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RESEARCH ARTICLE

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Monitoring a Norwegian freshwater crayfish tragedy: eDNA snapshots of invasion, infection and extinction

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Abstract

1. The European noble crayfish *Astacus astacus* is threatened by crayfish plague caused by the oomycete *Aphanomyces astaci*, which is spread by the invasive North American crayfish (e.g. signal crayfish *Pacifastacus leniusculus*). Surveillance of crayfish plague status in Norway has traditionally relied on the monitoring survival of cage-held noble crayfish, a method of ethical concern. Additionally, trapping is used in crayfish population surveillance. Here, we test whether environmental DNA (eDNA) monitoring could provide a suitable alternative to the cage method, and a supplement to trapping.
2. We took advantage of an emerging crayfish plague outbreak in a Norwegian watercourse following illegal introduction of disease-carrying signal crayfish, and initiated simultaneous eDNA monitoring and cage-based surveillance, supplemented with trapping. A total of 304 water samples were filtered from several sampling stations over a 4-year period. eDNA data (species-specific quantitative real-time PCR [qPCR]) for the presence of *A. astaci*, noble and signal crayfish within the water samples were compared to cage mortality and trapping.
3. This is the first study comparing eDNA monitoring and cage surveillance during a natural crayfish plague outbreak. We show that eDNA monitoring corresponds well with the biological status measured in terms of crayfish mortality and trapping results. eDNA analysis also reveals the presence of *A. astaci* in the water up to 2.5 weeks in advance of the cage method. Estimates of *A. astaci* and noble crayfish eDNA concentrations increased markedly during mortality and vanished quickly thereafter. eDNA provides a snapshot of the presence, absence or disappearance of crayfish regardless of season, and constitutes a valuable supplement to the trapping method that relies on season and legislation.
4. *Synthesis and applications*. Simultaneous eDNA monitoring of *Aphanomyces astaci* (crayfish plague) and relevant native and invasive freshwater crayfish species is well-suited for early warning of invasion or infection, risk assessments, habitat evaluation and surveillance regarding pathogen and invasive/native crayfish

status. This non-invasive, animal welfare friendly method excludes the need for cage-held susceptible crayfish in disease monitoring. Furthermore, eDNA monitoring is less likely to spread *A. astaci* than traditional methods. This study resulted in the implementation of eDNA monitoring for Norwegian crayfish plague and crayfish surveillance programmes, and we believe other countries could improve management strategies for freshwater crayfish using a similar approach.

KEYWORDS

crayfish plague, disease surveillance, environmental DNA, host–pathogen, invasive species, noble crayfish, signal crayfish, species-specific detection

1 | INTRODUCTION

Environmental DNA (eDNA) monitoring of aquatic systems is a rapidly advancing research field that promises improvements, not only to aquatic species conservation, but also for early detection of invasive species and harmful pathogens at low densities and at any life stage or season (Bohmann et al., 2014; Kelly et al., 2014; Strand et al., 2014). Water can be screened for the presence of micro- and macroorganisms by either a broad approach such as metabarcoding (Shaw et al., 2016; Valentini et al., 2016), or a targeted approach using species-specific quantitative real-time PCR (qPCR) or droplet digital PCR (ddPCR) (Doi, Takahara, et al., 2015; Doi, Uchii, et al., 2015; Strand et al., 2014; Thomsen & Willerslev, 2015). eDNA studies have been applied for detection of a wide range of aquatic macroorganisms including freshwater crayfish (Agersnap et al., 2017; Dougherty et al., 2016; Tréguier et al., 2014). Molecular detection and quantification of waterborne pathogens in environmental samples has been widely utilised for decades (Ramirez-Castillo et al., 2015).

The oomycete *Aphanomyces astaci* is native to North America and is an obligate parasite on American freshwater crayfish (Söderhäll & Cerenius, 1999). It is the causative agent of crayfish plague in susceptible European freshwater crayfish (Alderman, Polglase, & Frayling, 1987), and is listed among the world's 100 worst invasive species (Lowe, Browne, Buoudjelas, & De Poorter, 2004). *Aphanomyces astaci* infection is a notifiable disease both nationally in Norway (list 3, national disease; Vrålstad et al., 2017) and internationally (OiE, 2017). It causes a rapid decline in European crayfish populations, and is spread and maintained by invasive non-indigenous North American carrier crayfish that have rapidly established themselves in Europe (Holdich, Reynolds, Souty-Grosset, & Sibley, 2009). The pathogen invades the cuticle of all freshwater crayfish, but hyphal growth is inhibited by melanisation in resistant North American crayfish. In susceptible crayfish species, the hyphae grow deeper into tissues and organs, causing rapid death. The oomycete reproduces asexually via clonal flagellated zoospores that locate new crayfish hosts through weak chemotaxis. Zoospores can encyst and re-emerge several times, but both zoospores and cysts have a relatively short life span (2–8 weeks) dependent on water temperature (Söderhäll & Cerenius, 1999).

An *A. astaci* species-specific qPCR method is widely used for crayfish plague diagnostics and carrier status testing (Kozubikova, Vrålstad, Filipova, & Petrusek, 2011; OiE, 2017; Vrålstad, Knutsen, Tengs, & Holst-Jensen, 2009). The same method, which has been thoroughly tested and further developed (Makkonen, Strand, Kokko, Vrålstad, & Jussila, 2013; Strand et al., 2012), is used for eDNA monitoring for the presence of *A. astaci* zoospores and cysts in both small (Strand et al., 2011) and large water bodies (Strand et al., 2014; Wittwer et al., 2018). These studies have established that clinically healthy American crayfish emit a low number of *A. astaci* zoospores to the water regardless of season (Strand et al., 2012, 2014; Wittwer et al., 2018), while moribund infected susceptible crayfish emit huge numbers of infective zoospores (Makkonen et al., 2013).

Lake Øymarksjøen in the Halden watercourse is one of a few lakes in Norway hosting a population of the non-indigenous signal crayfish *Pacifastacus leniusculus*, which were introduced illegally around two decades ago, but not discovered until 2008 (Vrålstad, Johnsen, Fristad, Edsman, & Strand, 2011). The unknown presence of signal crayfish partly ruined long-term attempts to restock the lake with indigenous noble crayfish (*Astacus astacus*), following the first outbreak of crayfish plague in 1989 (Taugbøl, 2004). When the restocked population increased in number, a new large outbreak of crayfish plague occurred in 2005 (Vrålstad et al., 2009). The Norwegian Food Safety Authorities (NFSA) enforced a permanent closure of the Ørje water locks between Lake Øymarksjøen and Lake Rødenessjøen in an attempt to prevent upstream spread of *A. astaci* and signal crayfish (Vrålstad et al., 2011).

The noble crayfish population in Lake Rødenessjøen has been monitored every year since 2009 as a part of the national surveillance programme, using baited traps set at eight stations throughout the lake. During this period, the relative density of noble crayfish increased, and CPUE in 2014 ranged between 0.15 and 1.80 (Johnsen, Strand, & Vrålstad, 2017). In September 2014, both signal crayfish and noble crayfish were caught in the southern part of Lake Rødenessjøen just above the closed water locks. The Norwegian Environmental Agency (NEA) regarded the event as another illegal introduction of signal crayfish, since long-distance migration over land or through the closed locks was highly unlikely (Norwegian Environmental Agency, 2014). The illegally

introduced signal crayfish were confirmed *A. astaci* carriers, indicating the probable onset of a new crayfish plague outbreak in the local noble crayfish population. A crayfish plague surveillance programme commissioned by the NFSA was therefore conducted using live noble crayfish in cages to monitor the spread of the disease. Traditional cage experiments using noble crayfish as 'canaries in a coalmine' had been the sole method utilised for field monitoring of crayfish plague since it is introduction to Norway in the 1970s (Håstein & Unestam, 1972; Vrålstad et al., 2014). Decapod crustaceans are now covered by the Animal Welfare Act in Europe and the Law on Animal Welfare (LOV-2009-06-19-97) in Norway. Thus, the use of live crayfish for monitoring a lethal disease is of strong ethical concern. In addition to fatal infection with crayfish plague, cage-held crayfish are also subject to other causes of mortality such as moulting-associated cannibalism. Furthermore, cage-held crayfish commonly escape due to illegal human interference (Vrålstad et al., 2017). Previous studies have shown that eDNA monitoring of crayfish plague in large water systems is possible (Strand et al., 2014), but a direct comparison with traditional cage surveillance has not yet been performed.

In the present study, we took advantage of an emerging crayfish plague outbreak and compared traditional cage surveillance with eDNA monitoring using species-specific qPCR assays for targeted detection and quantification of *A. astaci* (Strand et al., 2014), noble crayfish and signal crayfish (Agersnap et al., 2017), from the same water samples. In addition, we used trapping data from 2014 and 2015 to compare and verify crayfish presence. We show that eDNA monitoring can reveal the presence of *A. astaci* in the water earlier than cages with live crayfish, and that the simultaneous monitoring of noble- and signal crayfish eDNA provides additional information on habitat status that otherwise must be obtained from separate CPUE surveys. Consequently, we propose that eDNA monitoring of the three species will prove a suitable, non-invasive and animal welfare friendly alternative to the traditional cage method.

2 | MATERIALS AND METHODS

2.1 | Study site

The study site (Figure 1) is part of the large Halden watercourse, which is 149.5 km long and consists of several lakes and connecting rivers and channels. The watershed covers 1,584 km^2 and consists of forests and farmland. The River Hølandselva flows into Lake Skulerudsjøen (surface area 1.7 km^2, retention time 0.05 year) which connects and flows into Lake Rødenessjøen (surface area 15.3 km^2, retention time 0.7 year). Ørje locks are located at the outlet and southern end of Lake Rødenessjøen (Figure 1). After the discovery of *A. astaci*-positive signal crayfish and infected noble crayfish close to Ørje locks (c.f. Table 2), the NFSA extended the crayfish plague control zone border in the Halden watercourse upstream of Ørje locks. The physical migration barriers (dams) in River Hølandselva (Figure 1) define the new boarder of the control zone. In the present study, the control zone of the watercourse is referred to as the 'infection zone' while the 'risk zone' refers to the remaining part of the watercourse as well as lakes and rivers with noble crayfish populations in close proximity to the infection zone (Figure 1). Several stations for cage surveillance and eDNA monitoring were established and monitored during subsequent years (2014–2017), covering the ongoing outbreak within the infection zone, and also monitoring selected sites of the risk zone (Figure 1). Trapping surveys were performed in Lake Rødenessjøen in 2014 and 2015, and catch per unit effort (CPUE; crayfish per trap night) data for signal- and noble crayfish were obtained. Figure 2 summarises the time line and frequency of the different monitoring methods.

2.2 | Traditional cage surveillance of crayfish plague

Four cage stations (1–4) were established on 1 October 2014 from upstream of Ørje locks in the south to Kroksund in the north of Lake Rødenessjøen. Each cage (one cage per station) containing 10 live noble crayfish was submerged a few metres from the lake- or river shore. The cage stations were located at sites with known crayfish presence and were readily accessible for frequent monitoring. Two additional cage stations (5 and 6) were established further upstream in the watercourse on 24 April 2015 (Figure 1). Crayfish were obtained from a local noble crayfish farmer. The captive crayfish were provided with shelter and were fed regularly with birch leaves and fish. Each cage was visually inspected twice weekly by local landowners who manually counted remaining live noble crayfish. Mortality in the cages was recorded and dead crayfish collected, frozen at −20°C and transported to the laboratory for crayfish plague diagnostics. Frozen crayfish were thawed, and tissue samples of eye, tail muscle and cuticle were subjected to DNA extraction using the QIAamp® DNA mini kit on a QIAcube automated DNA extractor (Qiagen) following the manufacturers protocol. Crayfish plague diagnostics were performed using an *A. astaci*-specific qPCR (Vrålstad et al., 2009), with modifications in the annealing temperature (Kozubikova et al., 2011). If crayfish plague was confirmed, the corresponding cage was removed from the watercourse. Cage surveillance lasted from September 2014 to October 2015.

2.3 | eDNA water sampling

Six stations for water filtration (eDNA stations) were established in conjunction with the cage monitoring (Figures 1 and 2) in 2014–2015. At each station, three replicate water samples were filtered on-site, with the exception of station 1 in 2014 (the signal crayfish invasion site) where extra water samples (3 × 3) were filtered from three sites in close proximity. Water samples were collected at 7- to 10-day intervals in October to November 2014 (Figure 2) to closely follow the initial phase of the outbreak. In total, 72 water samples were collected at stations 1–3 with an average of 6.9 L/filter. No eDNA samples were collected during winter due to ice coverage. In 2015, water samples were collected every second or fourth week from April to September (Figure 2) to follow upstream movement of the outbreak. In total, 120 water samples were collected at five stations

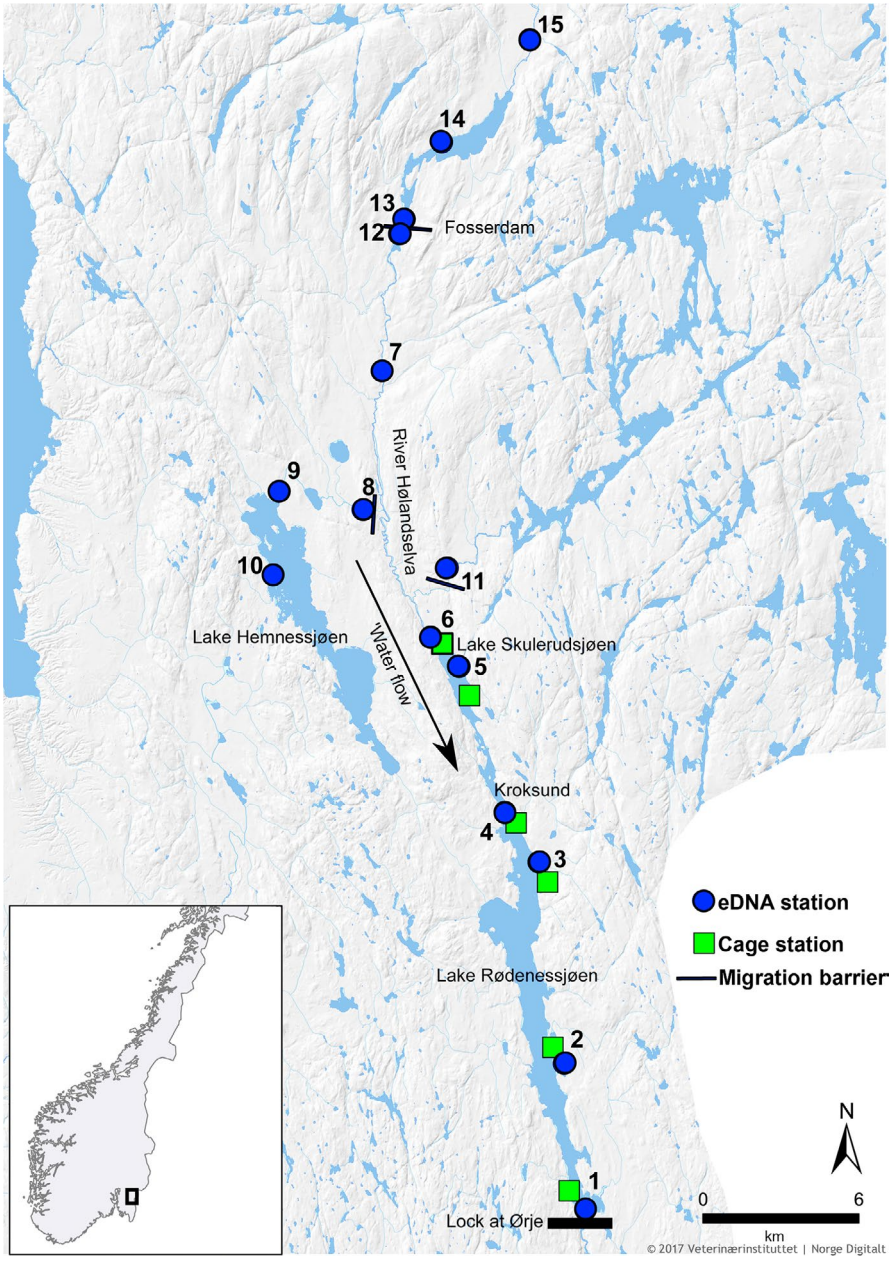

FIGURE 1 The study site includes parts of the large Halden watercourse in Norway with names for involved lakes, channels and rivers. Cage stations (green squares) and environmental DNA (eDNA) stations (blue circles) were established successively from 2014 to 2016 in a south–north direction, starting at the signal crayfish invasion site at Ørje locks (bold black line; station 1). Cage stations 1–6 and eDNA stations 1–7 and 12 are within the regulated infection zone, while the eDNA stations 8–11 and 13–15 are located in the risk zone, separated from the infection zone by migration barriers (bold black lines) such as dams and waterfalls

(stations 1, 3, 4, 5 and 6) from April to September with an average of 6.0 L/filter. As increasing focus was placed on upstream movement, station 2 was excluded after 2014. Additional stations upstream were established and sampled in June and August of 2016 and 2017 as part of a new crayfish plague monitoring programme (Figure 1 and 2). For cost-efficiency reasons, only two replicate water samples were collected per station. In total, 55 and 57 water samples were collected with an average of 3.3 and 4.0 L/filter in 2016 and 2017 respectively. Generally, for all stations, the water samples were taken upstream and at some distance (>20 m in the river and >200 m in the lake) to the nearest caged noble crayfish to avoid detection of eDNA from those crayfish. Between 1 and 10 L were filtered per sample depending on the turbidity of the water. The water samples were collected above the bed (~7 cm), 2–5 m from the shore, and filtered directly onto glass fibre filters (47 mm, 2 μm pore size, AP2504700 Millipore, Billerica, MA, USA) using a peristaltic pump (Masterflex L/S or E/S, Cole-Parmer, Vermon Hills, IL, USA) with Tygon tubing (Cole-Parmer) and an in-line filter holder (47 mm, Millipore). Each filter was transferred to a 15-ml sterile falcon tube, stored on ice in a cooling box until transported to the laboratory within 12 hr, and frozen at −20°C. The volume of the filtered water was measured and discarded on the shore at each site. Water samples were always collected in an upstream to downstream direction to avoid transferring *A. astaci* spores upstream. Also, stations outside the infection zone (risk zone) were always sampled before stations within the infection zone (Figure 1). Before filtration at each station, water was pumped through the hose and filter holder for a few minutes to rinse away remains of spores or eDNA from the previous upstream station, and

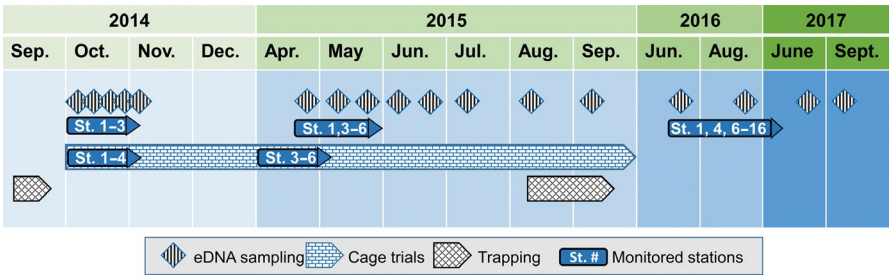

FIGURE 2 Timeline of the sampling methods and sampling frequency/effort. Involved stations (environmental DNA [eDNA] and cage) are indicated for different periods. eDNA was sampled at 10-day intervals in 2014 and at 2- to 4-week intervals in 2015. Cages with live noble crayfish were checked twice a week by local landowners. Trapping was conducted at eight locations in Lake Rødenessjøen in 2014 as part of the national surveillance of *Astacus astacus* and in 2015 extended trapping was conducted throughout the entire lake

to avoid filtering any disturbed sediments from the current station. After sampling of all stations within a zone (risk zone or infection zone), the tubing and filter holder were disinfected with 10% bleach for 30 min, followed by rinsing with 10% sodium thiosulfate, to remove DNA traces.

2.4 | Crayfish trapping—Catch per unit effort

Two extended surveys with baited traps were conducted in 2015 with the same methods as in the national surveillance programme of noble crayfish (Johnsen et al., 2017), using conventional two-funnel traps (mesh size 12 mm) baited with raw chicken (Figure 2). The first survey in August, comprised of 1,880 trap nights where traps were distributed at different sites (approximately 10 traps per site) covering most of the shoreline of Lake Rødenessjøen. The second, including 960 trap nights in August and September, covered the suspected signal crayfish invasion area. All equipment was disinfected after each sampling event. Permissions for trapping *A. astaci*-carrying signal crayfish were obtained from NEA and NFSA.

2.5 | eDNA analyses

DNA was extracted from filters using the CTAB (cetyltrimethylammonium bromide) extraction protocol described by Strand et al. (2014) with minor modifications (full protocol in Appendix S1). Briefly, the filters were freeze-dried, 4 ml of CTAB buffer was added and the filters were then fragmented using a pestle. The samples were frozen (−80°C) and thawed (65°), followed by addition of proteinase K and incubated at 65°C for 60 min. Chloroform was added, the sample was centrifuged and the supernatant (3 ml lysate) from each sample was divided into two 2-ml Eppendorf tubes for easier workflow resulting in two subsamples per filter (A & B; technical replicates). An additional chloroform step was performed, followed by isopropanol precipitation of DNA. The DNA pellet was washed with ethanol before resuspension in 100 µl TE buffer. During DNA extraction, an open tube with 200 µl of MilliQ water placed on the laboratory work bench was used as a laboratory work control. A tube with CTAB buffer (extraction blank control) followed the extraction protocol alongside the real samples. Separate laboratory rooms were used for pre- and post-PCR procedures (Agersnap et al., 2017) to minimise risk of laboratory-induced contamination.

The DNA samples were analysed using three different probe-based singleplex qPCR assays referred to as *Aphast*, *Astast* and *Paclen* (see Table 1 for a qPCR assay specifics). *Aphast* is the *A. astaci* qPCR assay adapted for detection and quantification in water (Strand et al., 2014), while *Astast* and *Paclen* represent qPCR assays for eDNA detection and quantification of noble and signal crayfish respectively (Agersnap et al., 2017). All qPCR analyses were run on an Mx3005P qPCR system (Stratagene); the *Aphast* setup followed Strand et al. (2014), while *Astast* and *Paclen* followed Agersnap et al. (2017) with the following modifications: we used 500 nM primer and 250 nM probe concentration and 60 s at 56°C for annealing/extension for both assays.

Standard dilution series for *A. astaci*, noble crayfish and signal crayfish were prepared using genomic DNA, according to Vrålstad et al. (2009) and Agersnap et al. (2017) (i.e. 'the Norwegian approach'). Four calibration points (standard dilutions ranging from ~20 pg/µl to ~3 pg/µl gDNA of *A. astaci*, and ~781 pg/µl to ~12 pg/µl gDNA of both crayfish species) were included in each qPCR run to generate a standard curve for quantification of eDNA in samples. Four technical qPCR replicates (i.e. two per subsample A and B) were analysed per water sample, two undiluted and two 10-fold diluted replicates. The presence or absence of qPCR inhibition was controlled by calculating the difference in cycle threshold (Ct) values (ΔCt) between the undiluted and corresponding 10-fold diluted DNA replicates, as previously described (Agersnap et al., 2017; Kozubikova et al., 2011). Briefly, the theoretical ΔCt value equals 3.32 in the absence of inhibition, but variation is expected due to minor inaccuracies in amplification efficiency, manual pipetting and other stochastic factors. We accepted a variance level of 15%, allowing for quantification in samples where the ΔCt is 3.32 ± 0.5 (range = 2.82–3.82) between the undiluted and 10-fold diluted replicates. If ΔCt was within this range, DNA copy numbers were calculated as the mean of the undiluted replicates and the 10-fold diluted replicates, the latter multiplied by 10. In case of inhibition (if ΔCt <2.82) the estimated eDNA copy number was based only on the 10-fold diluted DNA replicates, while if ΔCt > 3.82 (i.e. 10-fold dilution out of range), the estimation of eDNA

TABLE 1 Overview of the three species-specific assays used in the study, targeting *Astacus astacus*, *Pacifastacus leniusculus* (Agersnap et al., 2017) and the crayfish plague agent *Aphanomyces astaci* (Vrålstad et al., 2009). The target gene regions are mitochondrial genomic cytochrome oxidase 1 (CO1) and the nuclear genomic internal transcribed spacer (ITS)

Species	Assay	Target	Amplicon	Forward primer (5′–3′)	Probe (5′–3′)	Reverse primer (5′–3′)
Astacus astacus	*Astast*	COI	65 bp	GATTAGAGGAATAGTAGAGAG	FAM-AGGAGTAGGGACAGGATGAACT-BHQ1	CTGATGCTAAAGGGGGATAA
Pacifastacus leniusculus	*Paclen*	COI	65 bp	AACTAGAGGAATAGTTGAAAG	FAM-AGGAGTGGGTACTGGATGAACT-BHQ1	CCGCTGCTAGAGGAGGATAA
Aphanomyces astaci	*Aphast*	ITS	58 bp	AAGGCTTGTGCTGGGATGTT	FAM-TTCGGGACGACCC-MGBNFQ	CTTCTTGCGAAACCTTCTGCTA

copy number was based on the undiluted DNA replicates alone. If none or only one of the replicates was detected above limit of quantification (LOQ), further quantification was not performed and the result for the eDNA sample was reported as below LOQ (<LOQ) (see Table 1 for limit of detection (LOD) and LOQ specifics). A sample result was only regarded as positive if the overall detection (mean for all PCR replicates) was above LOD (Table 1). Following Kozubikova et al. (2011) and Agersnap et al. (2017), a cut-off was set at Ct 41, defining positive signals with a Ct value ≥41 negative (i.e. not detected). Environmental DNA copy numbers per litre water were calculated from the eDNA copy number quantified in the qPCR reactions according to Agersnap et al. (2017) using the equation: $C_L = (C_{rAB} * (V_e/V_r))/V_w$. Here, C_L represents the copies of eDNA per litre lake water, C_{rAB} represents the copies of eDNA in reaction volume summarised for subsample A and B, V_e represents the total elution volume after extraction, V_r represents the volume of eluded extract used in the qPCR reaction and V_w represents the volume of filtered lake water. The *Aphast* qPCR assay targets the multicopy ITS nrDNA-region (see Table 1). The spore concentrations for *A. astaci* (spores/L) were estimated according to Strand et al. (2011, 2014) using the equation: $C_L/138$, based on the estimation that one spore contains ~138 copies of the target DNA.

2.6 | Statistics

Estimated eDNA concentrations (C_L) from station 1, 3–6 in 2015 were $\log_{10}$ transformed and converted to first-order difference series to test for correlation between eDNA concentrations from the different species. Signal crayfish eDNA results were excluded from the correlation test, since signal crayfish eDNA was only detected at station 1 and at low concentration and frequency. Correlation was tested on the first-order difference series of eDNA concentrations (C_L) from noble crayfish and *A. astaci* using spearman rank correlation. The statistical tests were run in the software RStudio v. 1.1.456 (RStudio team, 2016) using R v 3.5.1 (R Development Core Team, 2018).

3 | RESULTS

3.1 | Cage surveillance versus eDNA monitoring

eDNA monitoring revealed the crayfish plague pathogen in the water earlier than the cage method. All three targets (*A. astaci*, noble crayfish and signal crayfish) were detected at low eDNA concentrations at station 1 on the first eDNA sampling date (3 October 2014; Figure 3), while 8 weeks passed before all noble crayfish were found dead in cage station 1 (*A. astaci* infection confirmed, Table 2). On 22 December 2014, all caged crayfish were dead due to crayfish plague at station 2 (Figure 3a, Table 2). Table S1 provides details for eDNA copy numbers for all targets, and *A. astaci* spore estimates.

We observed that presence/absence data, as well as fluctuation in eDNA concentrations, depicted to a large extent the

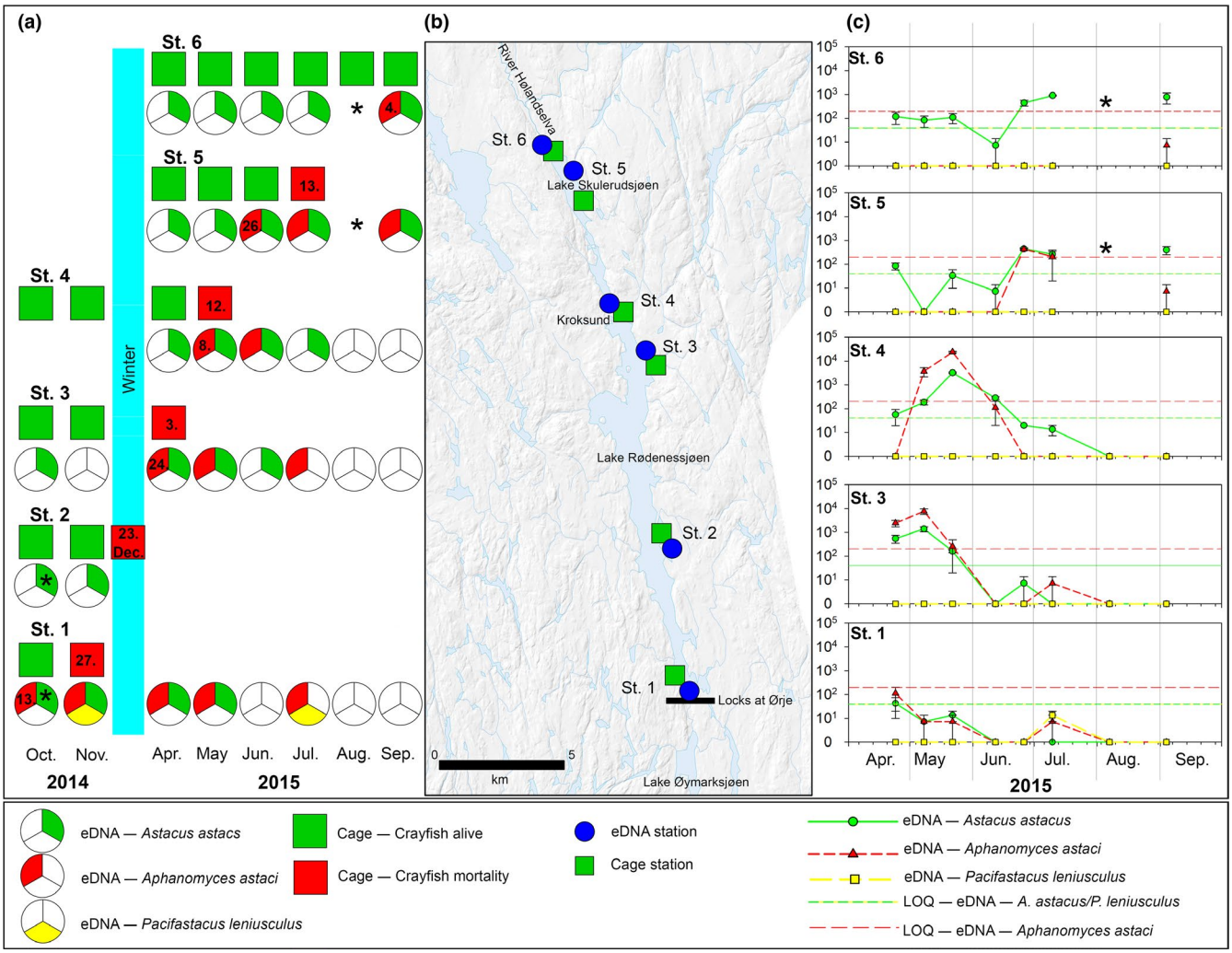

FIGURE 3 Comparison of environmental DNA (eDNA) presence/absence of *Astacus astacus*, *Aphanomyces astaci* and *Pacifastacus leniusculus* and mortality of caged *Astacus astacus* (a) at the cage and eDNA stations 1–6 in the Halden watercourse (b), with details for the eDNA concentration dynamics in the water quantified for *Astacus astacus* (green circles), *P. leniusculus* (yellow squares) and *Aphanomyces astaci* (red triangles) by qPCR (c). Triangular split circles (a) indicate detection of eDNA from *Astacus astacus* (green), *P. leniusculus* (yellow) and *Aphanomyces astaci* (red) per station in 2014 and 2015; these are not to be interpreted as pie charts. No detection is indicated with no colouring. The numbers (a) overlaying the circles indicate the date when *Aphanomyces astaci* was detected by eDNA, while the numbers overlaying the squares indicate the date for mortality in the cages caused by crayfish plague (i.e. *Aphanomyces astaci* infection). The circles and squares (a) depict the pooled results for the respective month. LOQ, limit of quantification. * Six samples from 2014 and another six from 2015 were excluded due to minor contamination in the controls

biological status of the crayfish and habitat in terms of freedom from disease, early infection, mortality and extinction. When the ice cover thawed in 2015, plague-induced mortality in the cage was observed at station 3 3 weeks prior to our first eDNA sampling event (24 April, Figure 3a, Table 2). Here, high levels of eDNA from *A. astaci* and noble crayfish were detected, with a further increase 2 weeks later, followed by a decline to trace amounts in the following weeks with no detection by August (Figure 3c). At station 4, only low levels of noble crayfish eDNA were detected on 24 April, while both noble crayfish and *A. astaci* were detected 2 weeks later (May 8th, Figure 3c). One week later, crayfish plague-induced mortality was observed in the cage (Figure 3a, Table 2). Concentrations of eDNA for both targets continued to increase and peaked on 22 May. Again, a rapid decrease followed, and by the end of June 2015, noble crayfish eDNA was detected only at low concentrations, while *A. astaci* was no longer detected (Figure 3c). From July to September 2015, noble crayfish eDNA was also undetectable. At station 5, only eDNA from noble crayfish could be detected in April and May, while *A. astaci* eDNA was also detected on 26 June. Noble crayfish mortalities in the cage were first observed 18 days later (Figure 3a, Table 2). Again, concentrations of eDNA from noble crayfish increased in parallel with eDNA from *A. astaci* during the outbreak period (Figure 3c). From July to August 2015, concentrations of eDNA from *A. astaci* decreased, while noble crayfish could still be detected. At station 6,

TABLE 2 Overview of noble and signal crayfish analysed with *Astacus astaci* quantitative real-time PCR (qPCR). Three tissues were screened per crayfish. Infection of *A. astaci* is reported at agent levels according to Vrålstad et al. (2009), which reflects increasing, semi-quantitative intervals of DNA concentrations found in the infected crayfish tissues. The shaded numbers indicate the numbers of crayfish with positive detection of *A. astaci* coloured according to the agent level. Only the highest observed agent level is included in the table regardless of tissue

							Agent levels								
							Negative		Positive						
Origin	Location	Date	NVI ref.	Crayfish species	# Analysed	*A. astaci* Prevalence	A_0	A_1	A_2	A_3	A_4	A_5	A_6	A_7	eDNA detection *A. astaci*
Trapping	Rødenessjøen south	12.09.2014	2014-23-237	Noble crayfish	5	57%	3	0	1	1	0	0	0	0	
Trapping	Rødenessjøen south	12.09.2014	2014-23-237	Signal crayfish	2	100%	0	0	1	1	0	0	0	0	
Cage	Rødenessjøen south	12.09.2014	2014-23-237	Noble crayfish	2	100%	0	0	0	0	0	1	1	0	
Ashore/Dead	Rødenessjøen south	29.09.2014	2014-23-265	Noble crayfish	2	100%	0	0	0	0	1	0	1	0	
Unknown	Rødenessjøen south	29.09.2014	2014-23-265	Signal crayfish	3	100%	0	0	1	1	1	0	0	0	
Cage monitoring	St. 1	27.11.2014[a]	2015-23-46	Noble crayfish	3	100%	0	0	0	0	1	1	1	0	03.10.2014
Cage monitoring	St. 2	22.12.2014[a]	2015-23-44	Noble crayfish	5	100%	0	0	0	0	0	0	2	3	-
Cage monitoring	St. 3	03.04.2015[a]	2015-23-88	Noble crayfish	3	100%	0	0	0	0	0	0	2	1	24.04.2015
Cage monitoring	St. 4	12.05.2015[a]	2015-23-121	Noble crayfish	3	100%	0	0	0	0	2	1	0	0	08.05.2015
Cage monitoring	St. 5	13.07.2015[a]	2016-23-8	Noble crayfish	5	100%	0	0	0	0	1	3	1	0	26.06.2015
Cage monitoring	St. 6	01.07.2015[b]	2016-23-7	Noble crayfish	3	0	3	0	0	0	0	0	0	0	04.09.2015

Note: [a]Mortality date of caged crayfish.
[b]Three crayfish died, but were negative for crayfish plague.

eDNA from noble crayfish was detected from April to September 2015 (Figure 3a,c), while eDNA from *A. astaci* was detected at low concentration in September samples. No crayfish plague-induced mortality of noble crayfish was observed in this cage (Table 2), and the eDNA concentrations of noble crayfish remained stable throughout the sample period. No eDNA from signal crayfish was detected at any station other than station 1 (Figure 3). The parallel increase and subsequent decrease in eDNA concentrations of *A. astaci* and noble crayfish correlated significantly (rho = 0.485; p = 0.0043, Figure 3c). Table S2 provides eDNA copy numbers for all targets, and spore estimates of *A. astaci* for 2015. Six samples from 2014 and another six from 2015 were excluded due to minor contamination detected in the laboratory work control or DNA blank control for these samples respectively (c.f. Figure 3).

3.2 | Trapping data versus eDNA

We found that trapping data and eDNA data are in agreement with regard to presence/absence results. At stations 2 and 3, noble crayfish eDNA was detected in 2014 (Table S1), corresponding well with the trapping of 135 noble crayfish (CPUE = 0.86) during the national surveillance programme the same year. In Lake Rødenessjøen, no traces of eDNA from noble crayfish were detected after July 2014 (Tables S1–S3). No noble crayfish were caught during August and September 2015, despite 2,840 trap nights, suggesting local extinction. At the invasion site (station 1), only 11% of the water samples analysed from 2014 to 2015 were positive for signal crayfish eDNA (Tables S1 and S2). The trapping surveys suggest that signal crayfish were restricted to the southern part of the lake at low density. Here, 110 signal crayfish were caught in 2015 using 960 trap nights

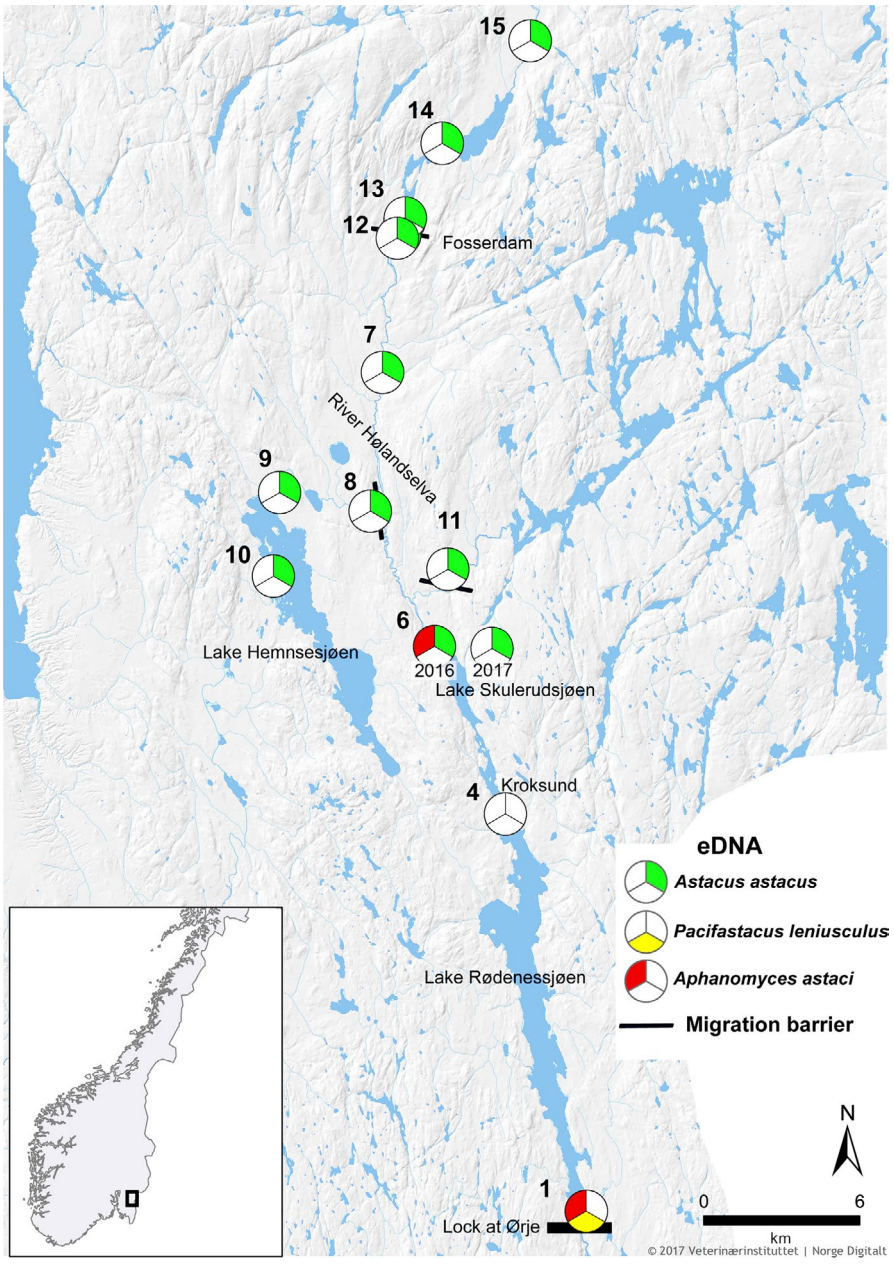

FIGURE 4 Triangular split circles indicate detection of eDNA from *Astacus astacus* (green), *Pacifastacus leniusculus* (yellow) and *Aphanomyces astaci* (red) per station in 2016 and 2017; these are not to be interpreted as pie charts. No detection is indicated with no colouring. Stations 1, 4, 6–7 and 12 are within the infection zone, while the stations 8–11 and 13–15 are located in the risk zone, separated from the infection zone by migration barriers (bold black lines) such as dams and waterfalls. The only change from 2016 to 2017 is found at station 6, where eDNA from *Aphanomyces astaci* was detected only in 2016

(CPUE = 0.12), and only large individuals were trapped (average 118.2 mm, *N* = 91), suggesting their recent release.

3.3 | Implementing eDNA monitoring

The comparative data obtained with eDNA monitoring and traditional methods (cages and trapping) convinced the authorities to officially include eDNA as a monitoring method. Thus, in 2016, eDNA was officially integrated into the national crayfish plague monitoring programme commissioned by NFSA. Cages were only used in the risk zone (data not shown), and cage surveillance was discontinued from 2017. The eDNA monitoring focus shifted to the River Hølandselva (station 6–7), and upstream locations (station 8–15) in addition to stations 1 and 4 (Figure 1). Several new stations (8–10, 13–15) were established in the risk zone to monitor potential spread. Noble crayfish eDNA was detected at all stations in the risk zone (Figure 4, Table S3), while no signal crayfish or *A. astaci* eDNA was detected here. In the River Hølandselva, eDNA from *A. astaci* and noble crayfish was detected at the outlet of the river in 2016 (station 6), while only eDNA from noble crayfish was detected further upstream in the river (station 7) (Figure 4). At station 4, eDNA of *A. astaci* and noble crayfish was no longer detected, and in 2017, all signs of *A. astaci* had disappeared from all stations with the exception of station 1 (Figure 4). At station 1, eDNA from signal crayfish and *A. astaci* was still detected (Figure 4). Table S3 provides details for eDNA detection frequency for all targets for 2016–2017.

4 | DISCUSSION

eDNA monitoring provides a reliable, non-invasive, ethical and animal welfare friendly alternative to cage monitoring for early detection of crayfish plague. During the predicted freshwater crayfish disaster in the Norwegian Halden watercourse, we demonstrated that eDNA monitoring can reveal the invasion of signal crayfish at low densities, as well as low numbers of waterborne infectious *A. astaci* spores 2–3 weeks prior to observation of mortality in cage-held susceptible crayfish. Furthermore, eDNA monitoring is less likely to spread *A. astaci* than traditional methods. As a direct consequence of the present study, eDNA monitoring has been adopted in crayfish plague disease management in Norway (Vrålstad, Rusch, Johnsen, Tarpai, & Strand, 2018; Vrålstad et al., 2017). We also confirmed the efficacy of simultaneous eDNA monitoring of three target organisms, represented in this study by a Red list species, an invasive species and a harmful pathogen, which has recently been demonstrated for invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen in the UK (Robinson, Webster, Cable, James, & Consuegra, 2018).

eDNA monitoring provides a snapshot of the crayfish and habitat status, such as invasion, infection and extinction. After the discovery of low signal crayfish eDNA levels (early invasion state), the repeatedly observed and significantly correlated increase and subsequent decline of eDNA from *A. astaci* and noble crayfish spanning only a few weeks at each station depict the acute disease situation (infection outbreak) followed by local noble crayfish extinction. Increased levels of noble crayfish eDNA during the crayfish plague outbreak could be caused by decay of dead noble crayfish, resulting in increased eDNA release to the ambient water. However, behavioural changes, such as uncoordinated spasmodic limb tremors (Alderman et al., 1987), loss of nocturnality (Westman, Ackefors, & Nylund, 1992), reduced escape reflex and progressive paralysis (OiE, 2017) make noble crayfish easier prey. Increased feeding on crayfish by predators may also contribute to increased eDNA shedding. The rapid decline and disappearance of *A. astaci* eDNA also supports previous studies showing that *A. astaci* has a short life span outside its host (Svensson & Unestam, 1975; Unestam, 1966). The rapid transmission of crayfish plague and the subsequent loss of noble crayfish throughout Lake Rødenessjøen (15.95 km^2), Lake Skulerudsjøen (1.82 km^2) and River Hølandselva from September 2014 to August 2015, demonstrates the devastating effect of crayfish plague on indigenous European crayfish populations (Holdich et al., 2009; Söderhäll & Cerenius, 1999; Svoboda, Mrugala, Kozubikova-Balcarova, & Petrusek, 2017). The rapid spread of *A. astaci* throughout the lakes can be facilitated by several factors, including an enormous bloom of infectious swimming zoospores produced from each dying crayfish individual (Makkonen et al., 2013), and wind driven currents leading to rapid spread from crayfish to crayfish in the population. Furthermore, fish feeding on diseased and dying crayfish act as long-distance vectors since *A. astaci* survive the passage through the fish gut (Oidtmann, Heitz, Rogers, & Hoffmann, 2002). However, despite the rapid spread throughout the two lakes, the outbreak was still active in River Hølandselva 1 year after initial infection. Advancement of spread then slowed, most likely due to slower upstream spread in a flowing river combined with the absence or very low density of noble crayfish, working as barriers for further spread. In fact, the crayfish plague seemingly burnt out, as it is no longer detectable in terms of eDNA in 2017.

Our study indicates that trapping data and eDNA data are comparable when used to measure the presence/absence, but do not always agree for measuring biomass. Relatively low CPUE measurements (0.15–1.8; Johnsen et al., 2017) correlated with a high frequency of positive eDNA samples for noble crayfish, while negative trapping results (2,840 trap nights) the following autumn were confirmed by negative noble crayfish eDNA results. These two factors together provided strong evidence for local noble crayfish extinction. Low densities of signal crayfish only at the invasion site (CPUE = 0.12) correlated with infrequent eDNA detection of signal crayfish in 11% of the samples, which demonstrates that it is possible to detect freshwater crayfish at very low densities in a large lake by means of eDNA. These results are similar to the study by Dougherty et al. (2016), where 10% of the eDNA samples were positive for the invasive freshwater crayfish *Faxonius rusticus* in a lake with a CPUE value of 0.17. Our results support the conclusions of Robinson et al. (2018) who detected endangered native crayfish in areas in which trapping failed, and suggested eDNA as suitable for detection of native and invasive crayfish and their infection status in a rapid, cost effective and highly sensitive way.

False negatives resulting from PCR inhibition are always a risk with environmental samples. The water in Halden watercourse is relatively turbid (e.g. Lake Skulerudsjøen and Lake Rødenessjøen had average secci depths of 1.2 and 1.6 m, respectively, in 2016). Filtering larger volumes of water might increase the risk of inhibition during PCR, due to the presence of PCR inhibitors such as humic acids. All our samples were run both undiluted and 10-fold diluted in order to account for PCR inhibition, and several samples showed signs of inhibition (difference in Ct values of <2.85). This may in some cases have led to underestimation of the actual eDNA concentration of some samples in this study. Additionally, the presence of low levels of eDNA from crayfish may be masked in some samples due to inhibition of the PCR reaction. Recent studies suggest that the use of ddPCR increases the detection rate of eDNA compared to qPCR, especially at low DNA concentrations, and is more robust against inhibition (Doi, Takahara, et al., 2015; Doi, Uchii, et al., 2015). ddPCR also offers absolute quantification and precise multiplexing (two or more targets in the same reaction) (Whale, Huggett, & Tzonev, 2016). Adopting the existing assays to develop a multiplex assay for eDNA detection of all three species in a single reaction would thus be beneficial. Additionally, future eDNA studies should also be designed to incorporate occupancy modelling to estimate the detection sensitivity using traditional surveillance and eDNA monitoring (Schmelzle & Kinziger, 2016).

An important goal of this study was to contribute to the reduction or replacement of live crayfish in crayfish plague monitoring. As a direct result, NFSA replaced cage surveillance of crayfish plague with eDNA monitoring, contributing to the 3Rs (replacement, reduction, refinement; https://www.nc3rs.org.uk/the-3rs) and improved animal welfare. From 2018, NEA has also implemented eDNA monitoring of noble crayfish and signal crayfish as a supplement to the traditional CPUE surveillance, which also increases the number of surveyed watercourses. As there is no cure for crayfish plague, it is essential to minimise the risk of spreading the pathogen to new areas. Since *A. astaci* is a notifiable disease in Norway, national legislation demands monitoring measures and control strategies to reduce the risk of further spread. Other countries in Europe may also choose to monitor crayfish plague, since this is also an OiE-listed, notifiable disease (OiE, 2017). Mitigation strategies in Norway include area restrictions, prohibiting crayfish trapping, increasing public awareness and mandatory disinfection of equipment. We advocate the use of the presented approach for early warning and targeted surveillance of non-indigenous crayfish species and crayfish plague in natural habitats, and for determination of the magnitude of an outbreak. It can also be used for improved conservation of indigenous crayfish, for example for assessing habitat status for crayfish restocking purposes or selection of Ark sites (Nightingale et al., 2017).

One of the primary benefits of eDNA monitoring in aquatic environments is the possibility for temporal and spatial monitoring of several organisms from the same eDNA samples. This approach is highly relevant for the study of other host–carrier–pathogen groups in marine and freshwater environments (Bass, Stentiford, Littlewood, & Hartikainen, 2015; Rusch et al., 2018). Additionally, recurrent sampling and long-time storage (e.g. biobank) of eDNA samples gives the possibility for retrospective analysis for other species of interest or even whole communities using environmental metabarcoding (Deiner et al., 2017). Environmental metabarcoding might even reveal emerging pathogens and/or invasive species that would go undetected unless specifically screened for, and could identify the causative agents for declines in other indigenous species. In the near future, technological advances will propel the eDNA monitoring concept forward, maturing from manually sampled eDNA snapshots to automated and continuous eDNA monitoring in real time.

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AUTHORS' CONTRIBUTIONS

D.A.S., S.I.J. and T.V. designed the study; D.A.S., J.C.R., S.A., W.B.L., S.W.K. and P.R.M. contributed to the method development; D.A.S., J.C.R., S.I.J. and T.V. carried out the fieldwork; D.A.S. and J.C.R. performed the molecular analyses; D.A.S., S.I.J. and T.V. drafted the manuscript, while all other authors contributed to and approved the final version.

DATA ACCESSIBILITY

Data available via the Dryad Digital Repository https://doi.org/10.5061/dryad.vf86jb2 (Strand et al., 2019).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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