* 97, no. 1: 75–83. https://doi.org/10.3354/dao02386.

Vrålstad, T., A. K. Knutsen, T. Tengs, and A. Holst-Jensen. 2009. "A Quantitative TaqMan® MGB Real-Time Polymerase Chain Reaction Based Assay for Detection of the Causative Agent of Crayfish Plague Aphanomyces Astaci." *Veterinary Microbiology* 137, no. 1–2: 146–155. https://doi.org/10.1016/j.vetmic.2008.12.022.

Weiperth, A., M. Bláha, B. Szajbert, et al. 2020. "Hungary: A European Hotspot of Non-Native Crayfish Biodiversity." *Knowledge and Management of Aquatic Ecosystems* 421: 43. https://doi.org/10.1051/kmae/2020035.

Wilcox, T. M., K. S. McKelvey, M. K. Young, et al. 2013. "Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity." *PLoS One* 8, no. 3: e59520. https://doi.org/10.1371/journal.pone.0059520.

Wilcox, T. M., K. S. McKelvey, M. K. Young, et al. 2016. "Understanding Environmental DNA Detection Probabilities: A Case Study Using a Stream-Dwelling Char *Salvelinus fontinalis*." *Biological Conservation* 194: 209–216. https://doi.org/10.1016/j.biocon.2015.12.023.

Wittwer, C., C. Nowak, D. Strand, T. Vrålstad, M. Thines, and S. Stoll. 2018. "Comparison of Two Water Sampling Approaches for eDNA-Based Crayfish Plague Detection." *Limnologica* 70: 1–9. https://doi.org/10.1016/j.limno.2018.03.001.

Wittwer, C., S. Stoll, M. Thines, and C. Nowak. 2019. "eDNA-Based Crayfish Plague Detection as Practical Tool for Biomonitoring and Risk Assessment of *A. astaci*-Positive Crayfish Populations." *Biological Invasions* 21: 1075–1088. https://doi.org/10.1007/s10530-018-1886-x.

Yates, M. C., D. J. Fraser, and A. M. Derry. 2019. "Meta-Analysis Supports Further Refinement of eDNA for Monitoring Aquatic Species-Specific Abundance in Nature." *Environmental DNA* 1: 5–13. https://doi.org/10.1002/edn3.7.

Yu, Y.-B., J.-H. Choi, J.-C. Kang, H. J. Kim, and J.-H. Kim. 2022. "Shrimp Bacterial and Parasitic Disease Listed in the OIE: A Review." *Microbial Pathogenesis* 166: 105545. https://doi.org/10.1016/j.micpath.2022.105545.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Results of sensitivity and Limits of Detection (LOD), reported as Ct-values, across laboratories, when targeting the different species, *Aphanomyces astaci*, *Astacus astacus* and *Pacifastacus leniusculus*. **Table S1:** A list of targeted assays developed for screening for specific species of crayfish and the crayfish plague pathogen (*A. astaci*) in eDNA (as of June 2025). **Table S3:** Compilation of variables used in the eDNA workflow across laboratories, covering the following methodological steps: sampling, DNA collection, filtrate handling, DNA extraction, and PCR. **Table S3:** Individual-level data for the crayfish included in the different experimental tanks and ponds. **Table S4:** Physiochemical meta data from tanks and ponds.

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