Environmental DNA (eDNA) monitoring of two different freshwater host-pathogen complexes in the interface between nature and aquaculture

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BY

JOHANNES. C. RUSCH

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Veterinærinstituttet

Norwegian Veterinary Institute

University of Oslo

Faculty of Mathematics and Natural Sciences

Department of Biosciences

Norwegian Veterinary Institute

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A PhD project is a marathon, not a sprint. Even though I am not an avid runner, I will use this metaphor to describe the long, arduous but exciting and rewarding road towards the winning post. When running a marathon, one travels to and through places one has never seen before. This thesis took me away from my native Austria to Oslo, with shorter and longer diversions to countries as diverse as Spain, Russia, Sweden, Czechia, the United States and to the beautiful lakes and rivers within Norway.

During the course of a marathon one also meets many people en route. Some run with you for a part of the way, others hand you water, give you words of encouragement, motivate you when your spirits are drooping or point you in the right direction. The final stretches of a marathon are indisputably the hardest part, a time of dwindling energy resources which can only be countered by steadfast determination. In my case the last year of this PhD thesis was undoubtedly influenced by the global covid-19 pandemic when much desired social interactions were reduced to a minimum.

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Summary

Environmental DNA (eDNA) monitoring methods are increasingly used as a supplement or substitute to conventional monitoring. This rapidly advancing research field promises improvements for aquatic species conservation and the detection of invasive species and pathogens. The eDNA dynamics of some groups of organisms like fish have been extensively studied, in particular fish of commercial interest or where there is a high invasive potential. However, there are still many knowledge gaps on eDNA dynamics and monitoring potential for rare and elusive species, and for host-pathogen complexes. The overarching goal of this thesis was to explore, develop and evaluate the potential of targeted eDNA detection and quantification as surveillance and biosecurity tool both in nature and aquaculture. For this purpose, we chose two dissimilar host-pathogen complexes, which are of economic importance and relevance regarding species conservation: The Atlantic salmon and the salmonid parasite *Gyrodactylus salaris* and freshwater crayfish with their obligate parasite *Aphanomyces astaci*. The salmon fluke *G. salaris* has caused significant damage to indigenous Atlantic salmon populations in Norway, and the Norwegian Government is working towards the eradication of this parasite. The oomycete *A. astaci*, carried and transmitted by American freshwater crayfish species, causes crayfish plague and is the largest threat to endangered European crayfish species, and is registered as a list 3 disease (national disease) in Norway. The same applies for *G. salaris*. In these host-pathogen complexes, fish shed much larger amounts of eDNA than crayfish as they are covered with a mucus layer. Conversely, the sporulating oomycete *A. astaci* is readily detectable using the eDNA methodology while the flatworm *G. salaris* assumingly only shed minute amounts of eDNA. Three main research questions were asked: 1) Can the eDNA methodology work equally well or better than conventional methods for biomonitoring of the host-pathogen models, particularly at low prevalence? 2) Can eDNA copy numbers serve as a proxy for host density and pathogen intensity? 3) How will environmental factors and organism biology influence the emission and detectability of host-pathogen eDNA?

We used both qPCR and ddPCR and drew upon already published species-specific assays or developed new ones where required (**paper I**, **III**, **IV**). For eDNA sampling, we adapted an already developed method but modified minor aspects like equipment (**paper III**) and storage of filter samples. Sampling of eDNA was conducted both under natural conditions in the field and under controlled conditions in an aquarium facility. We designed and conducted two mesocosm experiments to compare eDNA copy numbers with parasite intensity of *G.*

salaris on Atlantic salmon (**paper IV**) and to examine the influence of temperature, density and food availability on the detectability of eDNA of *A. astaci* and signal crayfish (**paper V**).

We showed that simultaneous eDNA monitoring of host-pathogen complexes is advantageous for biomonitoring purposes, but the outcome is highly dependent on the type of organism targeted and its biological traits (**paper I-V**). For the crayfish – *A. astaci* complex the eDNA methodology proved more sensitive and more animal welfare friendly than conventional methods, and simultaneous detection of crayfish provide information regarding noble crayfish population status (presence-absence) and potential threats from disease or non-indigenous crayfish. The method eliminates the need for live caged noble crayfish for disease monitoring, and detects the presence of crayfish down to very low population densities provided sufficient sampling effort (**paper II, III, V**). For the Atlantic salmon – *G. salaris* complex, results from our mesocosm experiment suggest that the eDNA methodology fails to detect parasite presence at low intensities with the same detection reliability as conventional methods (**paper IV**), but will nevertheless be a useful supplement to the work-intensive conventional methods (**paper I**). Field data also suggest a higher degree of detection success than we observed which is most likely due to experimental constraints in our study. We also developed assays for direct eDNA detection of specific mitochondrial haplotypes of *G. salaris.* These can differ in pathogenicity towards Atlantic salmon and may yield information on the origin of the infection. However, these assays targeting the mitochondrial *COI* gene are less sensitive than the nuclear ribosomal *ITS*-assay, which is better suited and more robust for presence-absence screening of *G. salaris*.

Estimations of biomass or relative abundance inferred from eDNA copy numbers are not straightforward as the amount of shed and detectable eDNA is substantially influenced by a multitude of factors. This poses a particular challenge for the detection of organisms that – through their very nature – shed less eDNA than others such as *G. salaris* or crayfish, of which the latter additionally spend considerable time buried beneath the substrate in their habitat. Life-cycle events play a major role in the eDNA dynamics. As dead crayfish emit more eDNA than live ones, a mass mortality event could be mistaken for a high density population and likewise, the capture of a dead free floating *G. salaris* specimen could be mistaken for high parasite intensity on fish. Furthermore, environmental factors heavily influence eDNA detectability even when the presence of the organisms remains unchanged. Our results show that changes in host density and pathogen intensity can be concealed by many other factors, rendering estimations of relative abundance highly challenging and for most practical purposes impossible. Here, detection frequency and probability of positive detection stand out as a better indicators of crayfish population density or *G. salaris* parasite intensity.

The shedding of eDNA does not happen in a uniform rate or manner and the source of eDNA also varies depending on the organism. The main source of eDNA of *A. astaci* are zoospores that are released at a relatively low rate from American carrier crayfish, but mass-produced during crayfish plague outbreaks. For *G. salaris*, there appears to be minimal shedding of eDNA from live parasites, leaving the main source of eDNA to be specimens that have become detached from their hosts, floating in the water. Its biology, including the clonal reproduction and parasitic nature, leave very few eDNA traces in the water from live, attached parasites. However, the rapid reproduction rate and near exponential growth of parasite numbers aid eDNA detection as we observed an increase of probability of positive detection per sample with increasing numbers of parasites. For the host, abraded cells and mucus constitute the main source of eDNA, and we did observe a generally high and relatively stable amount of eDNA from Atlantic salmon (**paper I, IV**). Due to the hard exoskeleton, crayfish shed substantially less eDNA than fish. Further, both temperature and food influenced the eDNA detection rates of crayfish and *A. astaci* (**paper V**). For *A. astaci* 20 °C was close to the upper temperature limit for sporulation, leading to drastically reduced eDNA detectability. The presence of food probably led to faster eDNA degradation through increased microbial activity, which greatly reduced the eDNA amount from crayfish. Here, live *A. astaci* spores were able to withstand this, and eDNA detection was not affected. Life-cycle events can significantly influence the released amount of eDNA. Crayfish release more eDNA during reproduction, moulting and death, and infections with *A. astaci* lead to increased sporulation, particularly during crayfish mass mortalities resulting from crayfish plague. Environmental factors, such as dilution effects and inhibitors in the water impact negatively on the eDNA detectability.

The differences in eDNA emission within and between the two host-pathogen models require special considerations for monitoring strategies with respect to water temperature and target organism biology. A consideration of life-cycle events may increase detection success. Sample numbers and volume required for high detection probability must be considered. For eDNA monitoring of host-pathogen complexes, we strongly recommend testing the samples for all relevant species, even if only one is of direct interest. This, and method considerations suitable for specific habitats, should guide the eDNA monitoring strategy. In conclusion, our results show that the amount of detectable eDNA can fluctuate in response to environmental or biological influences while the physical presence of the target organisms remains unchanged. Therefore, eDNA monitoring seems unsuitable for direct quantification of relative density or biomass, but is a powerful tool for presence-absence monitoring when the organism biology and ecology, along with environmental factors and habitat characteristics are taken into account.

Sammendrag

Miljø-DNA (eDNA) metoder brukes i økende grad som et supplement til eller erstatning for konvensjonelle biologiske overvåkingsmetoder. Dette er et forskningsfelt i rask utviking som vil kunne gi bedre beskyttelse av liv i vann, og bedre metoder for påvisning av patogener og fremmede arter. For noen grupper av organismer (f.eks. fisk) er allerede miljø-DNA dynamikk mye undersøkt, spesielt for fiskearter av kommersiell interesse eller med spesielt høyt invasjonspotensial. Imidlertid er det fremdeles mange kunnskapshull knyttet til dynamikk og overvåkingspotensial for miljø-DNA når det gjelder sjeldne arter, arter som er vanskelige å oppdage, også for vert-patogen komplekser. Det overordnede målet med denne avhandlingen er å undersøke, utvikle og evaluere potensialet for målrettet påvisning og kvantifisering av miljø-DNA som et miljøovervåkings- og biosikkerhetsverktøy, både i naturen og i akvakultur. For dette formålet valgte vi to vert-patogen komplekser, som både er økonomisk viktige og relevante for bevaring av arter: Atlanterhavslaksen og lakseparasitten *Gyrodactylus salaris* samt ferkskreps og deres obligatoriske parasitt *Aphanomyces astaci*. Flatormen *G. salaris* har forårsaket stor skade på den stedegne bestanden av atlantisk laks i Norge, og den norske regjeringen jobber for å utrydde denne parasitten. Amerikansk kreps er friske smittebærere av eggsporesoppen *A. astaci*, som forårsaker krepsepest og representerer den største trusselen mot truede, stedegne ferksvannskreps i Europa. Krepsepest er en liste 3 sykdom i Norge (nasjonal sykdom), det samme er *G. salaris*. I disse vert-patogen kompleksene skiller fisk ut mye større mengder miljø-DNA enn kreps, da fisk er dekket av et slimlag. Motsatt vil den sporulerende eggsporesoppen *A. astaci* lett oppdages med miljø-DNA-metoden, mens *G. salaris* antagelig bare utskiller ubetydelige mengder miljø-DNA. Vi stiller tre forskningsspørsmål: 1) Er miljø-DNA-metoden på nivå med eller bedre enn konvensjonelle metoder når det gjelder overvåking av vert-patogen komplekser, spesielt ved lav prevalens? 2) Kan antall kopier av miljø-DNA brukes som proxy for vertstetthet og patogenintensitet? 3) Hvordan påvirker miljøfaktorer og organismebiologien utskillelsen og påvisbarheten av verts- og patogen miljø-DNA?

Vi brukte både qPCR og ddPCR og benyttet allerede publiserte artsspesifikke analyser eller utviklet nye der det var nødvendig (**paper I, III, IV**). For miljø-DNA prøvetaking brukte vi en allerede utviklet metode med mindre modifikasjoner, inkludert prøvetakingsutstyr (**paper III**) og lagringsmetode for filterprøver. Vi tok miljø-DNA prøver både under naturlige feltforhold og under kontrollerte forhold i et forsøksakvarium. Vi designet og gjennomførte to mesokosmos eksperimenter for å sammenligne antall miljø-DNA kopier med forekomst av *G. salaris*

(parasittintensitet) på atlantisk laks (**paper IV**), og for å undersøke påvirkning av temperatur, tetthet og tilgang på mat for påvisbarhet av eDNA fra *A. astaci* og signalkreps (**paper V**).

Vi viste at parallel påvisning av vert-patogen komplekser ved bruk av eDNA kan være fordelaktig for overvåkningsformål, men utfallet avhenger sterkt av type målorganisme og dens biologiske egenskaper (**paper I-V**). Vi viste at miljø-DNA metoden for *A. astaci* var mer sensitiv og dyrevelferdsvennlig enn de tradisjonelle overvåkningsmetodene for krepsepest. I tillegg gir parallel miljø-DNA påvisning av kreps informasjon om tilstedeværelse eller fravær av edelkreps, samt potensielle farer som fremmede krepsearter. Metoden eliminerer behovet for bruk av levende kreps i bur, og med tilpasset prøvetakingsinnsats viser den forekomst av kreps selv med svært lave populasjonstettheter (**paper II, III, V**). For atlantisk laks og *G. salaris* viser resultatene fra mesokosmoseksperimentet at miljø-DNA metoden ikke oppdager lave forekomster av parasitten med samme pålitelighet som konvensjonelle metoder (**paper IV**). Den vil imidlertid være et nyttig supplement til de arbeidskrevende tradisjonelle metodene. Feltdata indikerer mer vellykket påvisningsrate enn våre observasjoner, noe som kan ha blitt forårsaket av eksperimentelle begrensninger i studiet. Vi utviklet også analyser for direkte påvisning av spesifikke mitokondrielle haplotyper av *G. salaris*, som kan avvike i patogenisitet mot atlantisk laks og muligens også gi informasjon om opprinnelsen til infeksjonen. Imidlertid er disse analysene, som påviser det mitokondrielle *COI*-genet, mindre sensitive enn analyse av *ITS* (nukleært ribosomalt DNA). Sistnevte er bedre egnet og mer robust for screening med tanke på fravær-tilstedeværelse av *G. salaris*.

Å estimere biomasse eller relativ tilstedeværelse på grunnlag av miljø-DNA-kopier er komplisert fordi mengden utskilt og påvisbart miljø-DNA påvirkes av en rekke faktorer i. Dette gir en spesiell utfordring for påvisning av arter som på grunn av deres iboende biologi utskiller mindre miljø-DNA enn andre, for eksempel *G. salaris* og kreps, hvorav sistnevnte tilbringer en betydelig del av tiden nedgravd i substratet. Livssyklusen spiller en stor rolle for miljø-DNA-dynamikken. Døde krepser skiller ut mer miljø-DNA enn levende, og derfor kan massedød forveksles med høy bestandstetthet. På samme måte kan et frittflytende dødt individ av *G. salaris* fanges på filteret og gi høy påvisning, som kan forveksles med høy parasittintensitet på fisk. Resultatene våre viser at endringer i vertstetthet og patogenintensitet kan kamufleres av mange faktorer. Dette gjør estimater av relativ tetthet utfordrende, og for i de fleste praktiske formål umulig. Påvisningsfrekvens og sannsynlighet for en positiv påvisning er derfor bedre indikatorer på populasjonstetthet av kreps eller parasittintensitet av *G. salaris*.

Utskilling av eDNA skjer ikke på samme måte for ulike organismer, og kildene til miljø-DNA varierer også avhengig av organismen. Hovedkilden til *A. astaci* miljø-DNA er zoosporer. De

slippes normalt ut i relativt små mengder fra Amerikansk bærerkreps, men produseres i store mengder under utbrudd av krepsepest hos Europeisk kreps. Levende *G. salaris*-parasitter utskiller tilsynelatende bare svært små mengder miljø-DNA, noe som betyr at hovedkilden til miljø-DNA er individer som flyter i vannet, løsrevet fra verten. På grunn av deres biologi, inkludert klonal formering og den parasittiske livsstilen, etterlater levende parasitter festet til verten veldig lite miljø-DNA-spor i vannet. Imidlertid favoriserer den raske formeringsraten og den nesten eksponentielle økningen av *G. salaris* populasjonen påvisning av miljø-DNA, slik at vi var i stand til å observere en økning i påvisningssannsynlighet med et økende antall parasitter. Fra verten er avslitte epitelceller og slimceller hovedkilden til miljø-DNA, og vi observerte generelt en høy og relativt stabil mengde eDNA fra atlantisk laks (**paper I, IV**). Krepsen på sin side skiller ut betydelig mindre eDNA enn fisk på grunn av sitt harde skall. Videre påvirket både temperatur og mat påvisbarhet av miljø-DNA fra kreps og *A. astaci* (**paper V**). For *A. astaci* var 20 ° C nær den øvre temperaturgrensen for sporulering, noe som førte til drastisk redusert miljø-DNA deteksjon. Tilgjengeligheten av mat førte trolig til økt nedbrytning av miljløDNA gjennom økt mikrobiell aktivitet, noe som kraftig reduserte mengde miljø-DNA påvist fra kreps. Imidlertid var levende *A. astaci*-sporer i stand til å motstå dette, og deres påvisbarhet ble derfor ikke påvirket. Hendelser gjennom livssyklus kan påvirke mengde miljø-DNA from frigis betydelig. Kreps skiller ut mer eDNA under reproduksjon, skallskifte og død, og infeksjon med *A. astaci* fører til økt sporulering, spesielt under massedød av kreps som følge av krepsepest. Miljøfaktorer, som fortynningseffekter og inhibitorer i vannet, påvirker eDNA-detekterbarheten negativt.

Forskjellene i frigjøring av miljø-DNA innen og mellom de to vert-patogen modellene krever spesielle vurdering i overvåkingsstrategier, særlig med hensyn på vanntemperatur og målorganismenes biologi. Å ta hensyn til hendelser i livssyklus kan øke påvisningssuksess. Antall prøver og prøvevolum som kreves for høy deteksjonssannsynlighet må vurderes. For overvåkning av vert-patogen komplekser anbefaler vi på det sterkeste å teste prøvene for alle relevante arter, selv om bare en er av direkte interesse. Dette, og hensynet til metoder tilpasset habitatet, bør være ledende for miljø-DNA overvåkingsstrategien. Oppsummert viser resultatene at påvist mengde miljø-DNA kan svinge sterkt på grunn av miljøpåvirkninger eller biologiske faktorer, selv om målorganismenes fysiske tilstedeværelse er uendret. Derfor ser det ut til at miljø-DNA metoder er uegnet for direkte kvantifisering av relativ tetthet eller biomasse. Det er imidlertid et kraftfullt verktøy for overvåkning av tilstedeværelse eller fravær, spesielt når målorganismenes biologi og økologi, sammen med miljøpåvirkninger og habitategenskaper tas med i betraktning.

Zusammenfassung

Die Verwendung von Umwelt-DNA (eDNA) findet zunehmend Verbreitung als Ergänzung zu – oder Ersatz für herkömmliche biologische Überwachungsmethoden. Dieses sich rasant entwickelnde Forschungsgebiet verspricht Verbesserungen im Schutz von Wasserlebewesen und im Aufspüren invasiver Arten und Pathogenen. Die eDNA-Dynamik einiger Organismengruppen (z.B.: Fische) wurde bereits intensiv erforscht, vor allem bei Fischarten von kommerziellem Interesse oder mit besonders hohem Invasionspotential. Es bestehen jedoch weiterhin viele Wissenslücken bezüglich der eDNA-Dynamik und dem Überwachungspotential von seltenen und schwer aufspürbaren Arten, sowie von Wirt-Pathogen-Komplexen. Die übergreifende Zielsetzung dieser Dissertation war es, das Potential zielgerichteter eDNA-Nachweise als Werkzeug für Umweltmonitoring und Biosicherheit, sowohl in der Natur als auch in Aquakultur aufzuzeigen und zu evaluieren. Zu diesem Zweck wurden zwei Wirt-Pathogen-Komplexe ausgewählt, welche wirtschaftlich bedeutsam und für den Arterhalt relevant sind: Der Atlantische Lachs und der Salmonidenparasit *Gyrodactylus salaris* sowie Flusskrebse und ihr obligater Parasit *Aphanomyces astaci*. *Gyrodactylus salaris* hat dem autochthonen Atlantik-Lachsbestand in Norwegen großen Schaden zugefügt und die norwegische Regierung ist bestrebt, diesen Parasiten auszurotten. Der Oomyzet *A. astaci*, welcher von amerikanischen Flusskrebsen mitgeführt und übertragen wird, stellt die größte Bedrohung für gefährdete heimische Krebsarten in Europa dar und gilt in Norwegen als Liste 3-Krankheit (nationale Bedrohung), wie auch *G. salaris*. Innerhalb dieser Wirt-Pathogen-Komplexe sondern Fische wesentlich größere Mengen an eDNA ab als Flusskrebse, da Fische von einer Schleimschicht überzogen sind. Im umgekehrten Fall lässt sich der sporulierende Oomyzet *A. astaci* ohne Weiteres mit der eDNA-Methode nachweisen, wohingegen der Plattwurm *G. salaris* vermutlich nur verschwindend geringe Mengen an eDNA absondert. Zur Erforschung stellten sich drei Hauptfragen: 1) Ist die eDNA-Methode herkömmlichen Methoden ebenbürtig oder sogar überlegen bei der Überwachung von Wirt-Pathogen-Komplexen, besonders bei geringer Prävalenz? 2) Kann die Anzahl der eDNA-Kopien stellvertretend für Wirtsdichte und Pathogenintensität herangezogen werden? 3) Wie beeinflussen Umweltfaktoren und die Biologie der Organismen die Absonderung und Nachweisbarkeit von Wirts- und Pathogen-eDNA?

Wir verwendeten sowohl qPCR als auch ddPCR und zogen bereits publizierte artsspezifische Assays heran oder entwickelten – wo notwendig – neue (**paper I**, **III**, **IV**). Zur eDNA-Probenentnahme benutzten wir eine bereits entwickelte Methode und modifizierten kleinere

Aspekte, wie Ausrüstung (**paper III**) oder Filterprobenlagerung. Proben wurden sowohl unter natürlichen Bedingungen im Feld, als auch unter kontrollierten Bedingungen in einem Aquariumslabor entnommen. Um die eDNA-Kopienanzahl mit dem Parasitenvorkommen von *G. salaris* auf Atlantischem Lachs zu vergleichen (**paper IV**), und um den Einfluss von Temperatur, Organismenanzahl sowie der Verfügbarkeit von Nahrung auf die Nachweisbarkeit der eDNA von *A. astaci* und Signalkrebsen zu untersuchen (**paper V**), wurden zwei Mesokosmosexperimente entwickelt und durchgeführt.

Wir zeigten, dass der simultane Nachweis von Wirt-Pathogen-Komplexen mittels eDNA vorteilhaft ist, wobei das Ergebnis von der Art des Zielorganismus und dessen biologischen Eigenschaften sehr stark abhängt (**paper I-V**). Die eDNA-Methode erwies sich als empfindlicher und tierfreundlicher als herkömmliche Methoden zum Monitoring des Flusskrebs - *A. astaci* - Komplexes. Zusätzlich dazu liefert der simultane Nachweis von Flusskrebsen Informationen über An- oder Abwesenheit von Edelkrebspopulationen, sowie potentielle Gefahren wie Seuchen oder nicht-autochthone Krebsarten. Die Methode schafft den Bedarf an lebenden Edelkrebsen in Käfigen ab und weist bei entsprechendem Beprobungsaufwand das Vorkommen von Flusskrebsen auch bei sehr geringer Populationsdichte nach (**paper II, III, V**). Für den Atlantischen Lachs – *G. salaris* – Komplex deuten die Ergebnisse des Mesokosmosexperiments darauf hin, dass die Methode ungeeignet ist, Parasitenvorkommen bei niedriger Anzahl mit derselben Verlässlichkeit wie herkömmliche Methoden nachzuweisen (**paper IV**). Allerdings stellt sie eine hilfreiche Ergänzung zu den arbeitsintensiven herkömmlichen Methoden dar (**paper I**). Daten von Feldversuchen weisen auf eine höhere Nachweisquote hin als in unseren Beobachtungen, was möglicherweise durch die experimentell bedingten Einschränkungen in unserer Studie verursacht wurde. Wir entwickelten außerdem Assays für den direkten eDNA-Nachweis spezifischer mitochondrieller Haplotypen von *G. salaris*, die sich in ihrer Pathogenität gegenüber Atlantischem Lachs unterscheiden und möglicherweise auch Informationen zum Ursprung der Infektion liefern können. Diese Assays, die auf das mitochondrielle *COI*-Gen abzielen, sind allerdings weniger empfindlich als das ribosomale *ITS*-Assay, welches für An- oder Abwesenheitsnachweise besser geeignet ist.

Es ist schwierig, von eDNA-Kopien abgeleitete Schätzungen der Biomasse oder relativen Häufigkeit anzustellen, da die Menge der abgesonderten und nachweisbaren eDNA von einer Vielzahl an Faktoren beträchtlich beeinflusst wird. Dies stellt eine besondere Herausforderung für den Nachweis von Arten dar, welche durch ihre inhärente Biologie weniger eDNA absondern als andere, wie z.B. *G. salaris* und Flusskrebse, von denen letztere eine beträchtliche

Zeit vergraben unter dem Substrat in ihrem Habitat verbringen. Der Lebenszyklus spielt bei der eDNA-Dynamik eine große Rolle. Tote Flusskrebse sondern mehr eDNA ab als lebende und daher könnte ein Massensterben mit einer hohen Populationsdichte verwechselt werden. Ebenso könnte ein am Filter aufgefangenes *G. salaris* Individuum mit einer hohen Parasitenintensität auf Fischen verwechselt werden. Unsere Ergebnisse zeigen, dass Veränderungen von Wirtsdichte und Pathogenintensität von vielen Faktoren verschleiert werden können. Dies macht Schätzungen der relativen Organismenanzahl herausfordernd und in den meisten praktischen Anwendungen unmöglich. Die Häufigkeit der Nachweise und die Wahrscheinlichkeit eines Nachweises sind daher geeignetere Indikatoren für Flusskrebs-Populationsdichten oder Parasitenintensitäten von *G. salaris*.

Die Absonderung von eDNA geschieht nicht gleichförmig. Ebenso variiert die eDNA-Quelle je nach Organismus. Die Hauptquelle von *A. astaci* - eDNA sind Zoosporen. Sie werden von infizierten amerikanischen Flusskrebsen normalerweise in relativ geringem Ausmaß freigesetzt, bei Ausbrüchen der Krebspest jedoch massenhaft produziert. Lebende *G. salaris*-Parasiten sondern augenscheinlich nur sehr geringe Mengen an eDNA ab, wodurch die Hauptquelle für eDNA einzelne Individuen sind, die vom Wirt abgelöst im Wasser treiben.
Durch ihre Biologie, inklusive der klonalen Vermehrung und der parasitischen Lebensweise, hinterlassen lebende, am Wirt befestigte Parasiten nur sehr geringe eDNA-Spuren im Wasser.
Die rasche Vermehrungsrate und der annähernd exponentielle Populationsanstieg begünstigen den eDNA-Nachweis allerdings, sodass wir eine Zunahme der Nachweiswahrscheinlichkeit mit zunehmender Parasitenzahl beobachten konnten. Von den Wirten stellen abgeschürfte Epithelial- oder Schleimzellen die Hauptquelle für eDNA dar und wir beobachteten deutlich eine generell sehr hohe und stabile Menge an eDNA von Atlantischem Lachs (**paper I, IV**). Flusskrebse sondern, bedingt durch ihren harten Panzer, wesentlich weniger eDNA ab als Fische. Darüber hinaus beeinflussten sowohl Temperatur, als auch Nahrung die eDNA-Nachweisbarkeit von Flusskrebsen und *A. astaci*. Für *A. astaci* lagen 20 °C nahe am oberen Temperaturlimit für die Sporulation, was zu einer drastisch verringerten eDNA-Nachweisbarkeit führte. Die Verfügbarkeit von Nahrung führte vermutlich zu einem vermehrten eDNA-Abbau durch mikrobielle Aktivität, welche die eDNA-Menge von Flusskrebsen stark verringerte. Lebende *A. astaci*-Sporen waren allerdings in der Lage, dem zu widerstehen und deren eDNA-Nachweisbarkeit war daher nicht beeinträchtigt. Ereignisse im Lebenszyklus können die abgesonderte eDNA-Menge beträchtlich beeinflussen. Flusskrebse sondern während der Reproduktion, Häutung und nach dem Tod mehr eDNA ab. Infektionen

mit *A. astaci* führen in Folge zu erhöhter Absonderung von *A. astaci*-Sporen, besonders bei krebspestbedingtem Massensterben. Umwelteinflüsse, wie Verdünnungseffekte und Inhibitoren im Wasser beeinträchtigen die eDNA-Nachweisbarkeit. Die Unterschiede der eDNA-Freisetzung innerhalb von und zwischen den beiden Wirt-Pathogen-Modellen verlangen eine besondere Berücksichtigung in Monitoringstrategien bezüglich Wassertemperatur und der Biologie des gesuchten Organismus. Eine Inbetrachtnahme der jeweiligen Lebenszyklen könnte den Nachweiserfolg erhöhen. Die Anzahl der Proben, die für eine hohe Nachweiswahrscheinlichkeit benötigt wird, muss ebenfalls in Betracht gezogen werden.

Wir empfehlen dringend, bei Wirt-Pathogen-Monitoring die Proben auf alle relevanten Organismen zu untersuchen, auch wenn nur einer davon von direktem Interesse ist. Dies, und die Auswahl von an das Habitat angepassten Methoden sollten die eDNA-Monitoringstrategie leiten. Zusammenfassend zeigen unsere Ergebnisse, dass die Menge an nachweisbarer eDNA auf Grund von Umwelteinflüssen oder biologischen Faktoren stark variieren kann, auch wenn die physische Präsenz der Zielorganismen unverändert bleibt. Daher scheint eDNA-Monitoring für eine direkte Quantifizierung der relativen Organismenzahl oder Biomasse zwar ungeeignet zu sein, sie ist jedoch ein leistungsstarkes Werkzeug zum Nachweis der An- oder Abwesenheit, besonders wenn Biologie und Ökologie der Zielorganismen sowie Umwelteinflüsse und Habitatbeschaffenheit berücksichtigt werden.

Abbreviations and definitions

Abbreviations:

BSA	Bovine Serum Albumin	**NFSA**	Norwegian Food Safety Authority (Mattilsynet)
COI	Cytochrome Oxidase Subunit 1	**NGS**	Next generation sequencing
CPUE	Catch per unit effort	**NICS**	Non-indigenous crayfish species
Cq	Quantification cycle	**NMBU**	Norwegian University of Life Sciences (Norges miljø- og biovitenskapelige universitet)
CR	Critically Endangered, (IUCN classification)	**NOK**	Norwegian Kroner
Ct	Cycle threshold	**nrDNA**	nuclear DNA
CTAB	Cetyl trimethylammonium bromide (used in CTAB DNA extraction buffer)	**NVI**	Norwegian Veterinary Institute (Veterinærinstituttet)
CytB	Cytochrome b	**OIE**	World Organisation for Animal Health
ddPCR	Droplet digital PCR	**OTU**	Operational taxonomic unit
dsDNA	double stranded DNA	**PCR**	Polymerase chain reaction
eDNA	Environmental DNA (DNA isolated from an environmental sample)	**PFU**	PCR forming units (amplifiable DNA copies)
EN	Endangered, (IUCN classification)	**qPCR**	Quantitative real-time PCR
eRNA	Environmental RNA (RNA isolated from an environmental sample)	**RAPD**	Random amplification of polymorphic DNA
IBOL	International Barcode of Life initiative	***rbcL***	Ribulose bisphosphate carboxylase large chain
ICS	Indigenous crayfish species	**SNPs**	Single-nucleotide polymorphisms
ITS	Internal transcribed spacer region	**ssDNA**	single stranded DNA
IUCN	International Union for Conservation of Nature	**SSRs**	Simple Sequence Repeats
LOD	Limit of detection	**SSU**	Small subunit
LOQ	Limit of quantification	**STR**	Short Tandem Repeats
LSU	Large subunit	**TE-buffer**	Tris EDTA buffer
matK	Megakaryocyte-Associated Tyrosine Kinase	**VU**	vulnerable, according to IUCN classification
MGB	Minor groove binder		

Definitions

Assay A chemical test to determine the presence or absence or more often the quantity of one or more components of a material.[1]

Agent level Semi-quantitative categories based on the estimated PFU values of *A. astaci* DNA in a tissue sample, ranked as low, medium or high agent levels in sample (the amount of pathogen in a tissue).[1] This is commonly also termed "pathogen load" (the amount of pathogen in a tissue).[2]

Cyst Protective coat surrounding resting cells, e.g. an encysted oomycete zoospore.[3]

DNA (deoxyribonucleic acid) One of the two forms of nucleic acid (composed of two complementary chains of nucleotides wound in a double helix) in living cells, the genetic material for all cellular life forms and many viruses.[4]

Ectoparasite A parasite that lives on the outside of its host's body.

Endoparasite A parasite that lives inside its host's body.

Epidemic An outbreak of a disease (especially an infectious disease) that affects a large number of individuals within a population at the same time.

Epidemiology the study of the occurrence of infectious diseases, their origins and pattern of spread through the population.[3]

Epizootic Epidemic disease amongst animals.[3]

Facultative parasite A parasite that can also live as saprotroph.

Hamulus A hook or hook-like process (as of a bone). [3]

Haplotype A set of linked genes or other genetic markers that are generally inherited together as a unit.

Haptor An attachment organ of flatworms. [3]

Host Any organism in which another spends part or all of its life, and from which it derives nourishment or gets protection. [3]

Indigenous species A species belonging to the locality; not imported; native. [3]

Infection Invasion of a tissue by endoparasites e.g. bacteria, viruses, fungi, protozoans, etc.[3]

Infection region A geographic area containing watercourses within which Atlantic salmon infected with *G. salaris* can naturally move, as defined by the Norwegian Environment agency.[5]

Infection zone Areas under special regulation as a result of earlier detection of *A. astaci*.[5]

Infrapopulation All the organisms of a single species of parasite within a single host at a particular time. [3]

Invertebrate: An animal that lacks a vertebral column (backbone).

Iteroparous Organisms that reproduce several or many times during a lifetime.

Non-indigenous (alien, exotic, non-native) species Opposite of indigenous species.[3]

Macroinvertebrates Any invertebrate or invertebrate larva whose size is measured in millimetres or centimetres rather than microscopic units. [3]

Microsatellite, Simple Sequence Repeats (SSRs) or Short Tandem Repeats (STRs) A type of simple sequence length polymorphism comprising tandem copies of, usually, di-, tri-, or tetranucleotide repeat units.[4]

Morphotaxonomy Classification of organisms according to their morphology. [3]

Mortality Death or death rate. [3]

Oligo (oligonucleotide) A short synthetic single-stranded DNA molecule.[4]

Obligate parasite A parasite that can only live as parasite. [3]

Oomycete Phylum of simple non-photosynthetic, saprobic or parasitic, unicellular or filamentous protists, now classified in the Stramenopila or the Chromista, formerly classifed as fungi. Unlike most fungi their cell walls contain cellulose. Sexual reproduction is oogamous and they reproduce asexually by motilezoospores. They include the water moulds (e.g. *Saprolegnia*), and the causative organisms of several important plant diseases, e.g. downy mildew of grapes (*Plasmopora*) and potato blight (*Phytophthora infestans*). [3]

Parasite/parasitic An organism that for all or some part of its life derives its food from a living organism of another species (the host). It usually lives in or on the body or cells of the host, which is usually harmed to some extent by the association. [3]

Pathogen Any disease-causing microorganism.

Pathogenic Causing disease, appl. a parasite (esp. a microorganism) in relation to a particular host. [3]

Prevalence The percent of a population being studied that is affected with a particular disease at a given time.[1]

Primer A short oligonucleotide that is attached to a single-stranded DNA molecule in order to provide a start point for strand synthesis.[4]

Probe A labelled (e.g. fluorophore) oligonucleotide designed to identify complementary or homologues molecules to which it base-pairs.[4]

Risk zone Remaining parts of the watercourse connected to infection zones as well as lakes and rivers with noble crayfish populations in close proximity to the infection zone.[5]

Saprotroph Any organism that feeds by absorbing dead organic matter.

Spore A small, usually unicellular, reproductive body from which a new organism arises, produced by some plants, fungi and protozoa.[4] In this thesis, the term *A. astaci* "spore" is used as a generic term for both zoospores and cysts, as qPCR or ddPCR is not able to discern between the two.

Vector Any agent (living or inanimate) that acts as an intermediate carrier or alternative host for a pathogenic organism and transmits it to a susceptible host. [3]

Virulence The ability to cause disease. [3]

Zoospore A motile, flagellated asexual reproductive cell in protozoans, algae and fungi. [3]

All definitions were, if not otherwise indicated, obtained from the Oxford Dictionary of Biology (Sixth Edition, 2008)

[1](*Gove 2000*); [2]Vrålstad et al. (2009); [3](*Lawrence 2005*) [4](*Brown 2002*) [5] (*Miljødirektoratet 2014a*)

List of Papers

This thesis is based on the following five papers and will be referred to in the text by their Roman numerals:

I. **Rusch JC**, Hansen H, Strand DA, Markussen T, Hytterød S, Vrålstad T (2018). Catching the fish with the worm: a case study on eDNA detection of the monogenean parasite *Gyrodactylus salaris* and two of its hosts, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Parasites & Vectors 11. doi: https://doi.org/10.1186/s13071-018-2916-3

II. Strand DA, Johnsen SI, **Rusch JC**, Agersnap S, Larsen WB, Knudsen SW, Møller PR, Vrålstad T (2019). Monitoring a Norwegian freshwater crayfish tragedy: eDNA snapshots of invasion, infection and extinction. Journal of Applied Ecology 56: 1661–1673. doi: https://doi.org/10.1111/1365-2664.13404

III. **Rusch JC**, Mojžišová M, Strand DA, Svobodová J, Vrålstad T, Petrusek A (2020). Simultaneous detection of native crayfish and invasive crayfish and *Aphanomyces astaci* from environmental DNA in a wide range of habitats in Central Europe. NeoBiota 58: 1-32. doi: https://doi.org/10.3897/neobiota.58.49358

IV. **Rusch JC**, Strand DA, Andersen T, Vrålstad T, Hansen H. Environmental DNA (eDNA) dynamics of the host-ectoparasite complex Atlantic salmon and *Gyrodactylus salaris* under experimental conditions. Manuscript

V. **Rusch JC**, Laurendz C, Strand DA, Johnsen SI, Edsman L, Andersen T, Vrålstad T. Exploring the eDNA dynamics of the host-pathogen pair *Pacifastacus leniusculus* and *Aphanomyces astaci* under experimental conditions. Manuscript

Introduction

Environmental DNA (eDNA)

Environmental DNA (hereafter eDNA) was a term first coined by Ogram and colleagues in 1987 while analysing microbial DNA from sediment samples (*Ogram et al. 1987*), but became widely used in the beginning of the 2000s (*Taberlet et al. 2012a*). In general, this term is used to describe DNA that can be "extracted from environmental samples (such as soil, water or air), without first isolating any target organisms" (*Taberlet et al. 2012a*) or "genetic material obtained directly from environmental samples (soil, sediment, water, etc.) without any obvious signs of biological source material" (*Thomsen and Willerslev 2015*). This methodology draws upon the fact that every organism sheds cells with genetic information (DNA) into its environment, or is invisibly present as in the case of microorganisms. Macroorganisms shed cells through various excretions as well as from abrasions of epithelial tissue or mucus layers, from body fluids, faeces or propagules such as gametes (*Valiere and Taberlet 2000, Valentini et al. 2009, Yoccoz 2012, Sint et al. 2015, Hänfling et al. 2016*). Enclosed within these cells is the DNA with the specific genetic signature of these organisms (*Alberts et al. 2002*). In the case of unicellular organisms or other multicellular microorganisms, the entire organisms or their propagules can be filtered and identified by means of eDNA analyses directly from the water. For a non-exhaustive overview of sources and influences on eDNA, see Figure **1**.

For several years now, researchers have been able to extract, amplify and analyse eDNA and assign it to the respective species. Environmental DNA analyses have been carried out in soil samples (*Taberlet et al. 2012b*), snow tracks (*Franklin et al. 2019*), crop surfaces (*Valentin et al. 2018*), sediments, faecal samples (*Dalén et al. 2004, Ruppert et al. 2019*) and air samples (*Johnson et al. 2021*). But commonly, as also in this thesis, eDNA analyses are conducted on water samples collected from aquatic environments (*Taberlet et al. 2018*), primarily on filtrates where pore size and filter type determine the water volume that is possible to sample and the size of the particles captured (*Strand et al. 2014, Turner et al. 2014, Jo et al. 2019, Jo et al. 2020*). eDNA is, in fact, also used to monitor infection rates during the SARS-CoV-2 pandemic by analysing wastewater samples (*Randazzo et al. 2020, Farrell et al. 2021*).

The principle of eDNA analyses relies on the polymerase chain reaction (PCR) (*Mullis et al. 1986*) in which the target DNA is amplified exponentially from an environmental sample and identified by means of genetic barcodes or other target-specific molecular markers. There are two common approaches for eDNA analyses (*Taberlet et al. 2012a, Deiner et al. 2017*). The

first approach involves species-specific detection of single species, or even genotypes/haplotypes within a species, using quantitative real-time PCR (qPCR) or droplet digital PCR (ddPCR). Both these methods rely on specific primers and probes that detect only the specific genetic motif unique to the target species (*Hebert et al. 2003, Hajibabaei et al. 2007, Taberlet et al. 2012a*). The second approach, eDNA metabarcoding, relies on amplification of specific loci using universal or taxonomic group selective primers followed by next generation sequencing (NGS) generating millions of reads. Here, more general or group-specific primers are used to amplify and sequence DNA from whole communities of organisms (*Holman et al. 2019, Ruppert et al. 2019*). The resulting sequence reads are compared to reference libraries (*Valentini et al. 2016, Liu et al. 2020*) through various pipelines. Metabarcoding aims at analysing whole communities or taxonomic groups on a broader scope (*Ficetola et al. 2008, Thomsen et al. 2012, Hänfling et al. 2016, Zizka et al. 2020*). It is now frequently used for assessing community structures and food webs (*Kennedy et al. 2020*), as a tool for monitoring biodiversity (*Sigsgaard et al. 2020*), and also for monitoring water-quality based on community composition (*Buss et al. 2015, Blackman et al. 2019, Sagova-Mareckova et al. 2021*). A third, less used approach for species monitoring is environmental metagenomics, involving shot-gun sequencing of all genetic material present in the eDNA sample (*Tessler et al. 2017, Fadiji and Babalola 2020, Thoendel et al. 2020*). In contrast to metabarcoding relying on a pre-selection of taxonomic groups with PCR-amplification and sequencing of a barcode-region, metagenomics using shot-gun sequencing reveals any gene present in the sample.

One of the challenges of metabarcoding is the reliance on reference libraries which are often incomplete, or the respective sequences have not been identified to species level (*Kwong et al. 2012, Curry et al. 2018*). This commonly results in a large fraction of "unknown" OTUs (operational taxonomic units). Further, sequence errors or choice of barcode regions that do not distinguish between closely related species or genotypes/haplotypes often prevent reliable species-specific detection and lead to the identification of OTUs on genus level. The cost per sample for metabarcoding surveys is similar to that of surveys based on morphological identification (*Buss et al. 2015, Elbrecht et al. 2017*) and is expected to decrease with technological advances.

Single species detection by qPCR or ddPCR is also affected by the reference problems in terms of the possibility for in-silico specificity testing. However, with good knowledge of the species in question, this approach is often used on one or few target species for which assays can easily be developed if they do not already exist. It is particularly useful for monitoring species of

specific concern, such as endangered and rare species (*Cardas et al. 2020, Mizumoto et al. 2020*), invasive species (*Miralles et al. 2016, Larson et al. 2020*) and pathogens (*Strand et al. 2011, Bass et al. 2015, Sieber et al. 2020*). Furthermore, it can also potentially provide information on abundance, density or biomass (*Jerde et al. 2011, Strand et al. 2011, Doi et al. 2015b, Lacoursière-Roussel et al. 2016, Doi et al. 2017, Tillotson et al. 2018, Capo et al. 2021*).

Single species detection using eDNA or eRNA as source is the state-of-the-art method in an increasing number of monitoring programs and environmental monitoring studies across the globe dealing with species from all domains of life (*Ruppert et al. 2019*). These include viruses (*Miaud et al. 2019, Bernhardt et al. 2021*), unicellular organisms (*Gomes et al. 2017, Vrålstad et al. 2017*), invertebrates (*Trujillo-González et al. 2019, Norris et al. 2020*), vertebrates (*Jerde et al. 2013, Hempel et al. 2020*), fungi (*Yan et al. 2018, Adamo et al. 2021*), algae (*Peters et al. 2018*) and plants (*Kuehne et al. 2020*).

Currently, the most common method for use in eDNA applications is either qPCR or ddPCR (*Wang et al. 2021*). Both methods offer important advantages over conventional PCR, with direct and specific detection of target DNA from any source or sample without the need of downstream sequence analyses. The use of probes in addition to specific primers offers higher specificity; less target DNA is needed for a reliable detection and identification – thus also making these methods more sensitive (*Vrålstad et al. 2009, Uchiyama et al. 2016*). Each method employs a fluorescent dye which is either measured at the end of each amplification cycle (qPCR) or after the entire PCR reaction in a separate device (ddPCR). Both methods provide information on quantification, but while qPCR relies on a standard curve for providing measures for relative quantification of DNA/target gene copy number, ddPCR offers the opportunity for absolute quantification of DNA/target gene number (*Quan et al. 2018*). In some cases, the quantification of DNA copies in a sample can be correlated to the number or biomass of a target species in the environment (*Jerde et al. 2011, Thomsen et al. 2012, Strand et al. 2014, Lacoursière-Roussel et al. 2016, Capo et al. 2019, Capo et al. 2021*).

At present, several well-established genetic markers ("barcode genes" or barcodes) are used for identification and delimitation of species and the marker of choice varies between different organismal groups. For fungi and oomycetes, the most commonly used gene DNA barcode is the multi-copied internal transcribed spacer region (*ITS*) of nuclear ribosomal DNA (*Schoch et al. 2012, Badotti et al. 2017*), which is a genetically variable spacer between the conserved ribosomal RNA genes *18S* (SSU), *5.8S* and *28S* (LSU) of nrDNA in Eukaryotes (*Hillis and Dixon 1991*). The most widely applied marker for animals is the mitochondrial cytochrome

oxidase subunit 1 (*COI*) gene (*Hebert et al. 2003, Waugh 2007, Badotti et al. 2017*) which is the preferred marker in the International Barcode of Life (IBOL) initiative. However, some studies have reported better results for discriminating between closely related species when using less conventional marker genes (*Minamoto et al. 2017*).

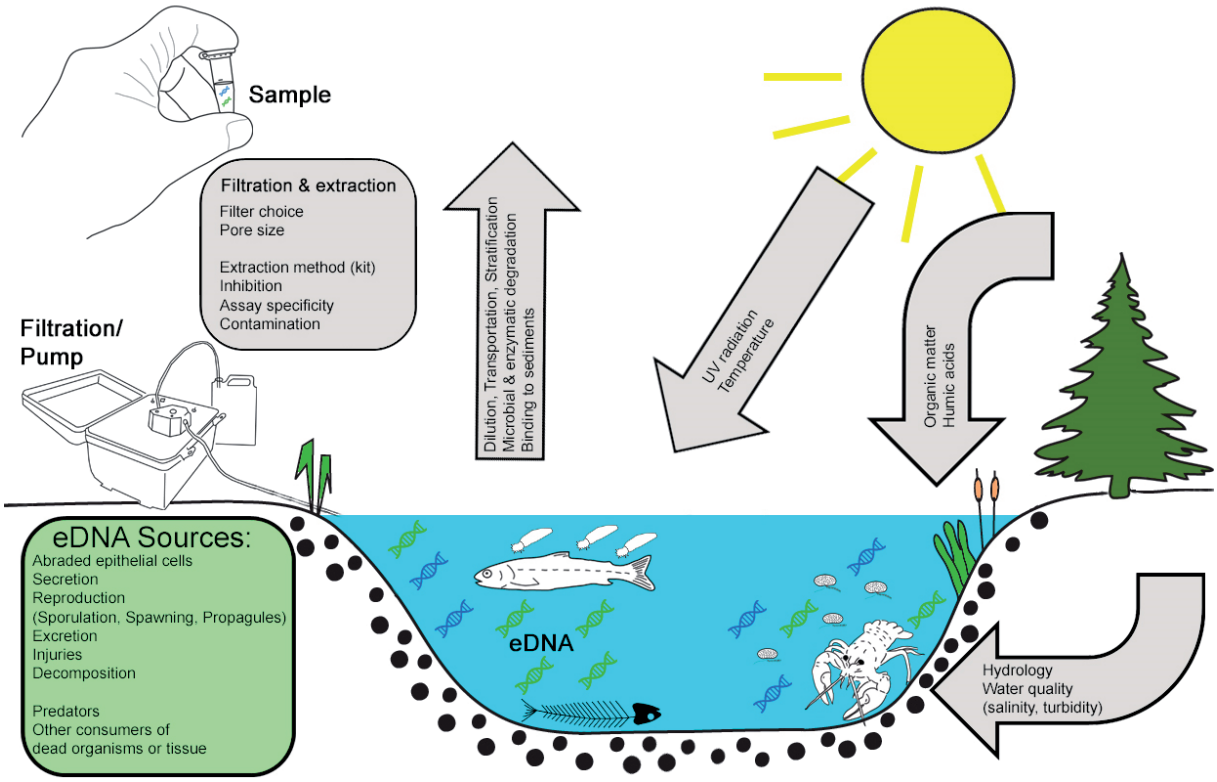

Figure 1: A non-exhaustive overview of sources (underlined in light green) and influences on environmental DNA (underlined in grey). Cells with DNA are shed by both living and recently deceased organisms, while microorganisms are represented as single- or minor multicellular units containing their DNA. In this figure, the organisms are represented by the targets of this thesis: salmonids and *Gyrodactylus salaris* as well as crayfish and *Aphanomyces astaci* zoospores. Environmental DNA can also stem from any other organism in the environment, such as (but not limited to) the plants depicted. Illustration by Johannes C. Rusch.

Biomonitoring

Traditional monitoring of species consists of a multitude of techniques and approaches. For aquatic animals, this can include trapping or catching the organisms with fishing-nets, electrofishing, visual observations and kick-net sampling for aquatic invertebrates (*Britton and Greeson 1989, Jerde et al. 2011, Rees et al. 2014, Vrålstad et al. 2017, Barnett et al. 2021, Hansen et al. 2021a*). For smaller aquatic organisms and microorganisms it might also involve plankton nets or water sampling (*Benson et al. 2019*) followed by microscopy and/or cultivation (*McDermott et al. 2014, Sagova-Mareckova et al. 2021*). These methods can be time-consuming, often rely on a steadily decreasing morphotaxonomic competence, and are sometimes difficult to conduct due to physical constraints within the habitat (*Bohmann et al. 2014*) or the nature and biology of the target species (*Pfleger et al. 2016, Hempel et al. 2020,*

Homel et al. 2021). For monitoring aquatic diseases and pathogens, the killing of numerous hosts for screening purposes is one of several methods used. This is often regarded as necessary both when examining and following disease outbreaks, and when demonstrating the likely absence of a disease pathogen (*Huver et al. 2015, Hytterød et al. 2017*). This thesis includes two relevant examples of monitored pathogens. In Norway, the surveillance for *Gyrodactylus salaris* is conducted using conventional methods, while for *Aphanomyces astaci* the use of eDNA has been implemented. In the case of *G. salaris*, juvenile salmon are caught, killed and examined after treatment of a river against the parasite for a period of a minimum of five years until the river can be declared free of the parasite (*Hytterød et al. 2020a*). Until recently, the spread and presence or absence of the crayfish plague agent *A. astaci* was monitored in Norway by keeping susceptible noble crayfish in cages and conducting molecular diagnostics on the carcasses to determine whether they died from the plague (*Vrålstad et al. 2017*). In many other countries, this might still be the consensus method. In comparison, the use of eDNA may have both advantages and drawbacks – which is a central topic for this thesis.

Especially in the case of aquatic environments, it is clearly an easier option to collect water samples (*Biggs et al. 2015, Sigsgaard et al. 2015*) than to resort to the aforementioned conventional methods. Early studies used small volumes of water (15-50 ml) and the DNA was often precipitated in sodium-acetate (CH_3COONa) prior to extraction and analysis (*Ficetola et al. 2008, Tréguier et al. 2014*). The current consensus is that water filtration with subsequent extraction of the DNA from the filters yields better results (*Hinlo et al. 2017, Spens et al. 2017, Troth et al. 2020*). However, many filter types and pore-sizes are used (*Goldberg et al. 2016*) and they seem to perform differently, depending on the respective target (*Strand et al. 2014, Fossøy et al. 2020, Jo et al. 2020*). To date, no "gold standard" filter type has been established (*Weigand et al. 2019*), but various filters for different purposes (species, habitats) work satisfactorily (*Fossøy et al. 2020*).

Although the number of studies examining eDNA is continuously growing (*Tsuji et al. 2019*), the knowledge gaps of the dynamics of eDNA are still large, also regarding host-pathogen complexes. In this thesis, two substantially different host-pathogen models that offer a unique possibility to compare eDNA dynamics are studied: the Atlantic salmon (*Salmo salar*) - *Gyrodactylus salaris* complex, and the freshwater crayfish (*Astacus astacus*) - *Aphanomyces astaci* complex. The expression "complex" is often used for species complexes, a group of closely related and morphologically indistinguishable organisms where taxonomic boundaries between them are unclear. In this thesis, the term complex is also used with regard to the

relationship between one or more host species and one or more pathogen variants. Specifically – and as described in the chapter below – several mitochondrial haplotypes of the monogenean parasite *G. salaris* have been recorded and the parasite can be found on other salmonid hosts, not only the Atlantic salmon. Likewise, several genotypes of the crayfish plague agent *A. astaci* can be distinguished (*Huang et al. 1994, Grandjéan et al. 2014*). On the Northern American continent, where *A. astaci* has co-evolved as a relatively harmless parasite with its original crayfish hosts, many genotypes exist.

Atlantic salmon – *Gyrodactylus salaris* complex

Atlantic salmon (Salmo salar)

Atlantic salmon (Salmoniformes, Salmonidae) is an anadromous fish species native to the northern hemisphere. In Norway they inhabit more than 400 watercourses (*Forseth et al. 2017*) and the country hosts a large proportion of the world's wild Atlantic salmon populations. After hatching in the springtime, the early life stages of Atlantic salmon (Alevin, fry, parr) occur in freshwater where they remain in the riverbed for the duration of between one and eight years (*Thorstad et al. 2010*) until they undergo a physiological and morphological change known as smoltification. This enables them to tolerate the higher osmotic pressure they will experience in saltwater. The geographical latitude, photoperiod and temperature as well as the nutrient richness of the river determine the time the fish require before they are sufficiently mature to leave their spawning grounds (*Thorstad et al. 2010*). Atlantic salmon populations show a substantial variability in their life histories regarding age and size (*Klemetsen et al. 2003*, *Thorstad et al. 2010*). After smoltification, these post-smolts weighing around 50 g spend up to five years in marine environments rapidly increasing in weight up to 25 kg. Between September and February, they return as adults to the freshwater rivers from which they originate to spawn (*Klemetsen et al. 2003*, *Thorstad et al. 2010*). Contrary to other salmon species, iteroparous Atlantic salmon are capable of returning to their spawning grounds multiple times in successive years (*Hansen and Quinn 1998*). However, some land-locked populations are non-anadromous and spend their entire life in freshwater such as populations in Lake Vänern (Schweden), Lake Saimaa (Finland) or River Namsen (Norway) (*Berg 1985*).

While salmon as a food product has turned from luxury item to commodity due to its ready availability through intensive aquaculture (*Ford and Myers 2008*), wild Atlantic salmon has seen a re-emergence of economic interest. Ecologically sensitive consumers and fishermen prefer wild salmon to farmed salmon (*Liu et al. 2011, Olaussen and Liu 2011*). Wild salmon is also an increasingly important species for angling tourism in Norway and elsewhere, both for

foreign and domestic tourists (*Liu et al. 2011*). In Norway, angling tourism is estimated to have a value between 300-500 million NOK annually (including ripple-effects) (*Myrvold et al. 2019*). Therefore, there is also an economic aspect to the importance of the conservation of Atlantic salmon.

Numbers of wild Atlantic salmon have been on the decline for decades and according to the IUCN red list the wild Atlantic salmon is classified as vulnerable in Europe (*Freyhof 2014*). Factors contributing to their decline are both anthropogenic influences such as construction work and/or damming for hydropower production in many salmon rivers as well as large scale salmon fishing and sportfishing (*Horreo et al. 2011, Forseth et al. 2017*). A threat to the genetic purity of wild Atlantic salmon are escapees from aquaculture that hybridise with wild salmon. In 2019 alone, more than 290,000 individuals escaped from net pens in Norway (*Fiskedirektoratet 2020*). Also, the increasing number of invasive pink salmon *Oncorhynchus gorbuscha* (Walbaum, 1792) may pose a threat to native Atlantic salmon stocks (*Sandlund et al. 2019*). Further threats come from the fact that the high number of farmed salmon in net pens increases the number of salmon louse, *Lepeophtheirus salmonis*, which also befall wild Atlantic salmon in the marine stage (*Forseth et al. 2017*). Another parasite, the ectoparasite *Gyrodactylus salaris*, infects Atlantic salmon in the freshwater stage. This parasite is a serious threat, and infections can result in a reduction of densities of juvenile salmon by up to 90 % in infected rivers (*Johnsen and Jensen 1991*).

As a popular and economically important fish species, Atlantic salmon was one of the earlier species to be incorporated into eDNA analyses. This includes research on salmon for monitoring purposes (*Atkinson et al. 2018*), as prey (*Parsons et al. 2005, Matejusová et al. 2008*), habitat preference and seasonal fish abundance (*Stoeckle et al. 2017, Lawson Handley et al. 2019*) and screening of fish markets (*Cline 2012*), to list only a few examples. Assays targeting both the *COI* gene (*Atkinson et al. 2018*) and the *CytB* gene (*Parsons et al. 2005, Matejusová et al. 2008*) are available.

Gyrodactylus salaris

The parasite *G. salaris* Malmberg, 1957 is a monogenean flatworm (phylum Platyhelminthes, Class Monogenea) of ~500 µm length (*Malmberg 1957*). The short generation span and direct life cycle of gyrodactylids can result in rapid growth of population on a susceptible host (*Bakke et al. 2007*). When born, these parasites already carry up to two successive generations inside

them, not unlike Russian matryoshka dolls (*Cable and Harris 2002, Bakke et al. 2007*). The reproduction may be sexual or clonal, the latter allowing a short generation time. With an already gravid daughter inside, the parasites can, upon successful transmission and attachment, immediately reproduce and start a new viable infrapopulation. A doubling rate of only a few days (*Jansen and Bakke 1991*) can lead to near exponential growth under favourable conditions and cause severe harm to fish populations (*Johnsen and Jensen 1991*) within a matter of weeks with parasite intensities exceeding 10,000 parasites per fish (*Jensen and Johnsen 1992*). The most common way of transmission is via direct contact amongst host fish, including transfer from a dead host, but transfer also occurs indirectly via drift in the water and/or via attachment to the substrate (*Bakke et al. 1992, Soleng et al. 1999a, Olstad et al. 2006*).

The parasite attaches itself to the host with a haptor, a specialized attachment organ consisting of a large disc with 16 peripheral articulated marginal hooks (see Figure **6**) and a single pair of ventrally orientated hamuli (*Bakke et al. 2007*). It feeds off the mucus layer that protects fish but also injures the host with the hooks while attached. Thus, it weakens the immune system of the host, leaving it vulnerable to potentially lethal secondary infections of bacterial or fungal nature (*Bakke et al. 2007*). It also has a detrimental effect on the osmoregulative capabilities of the fish (*Pettersen et al. 2013*).

To date over 400 species have been described from the genus *Gyrodactylus* and they generally display a degree of host-specificity, with 59% of species being recorded from single hosts (*Harris et al. 2004*). Furthermore, it has been documented (*Ziętara and Lumme 2002, Olstad et al. 2007*) that gyrodactylids are capable of host-switching and rapidly establishing populations on new susceptible hosts. It is important to note that the high number of gyrodactylids described on single hosts may also stem from the low number of species studied in detail, particularly with regard to host-specificity experiments.

Gyrodactylus salaris was first discovered on Atlantic salmon in Sweden in 1957 (*Malmberg 1957*) and has currently been verified to be present in 14 countries (*Paladini et al. 2021*). The natural distribution is assumed to comprise the eastern parts of the Baltic area including the drainages of the lakes Onega and Ladoga (Russia), as well as other rivers in Finland and Sweden that drain into the Baltic Sea (*Ergens 1983, Malmberg and Malmberg 1993, Anttila et al. 2008, Karlsson et al. 2020*). The first detection in Norway was made in 1975 (*Johnsen and Jensen 1986, Johnsen and Jensen 1991*) when *G. salaris* was discovered after a mass-mortality event in a hatchery in Møre and Romsdal County, (Western Norway) and in River Lakselva in Northern Norway in the same year. The parasite entered the country on imported fish from

hatcheries around the Baltic Sea (*Johnsen and Jensen 1991, Hansen et al. 2003, Karlsson et al. 2020*). A monitoring program was established and in the following five years, *G. salaris* was discovered in three more Norwegian rivers. A subsequently established "*Gyrodactylus* committee" initiated further research on *Gyrodactylus sp*. (*Johnsen and Jensen 1991*) which eventually led to the implementation of today's monitoring programs. The severity of infections with this parasite is acknowledged by its classification as a list 3 notifiable pathogen in Norway. It is also listed by the World Organisation for Animal Health (Office International des Epizooties, OIE).

The parasite is widely distributed across Fennoscandia (*Paladini et al. 2021*) and causes severe damage to populations of Atlantic salmon. However, salmon originating from the Baltic Basin generally display a higher resistance against infections with *G. salaris* as has been demonstrated both in the field (*Anttila et al. 2008, Lumme et al. 2016*) and during laboratory experiments (*Bakke et al. 1991, Bakke et al. 2004*). *Gyrodactylus salaris* seem to have a wider host specificity than other species of *Gyrodactylus* (*Bakke et al. 2002*), but this might also be due to the fact that this is the most intensively studied species. According to the OIE Manual of diagnostic tests for aquatic animals (*OIE 2019b*), the host species that fulfil the criteria for listing as susceptible to infection with *G. salaris* in addition to Atlantic salmon are rainbow trout (*Oncorhynchus mykiss*), Arctic charr (*Salvelinus alpinus*), brown trout (*Salmo trutta*), grayling (*Thymallus thymallus*), and North American brook trout (*S. fontinalis*). Rainbow trout is a particularly important carrier host and has been instrumental in the spreading of *G. salaris* (*Paladini et al. 2009, Hansen et al. 2016*). Since the 1970s, salmon in 51 rivers in Norway have been struck by infections with *G. salaris* but to date, infections remain in only eight rivers (*Hansen et al. 2021a*). This reduction of the number of rivers hosting the parasite was achieved through the implementation of extensive and expensive eradication programs. This treatment is not feasible in natural systems, where infections with *G. salaris* have instead been combatted with expensive and extensive eradication programmes using rotenone (*Sandodden et al. 2018*) that kills both the parasite and the host. Other treatments have been conducted with aqueous aluminium (*Soleng et al. 1999b, Hindar et al. 2015*) and the use of sodium-hypochlorite also is currently being tested as a potential method (*Hagen et al. 2014, Hagen et al. 2020*). Both these methods are more environmentally friendly as they do not seem to harm fish. The treatment of three infected areas (Lærdal, Driva and Vefsna) has been estimated to cost 275 million Norwegian kroner (~25 mio. €) (*Andersen et al. 2019*).

Molecular species determination of *G. salaris* has so far relied upon analysis of the *ITS* sequence (*Collins et al. 2010*) or sequencing of the *COI* gene (*Hansen et al. 2003, Meinilä et al. 2004, Hansen et al. 2006*). A closely related species, *Gyrodactylus thymalli* Žitnaň, 1960, found on grayling (*Thymallus thymallus*) cannot be distinguished from *G. salaris* on the basis of their respective *ITS* sequences. For diagnostic purposes, this poses a delicate problem since *G. thymalli* are considered benign towards Atlantic salmon. Analyses of the *COI* sequences of *G. salaris* revealed considerable genetic variation and were able to discern several mitochondrial haplotypes (*Hansen et al. 2003, Meinilä et al. 2004, Hansen et al. 2006, Hansen et al. 2007*) that in general fall into different well supported clades. These clades or groups of mitochondrial haplotypes corresponded well to geography in that there is a genetic difference between parasites found in different watersheds (*Hansen et al. 2003*). They are often also linked to host-specificity as haplotypes known from salmon are not found on grayling and conversely, haplotypes from grayling are not found on salmon. However, there was no support for the monophyly of all *G. salaris* haplotypes or of all *G. thymalli* haplotypes, i.e., *COI* cannot be used to distinguish unambiguously between the two species. More importantly, some of the currently known haplotypes can be both pathogenic and apathogenic and, therefore, it is not possible to infer potential virulence from specific haplotypes (*Hansen et al. 2007*). To date, three different variants of *G. salaris* characterized as haplotype A, B and F are known from Atlantic salmon in Norway (*Hansen et al. 2003, Olstad et al. 2007*). With the exception of finding a variant of haplotype F on Arctic charr (*Salvelinus alpinus*) in Southern Norway that proved to be non-pathogenic in experimental trials (*Olstad et al. 2007*), all these haplotypes are considered pathogenic and have caused epidemics in Norwegian rivers (*Hansen et al. 2003*).

The application of eDNA methods targeting *G. salaris* had not been tested before the beginning of this PhD-project, while a multitude of studies focus on the potential salmonid fish hosts of this parasite (*Matejusová et al. 2008, Wilcox et al. 2015, Atkinson et al. 2018*).

Freshwater crayfish – *Aphanomyces astaci* complex

Freshwater crayfish

Freshwater crayfish (Decapoda) are macroinvertebrates that can be found in both lotic and lentic freshwater systems on every continent except for Antarctica. These crustaceans are divided into two superfamilies called Astacoidea Latreille, 1802 and Parastacoidea Huxley, 1879 which inhabit the northern and southern hemisphere, respectively. The crayfish species dealt with in this thesis belong exclusively to the superfamily of Astacoidea which consists of the families Astacidae Latreille, 1802 and Cambaridae Hobbs, 1942. While representatives of the Cambaridae originate from the American continents, species of astacid crayfish are native

to both North America and Europe (*Holdich et al. 2009*). To date, more than 650 crayfish species have been described (*Crandall and De Grave 2017*).

Europe is home to only five indigenous crayfish species (often referred to as ICS): the noble crayfish *Astacus astacus* (Linnaeus, 1758), the narrow clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823), the thick clawed crayfish *Astacus pachypus* Rathke 1837, the stone crayfish *Austropotamobius torrentium* (Schrank, 1803) and the white clawed crayfish species complex *Austropotamobius pallipes* (Lereboullet, 1858). Based on analysis of molecular phylogeny, it has been suggested that both *A. torrentium* and *A. pallipes* could be species complexes (*Grandjean et al. 2002a, Grandjean et al. 2002b*). Currently nine distinct evolutionary lineages, of which the species status is currently undefined, have been observed in *A. torrentium* (*Lovrenčić et al. 2020*) but one clade has recently been proposed as separate species *A. bihariensis* (Pârvulescu 2019). In *A. pallipes*, at least four groups have been identified. However, no definitive consensus has yet been reached whether these represent separate species or phylogenetic lineages within one species (*Chiesa et al. 2011, Jelić et al. 2016*).

North America is the greatest hotspot for crayfish diversity with over 400 species, followed by Australia with more than 140. Many of these species are more colourful than the usually earthen coloured European crayfish and have, therefore, piqued the interest of aquarium owners since the 1980s (*Chucholl and Wendler 2016*). An estimated 120 species entered the transcontinental ornamental pet trade, which has been identified as a pathway for both invasive species and crustacean diseases (*Mrugała et al. 2015, Chucholl and Wendler 2016*). Non-indigenous crayfish species (often referred to as NICS) from aquariums have been released into European streams and lakes (*Patoka et al. 2016, Haubrock et al. 2021*). Others such as *Pacifastacus leniusculus* (Dana, 1852) and *Procambarus clarkii* (Girard, 1852) were released intentionally for aquacultural purposes (*Svärdson 1995*).

The introduction of non-indigenous crayfish species into Europe dates back to 1890 when *Faxonius limosus* (Rafinesque, 1817) was first introduced into Poland and Germany (*Kossakowski 1966, Müller 1978*). In 1959, 60 specimens of *P. leniusculus* were imported into Sweden from California (*Svärdson 1995*) and in 1973, *P. clarkii* was introduced into Spain from Louisiana (*Habsburgo Lorena 1978*). These three species, introduced before 1975, are generally referred to as “old” non-indigenous crayfish species within the astacological community (*Holdich et al. 2009*), whereas species introduced after 1975 are regarded as “new” non-indigenous crayfish species. All three “old” species were imported for aquacultural

purposes, to mitigate the huge losses suffered by native crayfish stocks due to the crayfish plague. Here it has to be acknowledged that the introduced species had not been identified as carriers and vectors of the disease before 1969 (*Unestam 1969*). However, after their introduction these three American crayfish species were spread, often without permits, within and between countries. This happened both through natural crayfish migration and illegal human-assisted movements (*Bohman et al. 2011, Bohman and Edsman 2011, Ruokonen et al. 2018, Jussila and Edsman 2020*). To date, there are more than 4,000 signal crayfish populations in Sweden and less than 1,000 noble crayfish populations from originally ~30,000 in 1900. This constitutes a decline of 97 % of the original population number (*Bohman and Edsman 2011*) and illustrates the impact which the introduction and subsequent (illegal) spreading of American crayfish species has on indigenous crayfish populations. In Sweden the noble crayfish is classified as critically endangered (CR) (*Artsdatabanken 2020*). Currently, there are 12 non-indigenous crayfish species with confirmed presence in the European waters (*Kouba et al. 2014, Weiperth et al. 2017*), including the three "American crayfish species. The "new" non-indigenous crayfish are two Australian species, *Cherax destructor* and *C. quadricarinatus*, North American species *Faxonius immunis*, *F. juvenilis*, *F. virilis*, *Procambarus acutus*, *P. alleni*, *P. virginalis* and the originally Central American species *Cambarellus patzcuarensis*. This number is, unfortunately, expected to increase as there are at least 25 non-indigenous crayfish species available in the European pet trade. Indeed, all the introduced species have featured on the pet market lists over the course of time (*Mrugała et al. 2015, Chucholl and Wendler 2016*).

Crayfish inhabit rivers, streams, brooks, ponds and lakes. They play an important role within their ecosystem due to their dietary habits and behaviour. They are regarded as useful indicators for water quality (*Sylvestre et al. 2002*) and as keystone species and ecosystem engineers since they significantly influence detritus processing and thus sediment dynamics and food webs in streams (*Usio and Townsend 2001, Usio 2002, Creed and Reed 2004, Reynolds et al. 2013*). Additionally, they are an important food source for other animals (*Englund and Krupa 2000*). Crayfish have also been defined as umbrella species with reference to the many co-dwellers in the water which benefit from their presence (*Reynolds et al. 2013*). They are also recognised both as an indicator species because their wellbeing indicates the health of their wider environment and as a flagship species due to their cultural heritage value (*Füreder and Reynolds 2003*).

In Europe, crayfish used to feature more prominently in central European diet (in such countries as Austria, Czechia, Germany) with records dating back to 1504 (*Füreder and Machino 1998*) but are still an important socio-economic factor in Fennoscandia (*Jussila and Edsman 2020*) and Spain (*Conde and Domínguez 2015*). In the southern states of the USA there is a thriving aquaculture industry (*Holdich 1993*) accompanied by a tradition of crayfish-related cultural events (*Romaire et al. 2005*).

In Norway the original distribution area of noble crayfish lies within Eastern Norway (Østlandet), in particular in Lake Steinsfjorden, which hosts the currently oldest known crayfish population in Norway, and in the Halden- and the Glomma watercourses, including tributaries and surrounding lakes (*Skurdal et al. 2013*). Although most noble crayfish populations in Norway are the result of human introductions some hundred years ago, research suggests that they could have entered Scandinavia via the Ancylus Lake 8,000 – 9,500 years ago (*Edsman and Schröder 2009, Johnsen et al. 2017*), and might therefore have colonised some waterbodies from Sweden after the last ice-age. Recent stocking events have extended the occurrence of this species to Central and Western Norway, and Norway hosts around 470 known populations of noble crayfish (*Artsdatabanken 2020*). Although the number of populations may at first glance suggest the contrary, this species is classified as endangered (EN) on the Norwegian redlist and vulnerable (VU) on the international IUCN red list (*Edsman et al. 2010, Henriksen and Hilmo 2015*). Here, the current evaluation is based on the possibly irreversible population reduction (criteria code: A) according to the 2015 Norwegian redlist (*Henriksen and Hilmo 2015*).

The first study with crayfish and eDNA was conducted in 2014 by Tréguier and colleagues (*2014*) who explored the possibilities of detecting the invasive freshwater species *P. clarkii* from water samples. Two years later, two more studies and species-specific PCR assays were published for the Japanese crayfish *Cambaroides japonicus* (*Ikeda et al. 2016*) and for another North American species, the rusty crayfish *Faxonius rusticus* (*Dougherty et al. 2016*). Since then, several studies have been published with assays for both indigenous (*Agersnap et al. 2017, Cai et al. 2017, Robinson et al. 2018, Atkinson et al. 2019, Troth et al. 2020*) and non-indigenous crayfish species (*Dunn et al. 2017, Larson et al. 2017, Cowart et al. 2018, Harper et al. 2018, Mauvisseau et al. 2018, Rice et al. 2018, Mauvisseau et al. 2019b, Chucholl et al. 2021*).

Apart from two assays targeting the *16S* sequence (*Robinson et al. 2018*) and *cytochrome c oxidase subunit III* sequence (*Geerts et al. 2018*), all currently published assays target the *COI* sequence. This sequence of this gene displays sufficient inter-specific variance to distinguish

successfully between indigenous and non-indigenous crayfish species. However, in regions with higher crayfish species biodiversity than Europe, closely-related species that often differ only marginally in the targeted DNA sequence can co-occur and thus complicate species-specific differentiation (*Larson et al. 2017, Mauvisseau et al. 2019b*).

Aphanomyces astaci

The biggest threat to noble crayfish and in fact any crayfish species indigenous to Europe is the oomycete *Aphanomyces astaci* Schikora, 1906. This fungal-like watermould is the causative agent of crayfish plague, a lethal disease to which all native European crayfish species are susceptible, although with exceptions addressed below. The high level of threat which this organism poses is evinced by its inclusion in the IUCN list of 100 of the world's worst invasive alien species (*Lowe et al. 2004*) and the OiE (*OIE 2019a*). In Norway it is registered as a list 3 disease in the "Regulation on animal health requirements for aquaculture animals and products thereof, prevention and control of infectious diseases in aquatic animals" (FOR 2008-06-17-819). *Aphanomyces astaci* was first described in 1903 by Shikora and confirmed as the causative agent of crayfish plague thirty years later (*Nybelin 1934*).

Aphanomyces astaci is a parasite of North American crayfish with a shared history of evolution *(Unestam 1969, Unestam 1972)*. Through co-evolution, crayfish in North America have developed defence mechanisms and immune responses against the pathogen and thus they can survive infections. In the event of an infection, hyphae of *A. astaci* penetrate the chitinous crayfish carapace and ramify into the cuticle from a germination plug (*Unestam and Weiss 1970*). In North American crayfish, the growing tips are encapsulated with melanin (*Cerenius et al. 2003, Cerenius et al. 2008*), and are often visible as black spots on the cuticle. In susceptible crayfish, the hyphae penetrate into the tissue and organs, causing the crayfish to die (*Alderman and Polglase 1988*). From hyphae in the cuticle, sporangia grow to the outside of the cuticle, forming primary spores that are released as spore-balls from which swimming zoospores emerge (*Andersson and Cerenius 2002, Vrålstad et al. 2006*). These zoospores, which constitute the infective stage, can survive through encystation for a short period of time in the absence of a host (*Cerenius et al. 1988*).

The pathogen can easily and unintentionally be spread from one waterbody to others. A mundane example could be the use of fishing gear which is improperly dried or not disinfected when the owner moves from one fishing ground to a second one. Throughout Europe, the spread of *A. astaci* has been facilitated unwittingly, either as a result of insufficient information or more recently due to the reckless disregard of existing regulations (*Jussila and Edsman 2020*).

Initially, this pathogen was introduced into Europe around 1859, where it was first noticed in the River Po in Italy (*Alderman 1996*). From there it spread and led to mass mortalities of native crayfish throughout the whole of Europe, wherever American crayfish species had been introduced (*Holdich et al. 2009*). By 1971, the first genotype of *A. astaci* that came to Europe (genotype "As") had reached Norway (*Vrålstad et al. 2014*). Within this period of over one and a half centuries since the initial introduction, several outbreaks of crayfish plague have occurred as a direct result of the importation of various North American crayfish species, most notably *P. leniusculus*, *F. limosus* and *P. clarkii* (*Holdich et al. 2009*). Analysis of random amplified polymorphic DNA (RAPD) was the first method used for the discovery of and distinction between several groups of genotypes of *A. astaci* (*Huang et al. 1994*). Group A (genotype "As") constitutes strains of the original invasion of which the American crayfish host species remains unknown. Groups B (Genotype "PsI") has been linked to *P. leniusculus* of North American origin. Group D (Genotype "Pc") and Group E (Genotype "Or") were originally isolated from the North American species *P. clarkii* and *F. limosus* respectively (*Grandjéan et al. 2014, Svoboda et al. 2017*). It is, therefore, widely accepted that *A. astaci* originated in North America, evolving crayfish species-specific genotypes. However, recent findings suggest the contrary that *A. astaci* is widely distributed and genetically diverse with a likely origin in the south-eastern US, with no clear species-specificity or geographical patterns (*Martín-Torrijos et al. 2021*). While the RAPD-method only enables genotyping of pure culture isolates, microsatellite analyses have been developed to determine genotype status directly from tissue samples (*Grandjéan et al. 2014)*. Another method targeting the mitochondrial DNA (mtDNA) genes also allows for detection and haplotyping of *A. astaci* from clinical samples (*Makkonen et al. 2018*). Recently, a further genotyping method was published on the basis of whole genome sequencing, which to a large extent confirms the originally described genotypes A-E (*Minardi et al. 2019*).

For diagnostic purposes, sequencing and/or qPCR targeting of the *ITS*-region that is commonly used as barcoding marker in fungi (*Schoch et al. 2012, Badotti et al. 2017*), has served as a golden standard for molecular diagnostics of crayfish plague from crayfish tissue samples for the past 15 years (*Oidtmann et al. 2006, Vrålstad et al. 2009*). It is recommended as diagnostic marker in the OIE diagnostic manual (*OIE 2019a*) and is more specific and sensitive than a qPCR assay targeting the GH18 chitinase family genes (*Hochwimmer et al. 2009*).

Current eDNA monitoring of *A. astaci* is carried out using the *ITS*-assay developed by Vrålstad et al. (*2009*) and modified by Strand (*2013*). This assay is 100 times more sensitive than the

GH18-gene qPCR assay (*Tuffs and Oidtmann 2011*) and detects down to one zoospore as this contains more than 100 DNA-copies of the *ITS*-region (*Strand et al. 2011, Tuffs and Oidtmann 2011*). The method was first tested for eDNA purposes by Strand and colleagues (*2011*) by applying the assay developed by Vrålstad *et al*. (*2009*) directly to water samples. Further investigation using this eDNA method on the ambient water of signal crayfish revealed steady sporulation of *A. astaci* in latent carrier crayfish and the influence of temperature on detectable spores (*Strand et al. 2012*). Analysis of the sporulation dynamics of *A. astaci* from susceptible noble crayfish disclosed an increase of spore production from infection to death (*Makkonen et al. 2013*). Furthermore, a comparison of depth filtration (5 L) and dead-end ultrafiltration (~100 L) (*Strand et al. 2014*) showed depth filtration to be less labour-intensive and the samples obtained were less prone to PCR inhibition.

Knowledge gaps

The body of knowledge regarding the application of eDNA for surveillance, inventories or the exploration and resolution of biological questions is constantly growing (see *Leese et al. 2016 and references therein*). Methods based on eDNA as representative unit for the organisms themselves are being considered as a supplement to, or even the possible replacement of, conventional biomonitoring (*Jerde et al. 2013, Biggs et al. 2015, Buss et al. 2015, Smart et al. 2015, Bylemans et al. 2016, Leese et al. 2016, Vrålstad et al. 2017*). Still, there are many knowledge gaps regarding the dynamics of eDNA in general, including host-pathogen complexes, such as eDNA emission and degradation rates in relation to the different but yet interconnected organisms, biological properties, and the different organism eDNA responses to environmental factors.

Several studies examine the downstream transport or stratification of eDNA (*Deiner and Altermatt 2014, Deiner et al. 2016, Rice et al. 2018, Lawson Handley et al. 2019*). Others have analysed the correlation between biomass of the target organism and eDNA quantity (*Takahara et al. 2012, Doi et al. 2015b, Doi et al. 2017, Fukaya et al. 2020*) and even the eDNA emission during various stages of life – and death (*Kamoroff and Goldberg 2018, Curtis and Larson 2020*). Further studies investigate the effect of ambient (water) temperature (*Buxton et al. 2017, Jo et al. 2019*) and other biotic and abiotic factors (*Pilliod et al. 2014, Stewart 2019*). All these studies have made valuable contributions towards a better understanding of eDNA. However, the focus is mostly on single (invasive) species or pathogens (*Agawa et al. 2016, Gomes et al. 2017, Trujillo-González et al. 2019, Sieber et al. 2020*). There is also a multitude of studies

focussing on salmonid fish hosts (*Matejusová et al. 2008, Wilcox et al. 2015, Atkinson et al. 2018*), but the eDNA dynamics of host-pathogen complexes have received less attention.

For the design and implementation of eDNA as a monitoring tool and to improve current eDNA-based monitoring approaches, more in-depth knowledge on the eDNA dynamics of host-pathogen complexes is required. Understanding detection limits and thresholds and organism-specific emission patterns will help evaluate and improve the efficiency of eDNA monitoring and help to determine how and when eDNA monitoring is applicable.

In this thesis, two completely different aquatic host-pathogen models were studied. For the two chosen host groups, salmonid fish and freshwater crayfish, the differences might yield fundamentally different outcomes. Fish have been shown to shed considerable amounts of eDNA, particularly from the mucus layer covering the scales (*Merkes et al. 2014, Klymus et al. 2015, Takeuchi et al. 2019*) compared to crustaceans (*Forsström and Vasemägi 2016, Fossøy et al. 2020, Crane et al. 2021*) that are covered with a hard chitinous carapace. Thus, eDNA monitoring is likely not to be equally efficient for the two groups, and sampling method adjustments will clearly be needed. Regarding the two chosen parasites or pathogens, both are undisputedly considerably smaller than their respective hosts in terms of biomass, but perhaps not in terms of eDNA detectability. Previous studies indicate that *A. astaci* is readily detectable using the eDNA methodology (*Strand et al. 2011, Strand et al. 2012, Makkonen et al. 2013, Strand et al. 2014*) as a result of (often massive) production of swimming, single-celled zoospores in the water. Conversely, no eDNA studies on *G. salaris* had been conducted when this thesis project started. Due to its size and biology, we expected that *G. salaris* sheds very little eDNA. Thus, the two models examined in this thesis consist of one host shedding large amounts of eDNA and an ectoparasite with presumed low eDNA emission, while the other host has presumed low eDNA emission coupled with an endoparasite shedding relatively large amounts of eDNA/spores. How these and other aspects influence the effectiveness and reliability of eDNA monitoring of the chosen host-pathogen complexes constitute knowledge gaps this thesis aims to reduce.

Thesis objectives

Principal objective

The overarching goal of this thesis was to explore, develop and evaluate the potential of targeted eDNA detection and quantification as surveillance and biosecurity tool for two highly different host-pathogen complexes (i.e. freshwater crayfish – together with *A. astaci* and Atlantic salmon – together with *G. salaris*). These host-pathogen complexes were chosen as models of relevance to natural aquatic habitats, aquaculture and aquarium trade. Special emphasis was placed on the detection of elusive targets with assumed differences in their emission or production of eDNA. To this end the following research questions were posed:

Research questions (RQ)

1. Can the eDNA methodology work equally well or better than conventional methods for biomonitoring of the host-pathogen models, particularly at low prevalence? (Papers **I-V**)
2. Can eDNA copy numbers serve as a proxy for host density and pathogen intensity? (Papers **IV, V**)
3. How will environmental factors and organism biology influence the emission and detectability of host-pathogen eDNA? (Papers **II, IV, V**)

In order to answer these questions, the following sub-goals were identified:

a. Develop, optimise and apply thoroughly validated species- and/or variant specific assays when these are missing for the target organisms studied in this thesis (Papers **I, III, IV**)
b. Perform proof-of-concept for eDNA detection and dynamics for the two aquatic host-pathogen complexes under field conditions (Papers **I, II, III, V**)
c. Determine the minimum-number of parasites per fish for reliable eDNA detection of *G. salaris* eDNA in water samples (Paper **IV**)
d. Explore whether meaningful semi-quantitative estimates of host number and/or pathogen load can be derived from eDNA copy numbers (Papers **IV, V**)
e. Explore if droplet digital PCR (ddPCR) offers advantages over qPCR for aquatic eDNA monitoring of these host-pathogen models (Papers **I, IV, V**)

Materials and Methods

This PhD project was conducted in collaboration with the research project TARGET (**II, V**), and the Norwegian surveillance programs for *Aphanomyces astaci* (**II, V**), and *Gyrodactylus salaris* (**I, IV**) at the Norwegian Veterinary Institute. The Central European study (**III**) was conducted in collaboration with the Charles University Prague, Czech Republic. The project applied and refined pre-existing filtering/pumping methods for eDNA sampling and sample storage. Where necessary, assays were developed or optimised for qPCR and/or ddPCR (**I, II, III**). The assays and methods were tested and validated in the field at several locations. With all methods in place, two infection trials with live animals were conducted in the shared aquarium facilities of the Norwegian University of Life Sciences (NMBU)/NVI. This section will provide a brief overview of the study areas, species examined and the methods applied in the **papers I-V** of this thesis.

Study areas

The locations for eDNA water sampling in this PhD project and in some cases also the source of biological material and live organisms for aquarium trials are shown in Figure **2** (Norwegian and Swedish locations) and Figure **3** (Central European locations). For the field study of *G. salaris* and its hosts (**I**) eDNA samples were collected from the Drammenselva watercourse. Here, live infection material of *G. salaris* was also collected for the infection study (**IV**). The field study of *A. astaci* and its hosts (**II**) followed a crayfish plague outbreak in the Halden watercourse. Here, live signal crayfish were captured for the aquarium study (**V**), in which also field samples from the Halden watercourse and the Swedish lake Stora Le were included. Finally, eDNA samples from a broad range of locations in Western and Central Europe comprised the sample material for **paper III** of this thesis.

Drammenselva watercourse:

The Drammenselva watercourse originates in central Norway and drains into the Atlantic Ocean in the Drammensfjord (Viken County) and lies within the so-called Drammen infection region (*Miljødirektoratet 2014a*). The Drammen infection region contains four of the eight remaining rivers in Norway where *G. salaris* is still present (*Hansen et al. 2021a*). This watercourse was infected in the 1980s via infected rainbow trout introduced to fish farms in the system. From these farms it presumably spread to the Lake Tyrifjorden and further to the rivers Drammenselva and Lierelva in the area known today as Viken County (*Mo 1991, Johnsen et al. 1999, Hansen et al. 2003*). All fish in all the farms that were found to be infected were culled and from that time on the presence of the parasite in this upstream area has been unknown but

presumed absent. The river Sandeelva further out in the Oslofjord was infected in 2003 and Selvikvassdraget was infected as late as in 2019 (*Hytterød et al. 2020b*). Døvikfoss below Lake Tyrifjorden is an absolute migration barrier for salmonids and has prevented upstream spreading of *G. salaris* into the northern parts of the Drammenselva watercourse. In **paper I**, water samples were taken along the watercourse for eDNA analysis to supplement standard surveillance methods for *G. salaris*, Atlantic salmon and rainbow trout in the Drammen infection region. For **paper IV**, live *G. salaris* specimens were obtained from River Lierelva for the infection trials.

Figure 2: A map of Norway and Sweden showing the locations where water samples were taken during this PhD project. Sampling locations are indicated by small black dots. The locations are the following: 1 - River Driva , 2 - Storåne, 3 - River Begna, 4 - Nes (2-4 Drammen watercourse), 5 - Fossersjøen, 6 - Dalstorpfoss, 7 - Hemnessjøen, 8 - Kroksund, 9 - Øymarksjøen (5-9 Halden watercourse), 10 - Lake Stora Le. Photos by Johannes C. Rusch.

Halden watercourse

The Halden watercourse is comprised of several lakes interconnected through rivers and channels in south-eastern Norway. The first outbreak of crayfish plague in this watercourse was reported in 1989 (*Taugbøl et al. 1993*). In 1995, noble crayfish were successfully re-stocked (*Taugbøl 2004*) but reintroduction efforts were thwarted in 2005 through another outbreak of crayfish plague in Lake Øymarksjøen and further downstream (*Vrålstad et al. 2009*). As a result, the water locks at Ørje were locked and this worked as an efficient infection and migration barrier against the signal crayfish, which had been discovered in 2008 in the southern part of the lake and appeared to constitute an established population with high *A. astaci* prevalence and infection load (*Vrålstad et al. 2011*). In 2014, signal crayfish that had presumably been illegally transported were discovered above the closed water locks in Lake Rødenessjøen (*Miljødirektoratet 2014b*). The national surveillance programme for *A. astaci,* which includes the Halden watercourse, has been carried out since the late 1980s (*Johnsen and Vrålstad 2017*) and since 2016 has utilised eDNA monitoring (*Vrålstad et al. 2017*). **Paper II** follows a crayfish outbreak and the progression through the Halden watercourse throughout a four-year period with both conventional surveillance methods and eDNA methodology. Infected signal crayfish were obtained from Lakes Øymarksjøen and Rødenessjøen for the mesocosm experiment in **paper V** and water samples were obtained for comparison with CPUE data in the same paper.

Stora Le

Lake Stora Le is located in western Sweden with parts of it reaching into south-eastern Norway and it is situated close to Lake Øymarksjøen of the Halden watercourse. Crayfish plague was suspected in 1989 after the discovery of dead noble crayfish on the Norwegian side (*Taugbøl et al. 1993*), and the outbreak in the Halden watercourse the same year is assumed to stem from infection transfer from its neighbouring lake Stora Le. However, no dead noble crayfish were found on the Swedish side of the lake and subsequent cage experiments at the outlet of the lake failed to detect the crayfish plague. Signal crayfish were first officially discovered in three localities in 2002, although they had been in the lake for several years before, according to local fishermen (*Jansson 2017*). Since 2004, yearly monitoring has been carried out to survey the spread and population development of the signal crayfish populations (*Jansson 2017*). For **paper V**, water samples and CPUE data were obtained from this lake.

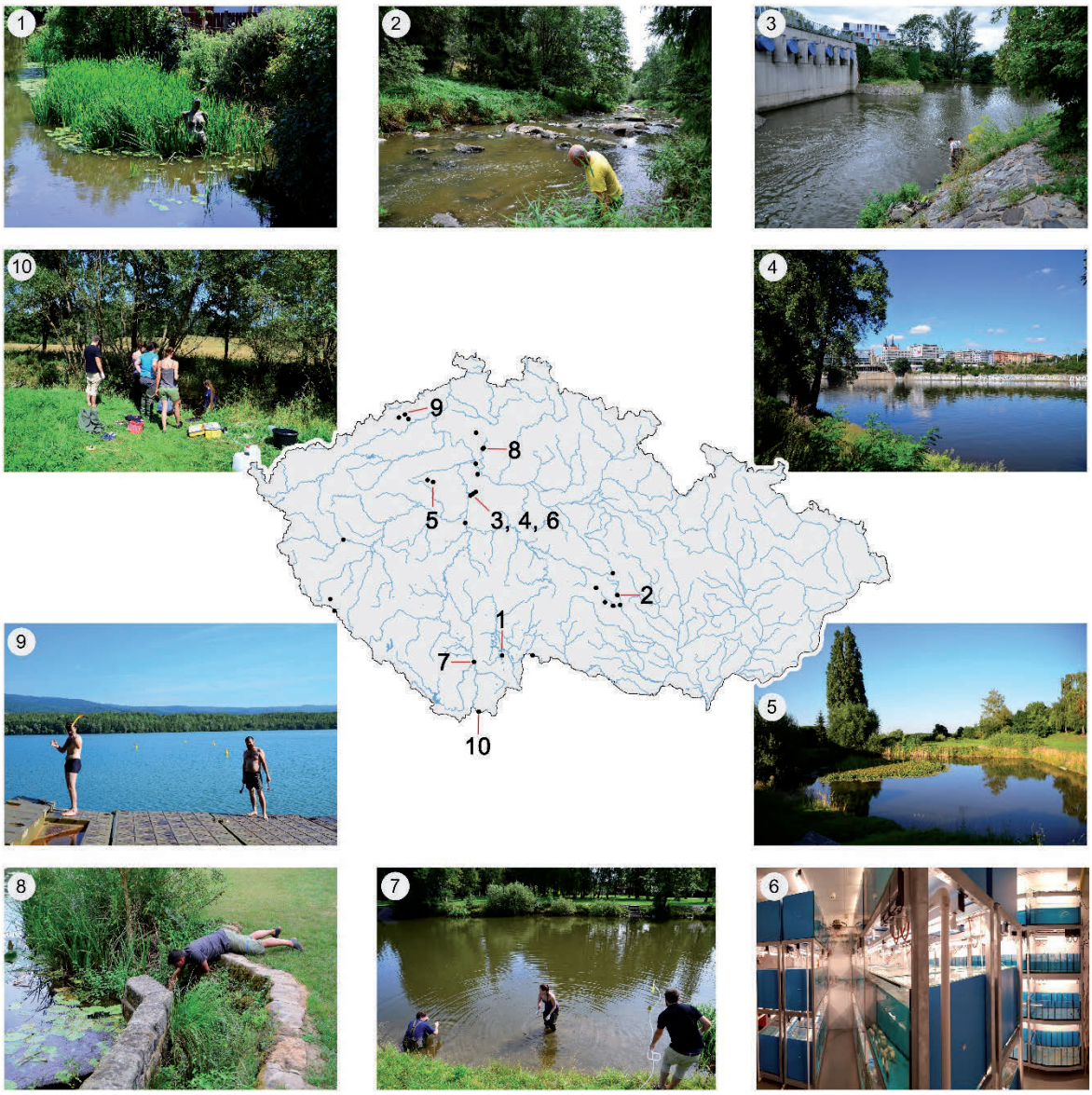

Figure 3: A map of Czechia showing the locations where water samples were taken during this PhD project. Sampling locations are indicated by small black dots. The locations with the closest town in brackets are the following: 1 - Zlatá stoka (Třeboň), 2 - Oslava (Velké Meziříčí), 3 - Rokytka (Prague), 4 - Vltava (Prague), 5 - Urban pond (Smečno), 6 - Aquariums at Czech University of Life Sciences (Prague), 7 - Malše (České Budějovice), 8 - Pšovka (Harasov), 9 - Barbora (Teplice), 10 - Malše (border with Austria). Photos by Johannes C. Rusch.

Central and Western Europe

Sampling in Central and Western Europe, with the main focus on the Czech Republic, was carried out in a total of 32 multifarious waterbodies ranging from large rivers to small brooks, from natural lakes to man-made reservoirs and from fishponds to flooded quarries. Czechia hosts three European crayfish species (noble crayfish *Astacus astacus*, stone crayfish *Austropotamobius torrentium* and the narrow-clawed crayfish *Pontastacus leptodactylus*) of

which the latter was introduced into the country in the late 19th century (*Štambergová et al. 2009*). Three North American crayfish species are also documented in the country. *Faxonius limosus* invaded the Elbe river in the 1960s (*Petrusek et al. 2006*) and *Pacifastacus leniusculus* was introduced for aquaculture purposes in 1980 (*Filipová et al. 2006*). Both species are widespread in some parts of the country. *Procambarus virginalis* has only recently been found and its occurrence is presumed a result from aquarium releases (*Patoka et al. 2016*). The crayfish plague first reached Czechia in the late 19th century (*Kozubíková et al. 2006*) and outbreaks have been recorded ever since (*Mojžišová et al. 2020*).

Additional water samples were taken from two lakes in Berlin with recently reported presence of *P. virginalis* and *F. limosus* (*Linzmaier et al. 2018*) (*A. Mrugała, pers. comm.*) and from a river in Budapest where *P. virginalis*, *F. limosus* and other non-indigenous crayfish species have been found (*Weiperth et al. 2017, Szendőfi et al. 2018, Veselý et al. 2021*). For the latter samples, water was filled into sterile 10 L cans by colleagues and transported to the laboratory while being kept cool and dark.

Animal trials

All experimental procedures involving live animals were conducted in accordance with the Norwegian Animal Welfare Act (Dyrevelferdsloven, LOV-2009-06-19-97) and EU regulations (EU Directive; 2010/63/EU). For crayfish trapping and acquisition as well as cage-surveillance with live crayfish in the Halden watercourse (**II**), all necessary permits were obtained through the collaborative projects. Here, the Norwegian Food Safety Authority, the County Governor and the Norwegian Environment Agency gave permits for sampling, capture and caging activities in crayfish plague infected locations (**II**), including the capture of live signal crayfish in the Halden watercourse for aquarium trials (**V**). The infection trial with *G. salaris* (**IV**) was approved by the Ethics Committee of the Norwegian Food Safety Authority (Mattilsynet) (FOTS ID 12081). Both the infection trial with *G. salaris* on Atlantic salmon (**IV**) and the temperature/density experiment on signal crayfish (**V**) were carried out in the secure research aquarium facility of the NVI/ (NMBU) in Oslo which is fully licensed to accommodate experiments on salmonids and decapod crustaceans. The water used in the aquarium facilities originated from Lake Maridalsvann, the drinking water reservoir of Oslo.

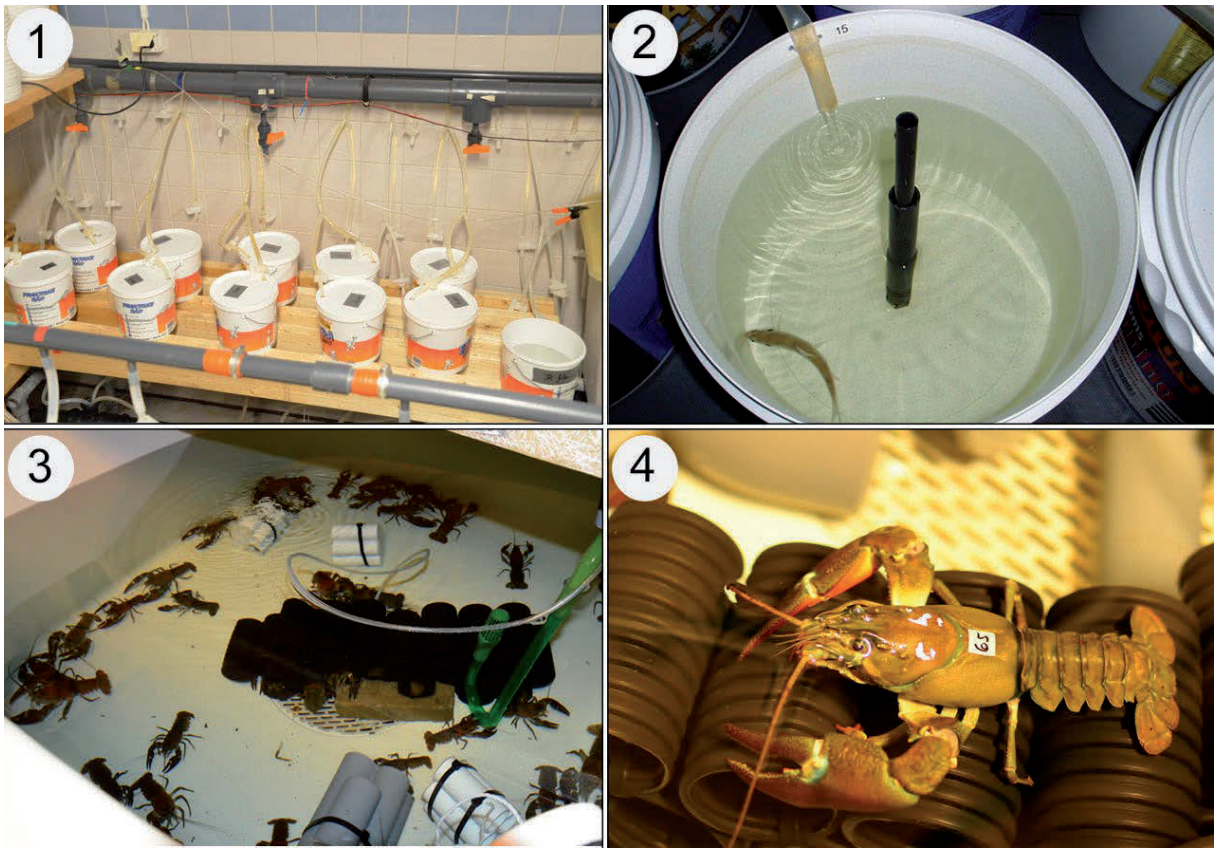

Figure 4: The top half of the figure (1&2) shows the experimental setup for **paper IV**. Ten buckets containing four litres of water and one juvenile Atlantic salmon each, eight of which were infected with *G. salaris* and two non-infected controls to the far left. Each bucket was individually supplied with constantly flowing fresh water. The bottom half of the figure is a photo of the communal crayfish tank used in **paper V**, in which the signal crayfish were housed and provided with shelter (3) and a signal crayfish with markings (4). Photos by Johannes C. Rusch (1,3,4) and Sigurd Hytterød, NVI (2).

Species

The following species were studied during the course of this thesis: Noble crayfish - *Astacus astacus* (native to Europe), Signal crayfish - *Pacifastacus leniusculus* (non-native), Marbled crayfish - *Procambarus virginalis* (non-native), Spiny cheek crayfish - *Faxonius limosus* (non-native), Crayfish plague agent - *Aphanomyces astaci* (non-native) (see Figure **5**). Atlantic salmon – *Salmo salar* (native), Rainbow trout - *Oncorhynchus mykiss* (non-native), Salmon fluke – *Gyrodactylus salaris* (native to the Baltic region) (see Figure **6**)

Figure 5: All crayfish species included in this thesis along with the crayfish plague agent. 1) Noble crayfish (*Astacus astacus*), 2) Signal crayfish (*Pacifastacus leniusculus*), 3) Marbled crayfish (*Procambarus virginalis*), 4) Spiny cheek crayfish (*Faxonius limosus*), 5) Hyphae balls of *Aphanomyces astaci* (Crayfish plague agent) in culture. Photos by Johannes C. Rusch.

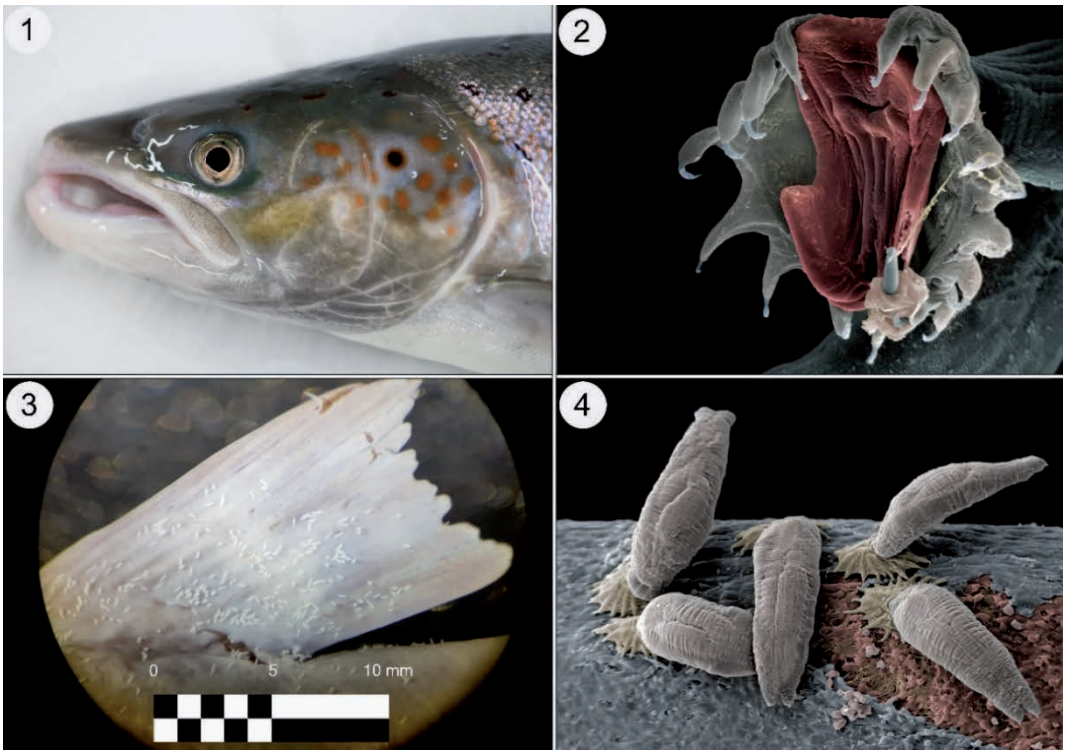

Figure 6: The host-pathogen complex Atlantic salmon and *Gyrodactylus salaris*: 1) Adult salmon from River Driva, 2) scanning electron microscopy (SEM) photo of the attachment organ (haptor) of *G. salaris* with the peripheral marginal hooks, 3) juvenile salmon highly infected with *G. salaris* (EtOH fixed), and 4) SEM image of *G. salaris* feeding on the mucus layer of Atlantic salmon. Photos: Sigurd Hytterød, NVI (1), Jannicke Wiik-Nielsen, NVI (2, 4) and Johannes C. Rusch (3).

Monitoring methods

Conventional methods

To determine the infection prevalence and intensity of *G. salaris* as well as to determine the species of fish present in the rivers analysed, electrofishing conducted by trained personnel was carried out (**paper I**). All fish captured for further examination were euthanised following the strict codes of practice in force in Europe and preserved intact in 96% ethanol for further examination. Visual inspection of the fish was carried out at a laboratory using a stereo-microscope to detect and count the *Gyrodactylus* specimens on the fish (see Figure **6**). The same counting procedure was used in **paper IV** to track the development and number of parasites per fish.

In **paper II** conventional cage monitoring was used for the surveillance of *A. astaci*. This was done as part of the surveillance program for *A. astaci* (*Vrålstad et al. 2017*), and involved 10 live noble crayfish per cage, provided with shelters and food and inspected twice a week by local landowners. If mortalities were recorded, dead crayfish were removed and stored on ice for further crayfish plague diagnostics at the laboratory. For population density estimates of crayfish (**II, V**), conventional trapping procedures were followed (*Johnsen et al. 2019*) in collaboration with ongoing surveillance of freshwater crayfish in Norway and Sweden. In the Central European study (**III**), hand-sampling was used to determine presence of crayfish. Where possible, snorkelling was also carried out.

The sampling and filtration of water

All eDNA samples were taken by filtering water through glass fibre filters (47 mm AP25 Millipore, 2 µm pore size, Millipore, Billerica, USA). This was done in one of two ways. Either using an in-line filter holder (Millipore) with tygon tubing (Masterflex) and a portable peristaltic pump (Masterflex E/S portable sampler, Masterflex, Gelsenkirchen, Germany) as first established by Strand *et al*. (*2014*) (**I, II, III, V**) or the filters were placed into filter cups (Nalgene Analytical Test Filter Funnel, 145-0045, Thermo Fisher Scientific, Waltham, USA) after removal of the original filter provided by the manufacturer (**III**). In the latter case, pumping was carried out by attaching the provided filter-cup adapter to a ¾ inch garden water hose and a drill-operated pump (Product code 1490-20, Gardena, Ulm, Germany). For the infection trial on Atlantic salmon (**IV**) the filter cups were attached to the tygon tubes and water was pumped using the aforementioned portable peristaltic pump.

When using the peristaltic pump (**I, II, III, V**), the front end of the tygon-tube was fastened to the inside of a plastic box to which a piece of diving-lead was fastened on the bottom. The

plastic box was later replaced with a stainless-steel box. These plastic / stainless-steel boxes were slowly sunk onto the bed of the brook or pond close to the centre. In the case of wider rivers or lakes, they were placed between 2 m and 5 m from the bank. To prevent the filter from being clogged by sediments from the bed of the waterbody, water was pumped through the tubes for several minutes prior to placing the filter into the holder. With the filter in place in the holder, water was then pumped through the filter using pressure. The filtered water was collected in a 5 L plastic can.

Samples taken with the filter cups (**III, IV**) were not obtained from the waterbed but rather from the middle of the water column (in the buckets used in the infection trial in **paper IV**) or from a short depth below the surface in streams. Here, the filters and filter cups and the interchangeable tube connected to the pump were submerged into the water. Since they were situated at the front end of the pumping system they filtered water using suction. As with the other pump, the filtered water was collected in a 5 L plastic can. During the *G. salaris* infection trial (**IV**), the filter cups were covered with a 50µ plankton mesh that had been attached using a glue gun. This measure was implemented to filter only cells and particles of *G. salaris* to get an idea of the sources of eDNA from this parasite other than the whole parasite itself. The mesh size was determined to be too small to let entire dead parasites through and onto the filter.

Figure 7: The two pump systems used during this PhD project. 1) The Masterflex portable peristaltic pump, 2) the in-line filter holder on top of a 5 L plastic can. 3) the drill-powered pump and 4) a filter being removed from the detachable filter-cups (4). Photos by Johannes C. Rusch.

Storage methods

Several methods to store the filters before the DNA could be extracted were used and further developed during the sampling period in this thesis. At first, filters were simply put into sterile 15ml "falcon type" tubes, stored on ice and frozen upon arrival at the laboratory (**I, II**). Prior to DNA extraction the filters were frozen to -80 °C and then freeze dried for 24 h in batches, using a freeze dryer (Heto drywinner, Thermo Fisher Scientific, Waltham, USA). The next method used was to put the filters into sterile falcon tubes already containing 4 ml of CTAB buffer which were stored on ice and frozen upon arrival at the lab (**III, V**). The last method is to fold the filters down the middle using a sterile single-use forceps and sealing them in a zip lock bag containing approximately 30 g of silica gel **(III**). This method eliminates the necessity for storing the filters on ice; they can simply be stored in an opaque box. The two latter methods are not dependent on a rapid return to the laboratory.

Molecular methods

DNA extraction and controls

In all papers the CTAB protocol used in Vrålstad *et al*. (2009) with the modifications suggested by Strand *et al*. (*2014*) was followed. This classic DNA extraction method was first described by Murray & Thompson (*1980*). The reason for choosing this older and more robust method over newer extraction kits is the large volumes of buffer required to extract DNA from the type of filters used during this project. DNA blank controls and laboratory environmental controls were included with each batch of extracted samples (see discussion of methods). In the aquarium experiments **(IV, V**), additional controls were taken from the inlet water. No negative field sample controls were included during eDNA sampling.

qPCR and ddPCR

Both quantitative real-time PCR (qPCR) and droplet digital PCR (ddPCR) rely upon the basic principles of PCR with amplification of a chosen target DNA fragment with primers. For species-specific or group-specific detection from environmental samples, the primers need to be designed for selective amplification of a DNA fragment with a unique signature (or barcode) for the respective species or group. However, while conventional PCR relies on DNA sequencing for further identification, the qPCR approach specifically detects unique (e.g. species-specific) DNA motifs with so-called hydrolysis probes which attach to single strand DNA (ssDNA) during the annealing phase (*Holland et al. 1991*). Hence, putatively specific

PCR products are detected only if the amplified sequence contains the complementary probe motif. The short TaqMan MGB probes used in the studies of this thesis are extremely sensitive to mismatches at the annealing site (*Yao et al. 2006*), which is particularly useful for design of unique diagnostic assays, e.g. the *A. astaci* qPCR assay (*Vrålstad et al. 2009*). The fluorescence emitted from the reporter dye is measured after each amplification cycle i.e. in real-time. The data output is essentially the quantification cycle (Cq) value, also called cycle threshold (Ct) value. This value indicates at which point the amount of fluorescence representing the amount of DNA is sufficient to cross a threshold line. By simultaneously running standard curves with a known amount of DNA copy numbers (or PCR forming units; (*Berdal et al. 2008, Vrålstad et al. 2009*)), it is possible to calculate the amount of DNA copies of the target gene (e.g. *COI*), or DNA-region (e.g. *ITS*) in a sample. The data output is given both as a numerical value and as a visualised amplification plot (Figure **8**). Quantitative PCR was used in **papers II** and **III** for analysis of eDNA samples and in **papers I** and **V** for comparison with ddPCR.

The ddPCR system relies on the same type of probes but prior to the actual PCR reaction, the entire reaction mix is partitioned into up to 20,000 micelles or "droplets" in an oil emulsion (*Baker 2012*). Instead of one reaction per well like in conventional PCR and qPCR, the ddPCR reaction occurs in every single droplet, potentially facilitating up to 20.000 reactions per well, if target DNA is present in the droplet. The absence, presence and intensity of fluorescence is measured after the end-point of PCR cycling (Figure **9**) as opposed to qPCR where it is measured after each respective cycle. Based on the number of positive droplets related to the total numbers of droplets, the analysis software calculates the absolute number of target DNA copies in a sample using Poisson distribution. All assays developed during the thesis were optimised for ddPCR. For all crayfish species (**paper III**), the commonly used *COI* marker (*Hebert et al. 2003, Waugh 2007, Badotti et al. 2017*) was targeted whereas the initial species-specific assay for *G. salaris* (**paper I**) was designed to target the *ITS* marker. The haplotype-specific assays for *G. salaris* (**paper IV**) also targeted the *COI* marker.

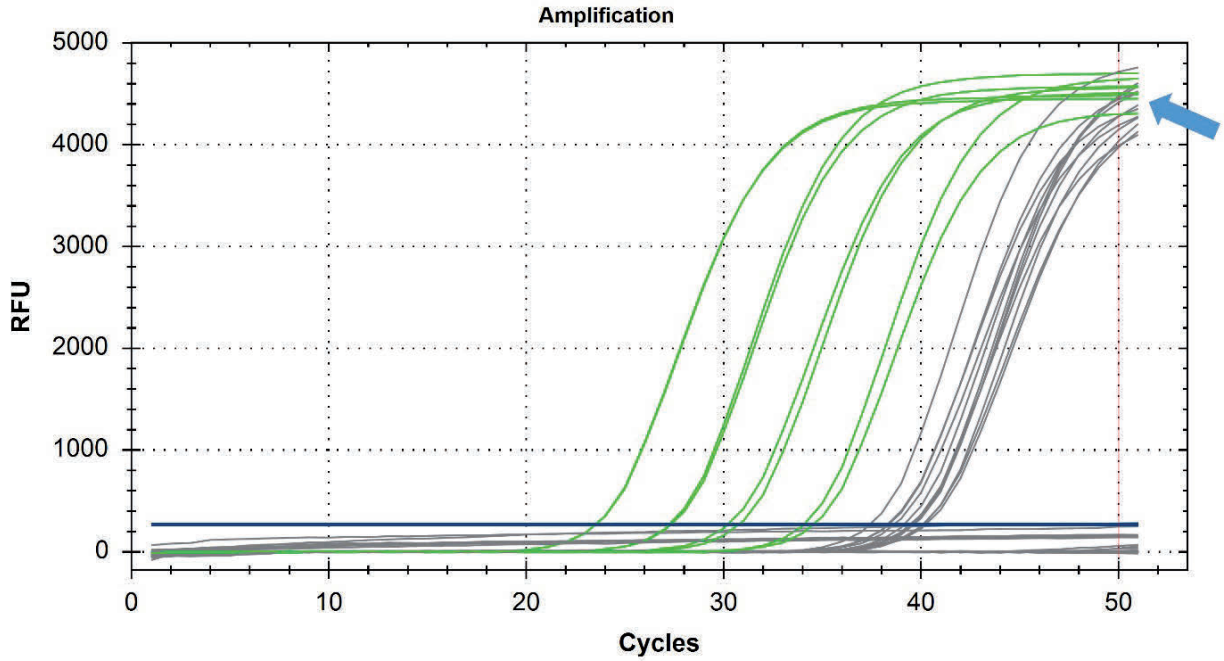

Figure 8: qPCR amplification plot of signal crayfish detection. The green lines represent the standards with a known concentration of DNA, used for calculating the DNA concentration in the samples. The grey lines represent the samples analysed. The horizontal blue line depicts the threshold which is used to determine the Cq/Ct value at which the DNA concentration in a reaction is sufficient to cross the threshold. The image was copied from the Biorad CFX software and slightly edited by adding a blue arrow and a light red vertical line to indicate "endpoint amplification".

Statistical methods and calculations

For the calculation of the number of eDNA copies/l in ddPCR samples we used a formula first published by Agersnap *et al*. (*2017*) (see **paper I** for details). All statistical analyses were carried out using R version 3.5.1 (**paper II**), version 4.0.2 (**paper IV**) and version 4.0.3 (**paper V**) (*R Core Team 2020*). In **paper II**, the estimated eDNA concentrations of the target species were tested for correlation using Spearman-rank correlation. The main statistical analysis in **paper IV** consisted of modelling the probability of positive detection of *G. salaris* in a sample as a function of mean parasite intensity. This was done using binomial linear models with logit links and the *glmmTMB* package (*Brooks et al. 2017*). We also calculated the number of samples required for a positive *G. salaris* detection at several infection intensities, representing low (100), medium (500) and high (1000) infection intensities respectively. For this purpose, we used the same approach as in the *dose.p()* function of the *MASS* package (*Venables and Ripley 2002*). In **paper V**, we statistically compared the qPCR and ddPCR results regarding their correlation. We also calculated the influence of temperature, density and food availability on the amount of detectable eDNA, again using generalized linear models and the *glmmTMB* package (*Brooks et al. 2017*). Furthermore, we used 3-level hierarchical occupancy models on data from field-samples to calculate the detection probability at different crayfish densities.

Occupancy modelling was carried out using the *msocc* package (*Stratton et al. 2020*) which was chosen due to its speed and efficiency. For visualisation of (statistics) results we used the *ggplot2* package (*Wickham 2016*).

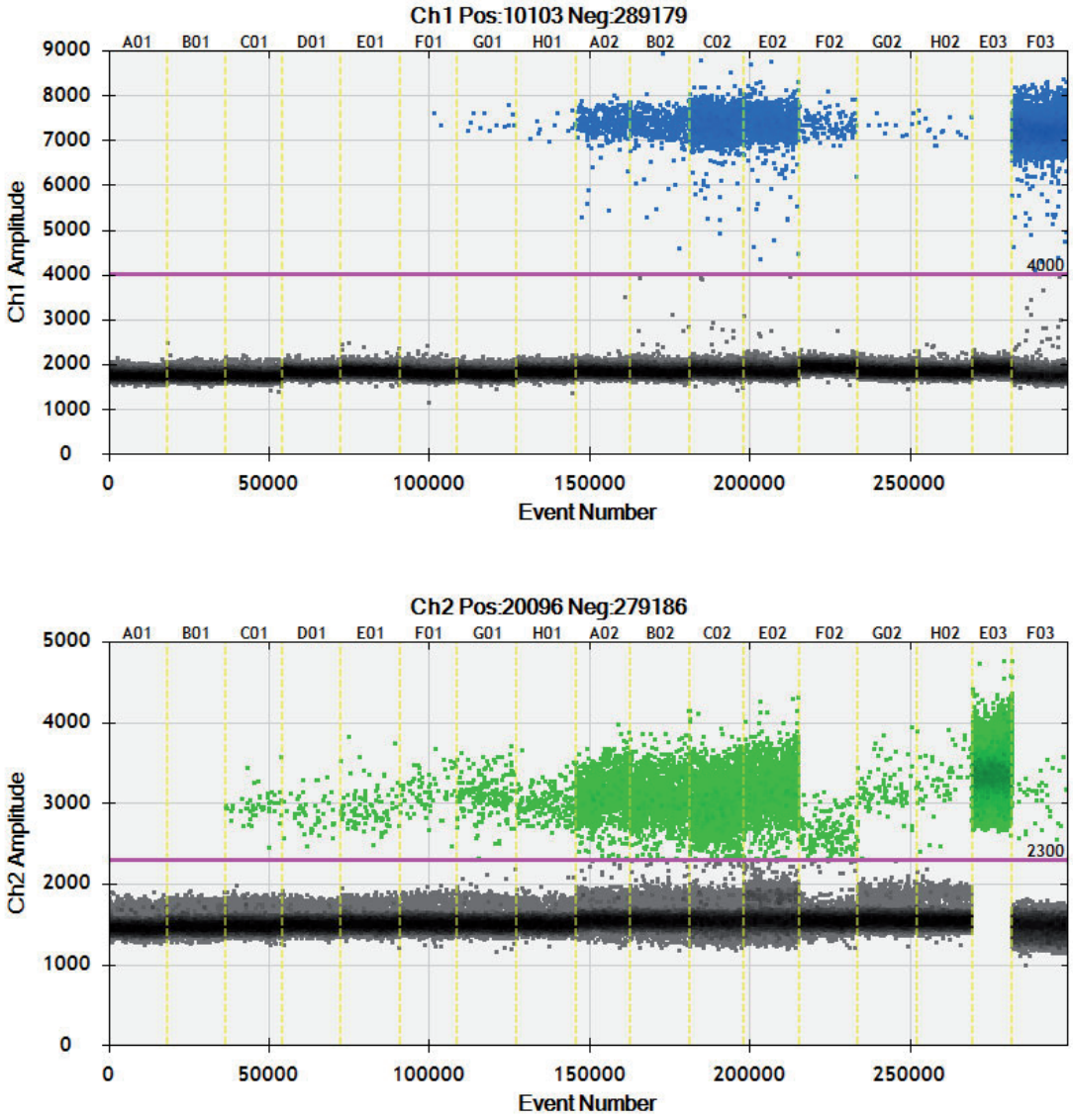

Figure 9: ddPCR amplification plot of a duplex reaction of *G. salaris* and Atlantic salmon. The blue dots represent droplets positive for *G. salaris*, the green dots represent droplets positive for Atlantic salmon. The dense band of grey dots represents negative droplets and the pink horizontal lines illustrate the threshold above which all droplets are deemed positive. Each dot displays the amplification amplitude after a defined number of cycles (45) and is the equivalent of the endpoint amplitude in qPCR. In Well E3, there is a noticeable absence of grey dots in the analysis for Atlantic salmon. This occurs when the amount of template DNA in the sample – in this case the positive control – is outside the dynamic range of the ddPCR. All droplets are positive for the target and there are no negative droplets.

Summary of Results

Paper I

In **paper I**, a newly developed species-specific ddPCR assay is presented which is capable of detecting eDNA from the aquatic parasite *G. salaris* in water samples. This is done in combination with two other assays derived from literature that can detect two of the parasite's hosts, the Atlantic salmon and the rainbow trout. The paper thus contributes to the development of species-specific assays and application of the eDNA method on a host-pathogen complex under natural conditions. **Paper I** also explores the advantages and challenges of the eDNA methodology in comparison to conventional monitoring. In the samples analysed for this study, we observed a decline of the eDNA signal with increasing distance from the source, in this case a rainbow trout farm. Extensive electrofishing in the sampling areas yielded no positive detection of rainbow trout. Positive detection of *G. salaris* occurred only in sampling areas known to be inhabited by infected fish and not in rivers and streams where the parasite has not been detected.

Paper II

This paper comprises the first study that follows a natural crayfish plague outbreak using both eDNA monitoring, cage surveillance and trapping simultaneously and contributes to the comparison of eDNA methodology and conventional methods for biomonitoring of the host-pathogen complex *A. astaci* – signal crayfish and *A. astaci* - noble crayfish. The results indicate a good correspondence between the eDNA monitoring and the biological status represented by both crayfish mortality in cages and information obtained through trapping. Like **paper I**, this study aims at the application of the eDNA method on a host-pathogen complex under natural conditions. Through eDNA monitoring, we were able to detect the presence of *A. astaci* in the water up to 2.5 weeks earlier than with the conventional cage method. Moreover, we were able to closely follow the eDNA dynamics of noble crayfish and *A. astaci* during a progressing outbreak. We observed a gradual increase of eDNA concentration of both targets culminating in a peak during mass mortality and followed by a rapid disappearance of any detectable eDNA of both noble crayfish and *A. astaci*. The resulting extinction of the noble crayfish population was confirmed by extensive trapping involving over 2,800 trap nights with zero crayfish caught, and corroborated the negative eDNA results.

Paper III

In this paper we present three newly developed and thoroughly validated qPCR assays for one indigenous (noble crayfish) and two non-indigenous American crayfish species (signal crayfish and marbled crayfish), two of which are optimised for the ddPCR platform. The importance of testing assays against a broad variety of DNA isolates of related crayfish species is highlighted, and we validated the specificity of qPCR assays against DNA isolates of most crayfish species documented from European waters. We further evaluated the presence of the crayfish plague agent *A. astaci* as well as its various crayfish hosts in a variety of aquatic environments, representative for crayfish habitats in Central and Eastern Europe. We detected eDNA of *A. astaci* together with several American crayfish but never in combination with noble crayfish. However, we also detected eDNA of both indigenous and non-indigenous crayfish co-occurring which could stem either from downstream transport of eDNA or syntopic presence of both species. Furthermore, we present a robust, easy-to-use and low-cost version of the eDNA sampling equipment.

Paper IV

This study attempts to evaluate the sensitivity and detection limits for eDNA monitoring and eDNA dynamics of the host-pathogen complex Atlantic salmon and *G. salaris*. In a 17-week challenge trial, Atlantic salmon were infected with *G. salaris*, kept in separate tanks and the parasite numbers were determined once a week. Corresponding water filter samples were taken weekly and analysed for the presence of *G. salaris* eDNA and the results compared to the parasite counts. We developed three ddPCR assays targeting the mitochondrial cytochrome C oxidase gene (*COI*), that detected and in part discerned between three mitochondrial haplotypes of *G. salaris* found in Norway. We were unable to relate parasite numbers directly to eDNA copy numbers. However, we determined the minimum amount of parasite individuals per fish required for positive detection (with 95 % confidence) of *G. salaris* eDNA under experimental conditions. When using the previously published nuclear ribosomal *ITS* ddPCR assay (**paper I)**, <200 parasites per fish were needed, while this number increased to nearly 300 when using the haplotype specific *COI* ddPCR assays. These numbers appear high when considering that we detected *G. salaris* in field samples (**in paper I**) at far lower intensities, so various technical, methodological and biological explanations are discussed here, and further in the thesis. We further established the amount of samples required for positive detection at varying levels of parasite intensity, under the same experimental conditions. The *ITS* assay was found to detect on average 10 - 17 fold more DNA copies than the *COI* assays when tested on tissue samples,

and detected *G. salaris* eDNA far more often in water samples than the *COI* assays. For general eDNA monitoring, the *ITS* assay is therefore recommended, while the *COI* assays can, provided there is sufficient eDNA in the sample, contribute to haplotype determination.

Paper V

Little is known about how population density and other environmental factors influence the detectability of the host-pathogen complex involving American crayfish and *A. astaci.* This study aims to examine the influence of temperature, density and food availability on the detectability of eDNA of *A. astaci* and signal crayfish in a mesocosm experiment. We also compared eDNA results with crayfish population density data (CPUE) from two lakes with varying signal crayfish density and *A. astaci* prevalence. Through the experiment we show that a limited set of factors can substantially change the amount of detectable eDNA of signal crayfish and *A. astaci* while their physical presence remains unchanged. In cold, clear water we observed eDNA quantities of both targets to increase far more than in a linear fashion with increased crayfish density. The presence of food decreased the detectability of crayfish eDNA but not of *A. astaci*. This was presumably due to increased microbial-induced eDNA degradation of shed (dead) cells from crayfish, while live spores resist such degradation. However, increased water temperature strongly reduced the detectability of *A. astaci* eDNA. The increased variability and complexity of influences under natural conditions suggests that reliable correlations between eDNA quantity and crayfish density is difficult to achieve. We also observed minimal correspondence between CPUE data and eDNA quantities in the field samples. We conclude that the eDNA methodology remains an effective tool for presence-absence monitoring of this host-pathogen complex but appears less suited for biomass quantification or population density estimation. As the host-pathogen complex is not uniformly influenced by environmental factors, we recommend a strategy of monitoring both targets, where the detection of one also points towards the presence of the other.

Discussion

Discussion of methods

Environmental sampling and filtration

A multitude of filter types with different membranes have been used in eDNA studies. The filters range from small pore sizes (e.g. "Sterivex^{TM}-GP" 0.22 µm (*Agersnap et al. 2017*) or nitrocellulose filters 0.45 µm (*Geerts et al. 2018*)) to larger pore sizes of 2 µm (*Strand et al. 2014, Anglès d'Auriac et al. 2019, Johnsen et al. 2020*) such as the filters used in this thesis. In studies within this thesis (**paper I-V)** AP2504700 glass fibre filters with 2 µm pore size were chosen in order to filter larger volumes of water. The filters are relatively thick, with a complex glass fibre mesh that offers a large holding capacity of particles (including smaller than 2 µm that are captured in the mesh) before they clog. Particularly when sampling for eDNA from the crayfish and *A. astaci*, filtering close to the lake bottom leads filters with smaller pore size to clog faster due to clay particles and sediments. We expected a higher chance of detecting targets that shed only little eDNA when sampling larger volumes of water (*Hunter et al. 2019*). In a recent study by Fossøy et al. (*2019*) calculations showed a higher probability of detecting *G. salaris* in 10 L water filtered through a 2.0 µm glass fibre filter than in 1 L water through a 0.45 µm cellulose filter, and Brannelly et al. (*2020*) found that detection probability of the amphibian pathogen *Batrachochytrium dendrobatidis* increased with the volume of water filtered. However, the filtration of large volumes can also increase the concentration of PCR inhibitors within the extracted sample (*Strand et al. 2014, Fossøy et al. 2018,* but see *Hunter et al. 2019*). We did not compare alternative filters, but overall we experienced that our choice of filter yielded satisfactory results regarding the eDNA detection.

Sampling location and distribution of sampling locations within a waterbody are important for successful detection of the target organism. Our sampling strategy included filtering water from near the lake bottom where crayfish dwell (**paper II, III, V**), and directly in the water column when targeting eDNA of Atlantic salmon and *G. salaris* (**paper I**). Comparisons of sampling at the bottom and at the surface of a lake have in some studies revealed a trend towards higher detection frequency of crayfish at the bottom (*Strand et al. 2014, Fossøy et al. 2020*). The simple explanation must lie in the proximity between the depth of water in which the organisms are to be found and the sampling depth, particularly in non-moving waterbodies. This is supported by other studies such as that conducted by Lawson Handley *et al.* (*2019*) who sampled a large lake in Northern England. They established that spatial distribution of eDNA

detection of several fish species closely reflected the ecology and habitat preference of the respective species. In rivers, the constant flow of water would be expected to churn eDNA (and other) particles within the water column and therefore the sampling depth within the water column would be of less consequence. Kamoroff and Goldberg (*2018*) conducted microcosm experiments on the distribution of eDNA of dead goldfish within the water column. They found the eDNA to be located at the bottom of the tanks, close to the source i.e. the dead fish. In natural lakes, outside of controlled experimental settings, stratification and subsequent seasonal turnover may, however, influence the location of eDNA. In general, we observed a good correspondence between eDNA results and the known presence of the pathogens and hosts (**paper I-V**). However, in **paper III**, we were unable to detect two crayfish species in both a lake and a river, although their presence had previously been confirmed by a third party. We further experienced that low pathogen intensity for *G. salaris* (below 100) could lead to negative eDNA results (**paper IV**). Also, *A. astaci* remained undetected by means of eDNA in the water in tanks with *A. astaci* positive signal crayfish kept in 20 °C water (**paper V**). Failure to detect the target species might be attributed to spatial mismatch (*Harper et al. 2018*) and/or a dilution effect due to small amounts of eDNA shed from crayfish or *G. salaris*. Level of precipitation prior to sampling, and associated dilution of eDNA concentration, may impact negatively on the detection success (*Johnsen et al. 2020*). In a relatively large river, the dilution effect can also be substantial and lead to detection failure. In our case, the failure to detect crayfish might also be explained by taking only two filter samples per location while occupancy modelling has shown that more eDNA samples are required for reliable qPCR detection of low-density crayfish populations (*Johnsen et al. 2020*). However, also in an experimental set-up with constant flow through, such as in **paper IV**, the dilution effect will impact on eDNA detectability. In our experiment eDNA from *G. salaris* was constantly depleted which probably explains the surprisingly low detection success. Abiotic factors, including UV light, temperature, turbidity, humic substances leading to inhibition, all impact on the eDNA detectability (*Stewart 2019*). In addition, a profound knowledge of the biology and ecology of the target species can be of paramount importance for sampling success, including temporal and spatial timing to encounter events of elevated eDNA emission. This is particularly important in the case of target-species that shed relatively little eDNA such as crayfish or *G. salaris* (see discussion of results).

On-site filtration is not always convenient or possible. In **paper III**, the samples from River Barat in Budapest, Hungary were delivered to us by colleagues where water had been collected

in the river in sterile containers and filtered later in the laboratory. Here, we detected the expected American crayfish species in both samples, indicating that the transport had not led to total eDNA degradation. A study by Curtis *et al*. (*2021*) has since examined the effect of storage time of water prior to sampling and concluded that a storage time of 1-2 days in a cooled and dark place does not negatively impact the yield of eDNA. This may prove useful for future studies when logistical constraints do not allow direct filtration on site. Another recent development that might serve as an alternative to on-site filtering is the use of filter membranes for passive collection of eDNA (*Kirtane et al. 2020, Bessey et al. 2021*) and 3D-printed passive samplers (*Verdier et al. 2021*), which perhaps could reduce sampling time and associated costs.

Controls and aspects of contamination

As recently pointed out by Sepulveda *et al*. (*2020b*) in a paper entitled "*The Elephant in the Lab (and Field): Contamination in Aquatic Environmental DNA Studies*", the rapid evolution of eDNA methods has resulted in knowledge gaps in smaller, yet critical details such as proper use of negative controls to detect contamination. In this thesis (**paper I-V**) we did include proper negative controls during the molecular laboratory work, following the same standards as diagnostic methods with the inclusion of a DNA blank extraction control, a laboratory environmental control and PCR negative controls (*Vrålstad et al. 2009*). We also included negative controls in the aquarium experiments (**papers IV, V**), either using separate "negative control buckets" with only clean water and non-infected fish as part of the experiment (**paper IV**) or filtered control water samples from a clean bucket filled with water from the pipes that fed into the aquarium room before the start of each experimental period (**paper V**). However, we did not include negative field samples, which is also regarded as important (*Carim et al. 2020, Sepulveda et al. 2020b*). Negative field controls can be samples of a presumed negative field site (*Spens et al. 2017*), or clean water brought on-site that is filtered before or after sampling (*Hunter et al. 2015*), to check for possible contamination from the filtration equipment.

The qPCR and ddPCR approaches are extremely sensitive, also in detecting minor contamination, and require only a few DNA copies of the target for a positive detection. Contamination problems in negative controls can therefore be more or less severe, depending on the quantity detected. We did, unfortunately, encounter minor problems with contamination in some of our controls. Below we address the problems for the papers concerned, explain how we solved them from case to case, in addition to providing potential explanations for the contamination issues. In **paper II**, minor contamination in either the laboratory environmental

control or the blank extraction control led to the decision that a total of twelve samples from a specific date were excluded. During the signal crayfish aquarium experiment (**paper V**), we detected low levels of contamination in some of the control water samples in week 5 and 6 of the experiment. Here, statistical tests on the effect of the contamination showed no difference in the significant factors when adjusting for the contamination, and we therefore included all samples in the analyses; the loss of data from two out of three weeks for the high-temperature experiment would have had a dramatic impact on the analysis. We presume the contamination did not stem from the inlet water but rather from inadequate cleaning of equipment. However, we cannot exclude the possibility that the contamination arose from the freeze-drying process of the filters prior to DNA extraction. Here, the falcon tubes were unscrewed for drying, and the lids simply placed on top of the tubes where they were prone to falling off. This procedure was deemed a potential contamination risk and abandoned. For the aquarium experiment with *G. salaris* infected Atlantic salmon (**paper IV**) we found three *G. salaris* specimens on one negative control fish, which was removed and the bucket was disinfected with chlorine after which a new uninfected fish was added. A possible explanation could be incomplete disinfection or mix-up in the use of gill-net, leading to unintentional infection of the control fish. The samples from that week's sampling event were nonetheless included in the analyses as the positive detection of a *G. salaris* specimen in the control bucket was not considered to influence the eDNA results in the rest of the buckets. In one sampling week we detected eDNA of *G. salaris* in the negative fish control samples where manual examination had not revealed any *G. salaris* specimen on the fish. Samples taken from all buckets that week were excluded from the statistical analysis. Here, we cannot exclude the possibility that contamination resulted from inadequate cleaning of the equipment, i.e. *G. salaris* specimens wedged in the pre-filter which had been glued onto the filter cup. To preserve its structural integrity, this mesh filter could not be scrubbed with a brush in order to clean it. In retrospect, *G. salaris* with its attachment hooks, might be particularly difficult to remove with disinfectants and cleaning procedures, and eDNA work with this parasite might require single-use equipment at all stages. The lack of negative field control samples is a weakness of ours and many other eDNA studies. It needs more consideration and points out the necessity of stringent, harmonised protocols in future eDNA monitoring work (*Sepulveda et al. 2020b*). However, we did take several precautionary measures to avoid field-related contamination. During field sampling for *A. astaci* and crayfish (**papers II, III, V**), water samples were always collected from upstream to downstream to avoid transferring *A. astaci* spores upstream. Stations outside the infection zones were always sampled before stations within the infection zone (**paper II**) and did in this respect

serve as a "negative" field site for *A. astaci* eDNA (but not for noble crayfish eDNA). Generally, before filtration at another station within the same waterbody, ambient water was pumped through the hose and filter holder for several minutes to rinse away any residual eDNA from the previous upstream location and as a precaution to avoid filtering disturbed sediments at the current station. After sampling of all stations within an infection zone or risk zone (**paper II**) or between different locations (**papers I, III**), the tubing and filter holder were disinfected with a 10% bleach solution. The same procedures were followed in the aquarium experiments. The contamination issues we encountered in the aquarium experiments may indicate that the disinfection protocols in some cases could have been insufficient. Goldberg *et al*. (see *Goldberg et al. 2016 and references within*) recommended 50% bleach solution for field disinfection of eDNA equipment. This is on the other hand problematic from a health and safety perspective and highlights the necessity of sampling equipment that is impervious to contamination. Single-use equipment like the filter cups used in **papers III & IV** could prove a viable solution.

Storage of filters and extraction

The first method used for storage of filters directly after sampling was to place them in a sterile 15 ml "falcon tube", store them on ice until arrival at the laboratory and then freeze them at -80 °C. The samples were then subsequently freeze-dried for 24 h. Due to the size of the freeze-drier, we were only able to process about 20 tubes at a time. As addressed above, this procedure was deemed a potential contamination risk and abandoned. The procedure subsequently adapted was to place the filters into 15 ml "falcon tubes" already containing 4 ml of CTAB buffer. When using the pumping setup with the filter cups where the filter is situated at the front end of the tube and water is filtered through vacuum, most residual water was removed from the filter through suction. However, when sampling from the bottom of the water column – for crayfish and *A. astaci* – water was pushed through the filter by pressure rather than vacuum and the filters were soaked with water upon removal from the filter holder. A comparison of dry and soaked filters revealed that the filters used (47 mm AP25 Millipore, 2 µm pore size) could retain up to 1 ml of water, thus diluting the 4 ml of CTAB in the tubes. We therefore adopted the method of Carim *et al*. (*2016*) where filters are placed in separate zip-lock bags containing ~30 g of desiccant (silica gel) using sterile single use forceps. This method prevents contamination and simplifies storage as the filters only need storage in an opaque container shielding the samples from sunlight without any need for cooling systems (*Allison et al. 2021*).

All DNA extractions were carried out using a CTAB DNA precipitation protocol (see **paper II**). No further DNA cleaning steps to remove PCR inhibitors, such as humic substances (*Wilson*

1997) with commercial kits were conducted, based on the experience from Strand (*2013*) where clean-up kits resulted in a loss of DNA with no extra benefits to increased detectability of targets. For future research, testing of more modern and automated extraction protocols may prove beneficial. A recent report (*Fossøy et al. 2020*), where the same filter type was used as we used throughout this project, found that by using different extraction methods, the DNA yield and thus the detectability of fish and crayfish could be increased. An automated extraction protocol would also significantly reduce hands-on time required for sample preparation in the laboratory (*Kessler et al. 2001*). For further eDNA research and monitoring programs involving freshwater crayfish or *G. salaris*, it will be particularly important to evaluate and optimise DNA extraction protocols. In particular the results of **paper IV**, where close to 200 parasites on a fish were required for a reliable qPCR detection of *G. salaris* eDNA, call for a re-examination using other DNA extraction methods.

qPCR versus ddPCR

During the course of this project, both qPCR and ddPCR were used. One of the sub-goals of this thesis was to explore if ddPCR offers advantages over qPCR for aquatic eDNA monitoring for the studied host-pathogen models (**papers I**, **IV**, **V**). The main clear advantage which is highlighted for ddPCR is the absolute quantification offered by this method, which eliminates the need for standards (*Baker 2012*) and thus increases the sample per PCR plate ratio. **Paper V** contains a direct comparison between ddPCR and qPCR where results from both the mesocosm experiment and field samples were subjected to a correlation test. For the mesocosm experiment, we observed a significant positive correlation between the two methods for both signal crayfish and *A. astaci* analyses. However, when comparing the results of the field samples we observed only a weak positive correlation for the *A. astaci* analyses while the correlation between ddPCR and qPCR results for signal crayfish was poor. Similar observations were made by Johnsen *et al*. (*2020*) for noble crayfish. However, these results are not independent as they were obtained on the same ddPCR machine at NVI. These results, that are in favour of qPCR, are contrasted by studies examining organisms other than crayfish (*Mauvisseau et al. 2019a, Wood et al. 2019, Banks et al. 2021, Brys et al. 2021*) which report a higher sensitivity of ddPCR compared to qPCR. Due to the separation of the reaction-mix into thousands of droplets, ddPCR has been reported to be more robust against inhibition (*Doi et al. 2015a, Wood et al. 2019, Capo et al. 2021*). However, during this thesis and in other reports, ddPCR was shown to be susceptible to inhibition, particularly in freshwater crayfish habitats (*Johnsen et al. 2020, Porco et al. 2021*). A possible explanation for the discrepancy of results and the poorer performance on field samples could indeed be inhibition. Certain types

of qPCR mastermix are designed for robustness against inhibition (*Strand et al. 2011*) such as "Taqman Environmental Mastermix 2.0" used throughout this thesis, whereas ddPCR relies on the partitioning of the sample into droplets to overcome this and currently no dedicated mastermix against inhibition is available. We encountered these issues only for eDNA samples from natural habitats of the crayfish - *A. astaci* host-pathogen complex, and not for *G. salaris* and Atlantic salmon. Most Norwegian crayfish habitats are surrounded by boreal coniferous forests which are known for releasing a substantial amount of humic acids into the water (*Meili 1992*). The reason for the lower detectability of crayfish with ddPCR, therefore, may well lie with the habitat type and subsequent inhibition, rather than the organisms themselves. A study by van Bochove *et al*. (*2020*), that found eDNA of amphipod crustaceans to degrade faster when organic matter was added to the aquarium water, supports this assumption. Thus, ddPCR offers no obvious advantages over qPCR for the eDNA detection of the freshwater crayfish – *A. astaci* host-pathogen complex. On the contrary, we obtain better results using qPCR.

The rivers and streams we sampled for evaluating the presence of eDNA from *G. salaris* and the hosts, on the other hand, were mostly clear and pristine. Samples from the relatively clear Drammen watercourse (**paper I**) were not subject to substantial amounts of inhibitors such as humic acids. Here we detected rainbow trout (in one part of the watercourse) at very low levels of 22 eDNA copies/l. In another infected part of the watercourse, we detected 560 eDNA copies/l of *G. salaris* and high levels of Atlantic salmon (10160 eDNA copies/l). While testing the *G. salaris ITS* assay, we also observed a higher specificity in the ddPCR system with no cross-amplification of *G. derjavinoides*, in contrast to what we observed with the qPCR system. Thus, for *G. salaris* and its hosts, the ddPCR system does not encounter the same challenges as for freshwater crayfish, and even discriminates between *G. salaris* and *G. derjavinoides*. In this respect ddPCR offers some advantages above qPCR for this host-pathogen complex.

Not all qPCR assays are easily transferred to the ddPCR platform. We experienced that well established qPCR assays for several of the crayfish species developed by Agersnap *et al*. (*2017*) were not transferable to the ddPCR platform, creating the need for further development of new functional ddPCR assays (**paper III**, **V,** (*Johnsen et al. 2020*)). From a cost-benefit perspective, this had a downside as we did demonstrate that the ddPCR approach was not superior to the qPCR approach for freshwater crayfish. Furthermore, ddPCR comes with an increased cost due to additional consumables (and more expensive reagents) (*Yang et al. 2014*). Droplet digital PCR also substantially increases the workload after plate preparation since it requires the step

of droplet generation which can take up to 45min for 96 wells prior to thermocycling and the step of droplet reading after thermocycling, also adding up to an hour to the process. Depending on the questions addressed and the type of organisms targeted, ddPCR can offer advantages over qPCR. However, we recommend a thorough cost-benefit evaluation and assay testing before making the final decision between ddPCR and qPCR.

Discussion of results

eDNA biomonitoring of the host-pathogen models compared to conventional methods

The first research question (**RQ-1**) explores whether the eDNA methodology can work equally well or better than conventional methods for biomonitoring of the host-pathogen models, particularly at low prevalence. To explore this, it important to understand the nature of information provided by conventional methods. Electrofishing with subsequent examination of 30 fish from each river under a stereomicroscope (*Hansen et al. 2021a*) has a high detection rate of *G. salaris* (probably near 100%) on susceptible Atlantic salmon in Norwegian rivers. This is because the population growth is not controlled on these salmon populations resulting in a high number of parasites on individual fish and a prevalence of near 100% within short time. The examination and counting of parasites on the fish also provides direct insight into the prevalence, intensity and infection site of the parasite specimens. As different species show different site specificity (*Bakke et al. 2007*) the actual location of parasites on the fish (e.g. fins versus body) can provide an indication on the species present (*Jensen and Johnsen 1992*) and the time since introduction can potentially be inferred from the prevalence and intensity data obtained (a small number of parasites point to a recent introduction). For diagnostics, a few parasites are removed from a subset of the analysed fish and DNA isolates of these parasites are subjected to PCR and subsequent sequencing of the ribosomal internal transcribed spacers (*ITS*) and mitochondrial cytochrome oxidase 1 gene (*OIE 2019a*). In addition to species determination, the *COI* sequence data can in some, but not all, instances give an indication of whether a particular strain is pathogenic or not (*Hansen et al. 2007*). For *A. astaci*, cage-based monitoring provides information on presence of the pathogen in the water, but only after infection and subsequent death of the susceptible caged noble crayfish, followed by confirmation of the crayfish plague diagnosis by molecular analyses of infected crayfish tissue (*Vrålstad et al. 2017*). Finally, freshwater crayfish trapping yields information on crayfish population density as well as population structure (*Johnsen et al. 2019*). In this chapter, the benefits and shortcomings of the eDNA methodology are discussed and compared to the

conventional methods based on results obtained from experiments and field studies conducted in this project, as well as the current state of knowledge.

To conduct the experiments and field studies, we needed thoroughly validated species-specific or even variant-specific assays for the target organisms of this thesis (sub-goal a), which was a major focus in many of the papers. We developed an *ITS* ddPCR-assay for eDNA monitoring of *G. salaris* (**paper I**) while for the detection of Atlantic salmon and rainbow trout we relied on published assays (*Matejusová et al. 2008, Wilcox et al. 2015*). In **paper III**, we developed new assays for eDNA monitoring of *Astacus astacus*, *Pacifastacus leniusculus* and *Procambarus virginalis* which were optimised for ddPCR analysis, and we drew upon a published assay for *Faxonius limosus* (*Mauvisseau et al. 2018*). Our results highlight the necessity of stringently tested species-specific assays, both in-silico and in-vivo, as the amplification of closely related non-target organisms may lead to false-positive detections. We were able to increase the specificity of the assay for *F. limosus* (*Mauvisseau et al. 2018*) but still observed amplification of a closely related species (*F. virilis*) (**paper III**). False-positive amplification is particularly problematic with parasites of economic concern like *G. salaris,* but also for species conservation in areas with a higher crayfish biodiversity than Europe. Recently, a newly described *Aphanomyce*s species isolated from noble crayfish in Finland, *A. fennicus* (*Viljamaa-Dirks and Heinikainen 2019*) has been shown to interfere with the used qPCR assay for *A. astaci* (*Vrålstad et al. 2009*) due to 100% *ITS* sequence homology in the MGB-probe site of the assay leading to false positive amplification (*Viljamaa-Dirks and Heinikainen 2019*). Here, an adjusted *ITS* assay, or a new assay targeting a more variable gene marker is required. This challenge was not met within this thesis, and judging by the relatively large amount of negative eDNA results, including in non-infected sites with noble crayfish (**paper II**), there is little reason to believe that the noble crayfish associated oomycete *A. fennicus* is abundant or has led to false positive results in the screened habitats. On the contrary, there is a good match between *A. astaci* positive samples and known presence of the pathogen (**paper II, III, V**).

For practitioners and stakeholders, information on the quality of an assay is imperative for comparison of assays and interpretation of results. Recently, Thalinger *et al*. (*2021*) proposed a five-level validation scale for eDNA assays, based on 122 variables and 546 published single species assays. Standards like these can help determine whether published assays are suitable for implementation in general monitoring programs or if further optimisation is required.

In **paper IV**, we extended the scope beyond the species-specific assays and developed haplotype specific ddPCR assays for *G. salaris* variants since it is impossible to distinguish

between *G. salaris* and *G. thymalli* using *ITS* as a marker (the *ITS*-assay). This is a problem in rivers in Norway and elsewhere inhabited by both Atlantic salmon and grayling and therefore highlights the need for more specific assays. Further, it is sometimes important also to distinguish between genetic variants or mitochondrial *COI* haplotypes of *G. salaris*, since some haplotypes can differ in pathogenicity towards Atlantic salmon (*Hansen et al. 2007*), and information on the origin of the infection (*Hansen et al. 2003*) might be obtained from the eDNA analyses directly. While we succeeded in developing two assays that specifically detected haplotypes A and F, we were unable to design an assay specifically detecting haplotype B. To solve this problem, we designed an assay that amplifies both haplotypes A and B. In the event of a positive detection with this assay, a second subsequent analysis with the assay for haplotype A would clarify which of the haplotypes is present.

To compare the eDNA methodology with conventional methods, it was important to first perform a proof-of-concept for eDNA detection for the two aquatic host-pathogen complexes under field conditions (sub-goal b). Strand *et al* (*2011*) demonstrated a high recovery of *A. astaci* spores in water samples and later provided a proof-of-concept for *A. astaci* water monitoring in large freshwater systems (*Strand et al. 2014*), and Tréguier *et al*. (*2014*) were the first to demonstrate eDNA monitoring for freshwater crayfish. Robinson *et al*. in (*2018*) demonstrated simultaneous detection of *A. astaci* and signal crayfish, a proof-of-concept for simultaneous monitoring that was also fully demonstrated in this thesis for *A. astaci* and several of its crayfish hosts (noble crayfish, signal crayfish, spiny-cheek crayfish and marbled crayfish; **paper II-III**). For the host-pathogen complex Atlantic salmon – *G. salaris*, **paper I** provides the first proof-of-concept in the infected River Lierelva. Here, we also tested water samples for the combination of Atlantic salmon – *G. salaris* and Rainbow trout – *G. salaris* in the presumed non-infected part of the Drammen watercourse. Other studies have later also reported the successful simultaneous detection of Atlantic salmon and *G. salaris* (*Fossøy et al. 2019, Hansen et al. 2021b*) where the observed pattern of eDNA concentrations was consistent with the parasite abundance observed by conventional methods. It is to a certain extent possible to detect and discriminate between *G. salaris* haplotypes with eDNA monitoring (**paper IV**), as is done with conventional monitoring when isolates are sequenced. However, the *ITS* assay was shown to be far more sensitive for this purpose. When tested on tissue isolates, the *ITS*-assay was more than 10-fold more sensitive than the haplotype specific *COI* assays, and in field samples the *ITS* assay detected up to 2500 DNA copies while the corresponding *COI*-analysis remained negative (**paper IV**). Similar observations were made by Minamoto *et al*. (2017),

Dysthe *et al*. (2018) and Moushomi *et al*. (2019), who observed higher copy numbers when targeting nuclear markers compared to mitochondrial markers. This is also reflected by the calculations of parasites required per fish for positive detection (**paper IV**), where 1.5 times more parasites were required when using the *COI* assay compared to the *ITS* assay. Based on the lower sensitivity of assays targeting the mitochondrial marker *COI*, and particularly when considering the low eDNA emission rate of *G. salaris* (see below), it appears the *ITS* assay for *G. salaris* is better suited and more sensitive for routine monitoring of water samples. In the event of positive eDNA detection, the haplotype specific assays can nonetheless, provided there are sufficient eDNA amounts in the sample, determine the haplotype and exclude the possibility of false positives caused by *G. thymalli*. Furthermore, the assays can be used for haplotype determination on tissue isolates from *Gyrodactylus* specimens obtained from infected fish as an alternative to more expensive DNA sequencing.

For the interpretation of any eDNA results, it is important to consider the possibility of downstream transport of eDNA (*Stewart 2019*). We detected rainbow trout eDNA only at localities where the positive detections could be attributed to eDNA release from land-based rainbow trout farms up to 25 km further upstream (**paper I**). Downstream transport of eDNA has been observed in other studies on freshwater invertebrates and transport distances are reported between 1.7 km (*Wacker et al. 2019*), 3 km (*Wittwer et al. 2019*), 7 km (*Chucholl et al. 2021*) and up to 10 km downstream (*Deiner and Altermatt 2014*). Downstream transport of eDNA can sometimes aid detection of aquatic organisms, especially when the exact location is unknown and sampling is carried out solely to determine presence or absence. However, it may also confound the results. We occasionally detected eDNA of both native and non-native crayfish species in the same sample (**paper III**). While this could indicate syntopic presence of both species, it is more likely attributed to downstream transport of eDNA, thus eDNA from two separated populations might merge aided by the flowing water. Additionally, since eDNA is shed also by dead and decaying organisms (*Turner et al. 2015, Kamoroff and Goldberg 2018*) this method cannot accurately distinguish between live and dead organisms (*Darling and Mahon 2011, Bohmann et al. 2014*). Nevertheless, due to rapid degradation of DNA originating from dead cells, eDNA results yield relatively reliable snapshots of what organisms are present or have recently been present in a habitat (*Bracken et al. 2019*). This was demonstrated in **paper II**, where eDNA from noble crayfish and *A. astaci* first increased during the on-going crayfish plague outbreak which induced mass mortality, but then disappeared from the system after around 8 weeks.

We observed a good correspondence between the results of conventional methods and eDNA monitoring for *A. astaci* and freshwater crayfish (**paper II**). We were even able to detect the presence of *A. astaci* eDNA in the water up to 2.5 weeks earlier than with the traditional cage method, suggesting that eDNA monitoring can be superior to the cage method for early warning. Caged noble crayfish do not only succumb to the crayfish plague once infected with *A. astaci* but are also frequently released due to vandalism of the cages or they might fall victim to cannibalism (*Vrålstad et al. 2017*). Decapod crustaceans are protected by the Norwegian Law on animal welfare (LOV-2009-06-19-97) and thus the use of live animals for disease monitoring posed substantial ethical concern. The non-invasive nature of eDNA sampling combined with the possibility of earlier detection compared to the conventional method has not only proven eDNA monitoring to be a more suitable surveillance method (*Wittwer et al. 2018*) in this instance. It has, in fact, led to the replacement of cage-based crayfish plague monitoring in favour of eDNA monitoring in Norway (*Vrålstad et al. 2017*). Although not published before 2019, it was the comparative studies and results of **paper II**, prior to its publication, that were instrumental for the adaptation of eDNA methods and termination of cage experiments in the *A. astaci* surveillance in Norway. Further, the concept of simultaneous eDNA monitoring of A. *astaci* and freshwater crayfish (noble crayfish and signal crayfish) has been implemented as a more holistic approach and supplement to trapping in the Norwegian surveillance program for freshwater crayfish (*Johnsen et al. 2019*). The same eDNA monitoring concept for *A. astaci* and freshwater crayfish has recently been implemented in Ireland (*Swords et al. 2020*).

The results of **paper II** and **III** in particular highlight the importance of screening for both the host (or vector) and the pathogen. In **paper II**, signal crayfish eDNA was detected even at very low population densities (CPUE 0.12) at the site where the crayfish plague outbreak emerged, while noble crayfish eDNA was consistently detected alone in all sampled sites that were unaffected by the outbreak. The simultaneous detection of *A. astaci* – signal crayfish – noble crayfish eDNA forecasted the imminent outbreak, and the peak and subsequent decline of *A. astaci* – noble crayfish eDNA depicted the on-going mortality event and subsequent eradication. In **paper III**, we found eDNA of various American crayfish in 65% of all samples but in only 29% of those did we simultaneously encounter eDNA of *A. astaci*. In one sample we detected eDNA of *A. astaci* but no American host crayfish. Furthermore, we found eDNA in only four of eight locations where analysis of crayfish tissue had confirmed presence of *A. astaci*. Since *A. astaci* is an obligate parasite that is only able to survive outside of a host for a few weeks (*Söderhäll and Cerenius 1999*), the presence of this pathogen also suggests the

presence of an American crayfish host. To date, there are only few studies that report American crayfish populations in Europe free of *A. astaci*-infections (*Skov et al. 2011, Schrimpf et al. 2013*). However, the prevalence of *A. astaci* can vary (*Schrimpf et al. 2013, Strand et al. 2014*) and thus it may not always be revealed in eDNA samples. For management purposes, a positive eDNA detection of American crayfish would therefore be more likely to indicate a low-prevalent presence of *A. astaci*, even if no *A. astaci* eDNA is detected. Likewise, in periods when crayfish eDNA is less abundant as a result of biological or environmental constraints (see below), a positive detection of *A. astaci* alone could suggest the presence of an American crayfish species, unless the detection stems from a crayfish plague outbreak and is spreading in a native crayfish population.

Analysing samples for eDNA of both the host and the pathogen provides particularly valuable information in the case of parasites that are linked to hosts or vectors as is the case with *A. astaci* and American crayfish. However, such analyses can prove more challenging when trying to establish early stages of invasions and infections on previously disease-free populations. This is the case with *G. salaris* and Atlantic salmon and also with *A. astaci* and noble crayfish. In Norway more than 400 rivers have confirmed populations of Atlantic salmon (*Forseth et al. 2017*), but after successful eradication programs, *G. salaris* remains confirmed in only eight (*Hansen et al. 2021a*). For noble crayfish, there are around 470 populations in Norway that have not been struck by *A. astaci* (*Johnsen et al. 2019*). In both cases, the surveillance challenge is how to detect an early infection of the pathogen in time to implement measures to reduce and control the further spread. Here, risk-based monitoring of areas with elevated risk for infection from neighbouring lakes or rivers is an important strategy. When using eDNA monitoring, a positive eDNA detection of the host in the absence of the pathogen is a relatively good confirmation of a desired habitat status (**paper II**; (*Vrålstad et al. 2017*)).

For *G. salaris*, proof-of-concept studies under field conditions show promising results (**paper I**, (*Fossøy et al. 2019, Hansen et al. 2021b*)), but they give only limited indication of the parasite intensity required to obtain positive eDNA results. **Paper IV** therefore aimed at determining the minimum number of parasites per fish for reliable eDNA detection of G. *salaris* (sub-goal C). An increasing number of eDNA studies is using statistical modelling to calculate the probability of detecting the target organism (*Dougherty et al. 2016, Dorazio and Erickson 2018, Doi et al. 2019, Fossøy et al. 2019, Da Silva Neto et al. 2020, Johnsen et al. 2020, Sieber et al. 2020*). Through statistical analysis of the data from the infection trial, we calculated that 185 (±50) and 290 (±80) parasites per fish were required for a 90% detection probability when using

assays targeting the *ITS* and *COI* marker respectively. However, these calculations are based on samples where plankton mesh pre-filters were used and from buckets with a constant, albeit low flow-through of water. Thus, any accumulating eDNA was constantly diluted and the apparently largest source of *G. salaris* eDNA in the water-column, free-floating parasites of large parts thereof, was not captured on the filters. This may explain the surprisingly high numbers required for detection in our study (**paper IV**), while studies on field samples report better and more consistent detection (*Fossøy et al. 2019, Hansen et al. 2021b*) (**paper I**). Nonetheless, these high parasite numbers needed for reliable detection (**paper IV**) question whether eDNA monitoring with the currently used methods can reliably detect *G. salaris* at low prevalence, such as in populations with less susceptible reservoir hosts (*Paladini et al. 2014*) or early infection stages on Atlantic salmon. While the metric of "parasites per single fish" may not be of direct relevance for management purposes where parasite prevalence and intensity are estimated from a sampled subset, it provides insight into the number of both parasites and samples required for positive detection and it highlights the necessity of adequate sampling strategies. To gain a better picture of the practical implications, we also calculated the number of samples required for positive detection depending on the infection load. To detect *G. salaris* at low infection rates (i.e. 100 specimens per fish) under experimental settings with 90% detection probability, three and six 1L filtered water samples are required for the *ITS* and the *COI* assay, respectively. These results correspond to other studies containing similar calculations (*Johnsen et al. 2020, Sieber et al. 2020*) which also indicate that more than one sample is required for successful detection of low-prevalent targets.

Proof-of-absence cannot be established on the basis of eDNA monitoring alone, particularly not for rare or elusive targets such as *G. salaris*, *A. astaci* and freshwater crayfish (as observed in **paper II-V**). This is mostly a matter of low prevalence or low population density, but may also result from environmental factors and biological traits reducing the eDNA detection success (see below). On the contrary, fish eDNA is more readily detected and thus sampling volumes are commonly lower (*Jerde et al. 2011, Jerde et al. 2019, Ahn 2020, Mizumoto et al. 2020*). We made corresponding observations for Atlantic salmon, where the eDNA signal was strong and relatively stable both in field samples (**paper I**) and in the infection trial (**paper IV**). To sum up, we show that simultaneous monitoring of host-pathogen complexes is advantageous with the eDNA methodology, but the performance of the method is highly contingent on the type of organism targeted and its biological traits, particularly with regards to organisms that shed only minute amounts of eDNA. It can in some cases outperform conventional methods in

terms of sensitivity as was exemplified by the detection of *A. astaci* 2.5 weeks prior to conventional methods and offers a non-invasive, animal welfare friendly alternative. However, for the Atlantic salmon – *G. salaris* complex the eDNA methodology fails to detect parasite presence at low intensities. Here, eDNA monitoring currently seems a promising supplement that can be combined with the conventional methods. The same applies to freshwater crayfish, where eDNA monitoring can substantially enlarge the surveyed area compared to the time consuming trapping and thereby broaden the yearly coverage of presence-absence monitoring.

eDNA copy numbers as a proxy for host density and pathogen intensity

Several studies in recent years have demonstrated the possibility to quantify biomass and abundance of various fish species, inferred from eDNA copy numbers (*Takahara et al. 2012, Takahara et al. 2013, Doi et al. 2015b, Doi et al. 2017, Capo et al. 2019*). However, estimations of relative abundance are not straightforward as several considerations have to be taken into account (*Bohmann et al. 2014*). The degradation rate of eDNA can be influenced by environmental factors (*Dejean et al. 2011, Foote et al. 2012*) which may also influence the metabolism of target organisms and thus their eDNA shedding rates (*Deagle et al. 2010, Murray et al. 2011*). In this thesis we attempted to determine whether this is also possible with two host-pathogen complexes of economic and environmental concern. To answer whether eDNA copy numbers can serve as a proxy for host density and pathogen intensity, it is important to ascertain whether meaningful semi-quantitative estimates of host number and/or pathogen load can be derived from eDNA copy numbers. Strand *et al*. (*2011, 2012, 2014*) had previously established that eDNA copy numbers could be used to estimate *A. astaci* spores per litre water based on the assumption that each spore contained a package of ~140 *ITS* copies (estimated by *Strand et al. 2011*). As there are many studies available on the quantification of fish biomass, the question thus has been least explored for *G. salaris* and the freshwater crayfish regarding the studied host pathogen complexes in this thesis.

We did not attempt to determine the average *ITS* or *COI* copy number of *G. salaris* specimens, as has been done for *A. astaci* spores (*Strand et al. 2011*), but instead endeavoured to correlate detectable eDNA copies with the parasite intensity (number of parasites) per singular fish. The rapid, albeit not exponential increase of parasite numbers on the fish in the infection experiment (**paper IV**) did not directly result in a corresponding consistent increase of detectable eDNA copies in the water. Both high and low eDNA copy numbers in the water were observed at both high and low parasite intensities. This could perhaps reflect that a body part of one (dead) *G.*

salaris individual captured on the filter could lead to rather high copy numbers even at low parasite intensities while many live *G. salaris* individuals attached to the fish emitted comparatively less eDNA. However, we observed that above a certain threshold of individuals, a consistent detection was occurring while detections at low intensities were highly random (**paper IV**). As we were unable to correlate parasite numbers directly with eDNA copies, we instead attempted to determine statistically the amount of parasites required for positive detection (see above) and to calculate the amount of samples required (under experimental conditions) for positive detection at varying parasite intensities. With theoretical densities of 100, 500 and 1000 parasites per fish (representing low, medium and high infection intensity), three samples were required for a 90% detection probability with the *ITS*-assay, but at medium and high intensities, one sample (1L filtered water) was sufficient for both the *ITS* and the *COI* assay. For the fish hosts, we observed a relatively high amount of eDNA copies which initially exceeded that of the parasites over 6000-fold but the discrepancy decreased with increasing infection intensity. Generally, there was a high variation of copy numbers for both targets. In nature, where the turnover of *G. salaris* individuals is relatively high, particularly at high parasite intensities, there might well be a better correspondence between infection intensities and eDNA copy numbers in the water. Hansen *et al.* (*2021b*) observed high amounts of *G. salaris* eDNA in water samples at sites with known infected fish populations in the Drammen river, and decreasing eDNA quantities with increased distance from the infected population both for *G. salaris* and Atlantic salmon.

Previous studies (*Forsström and Vasemägi 2016, Rice et al. 2018*) have shown that crustaceans are more challenging to detect than fish, for example, that shed multiple sources of eDNA into the water (*Jo et al. 2019*). Also, the direct comparison between fish eDNA and crayfish eDNA within the same water sample yielded substantially higher concentrations of fish eDNA and thus also a higher detection rate (*Fossøy et al. 2020*). In **paper II**, we could observe a pronounced increase and subsequent decline of eDNA copies per litre of both noble crayfish eDNA and *A. astaci* eDNA, correlated to the crayfish plague outbreak moving upstream in the Halden watercourse. The increased levels of noble crayfish eDNA during the crayfish plague outbreak were assumed to be caused by decay of dead crayfish or the consumption of crayfish by predators, both resulting in an increased release of eDNA into the ambient water. A recent study by Curtis and Larson (*2020*) supports the assumption that crayfish emit very low amounts of eDNA. They placed 15 crayfish carcasses in an enclosure within a stream and sampled downstream for 28 days and failed to detect any eDNA of the crayfish. However, their

observations and our findings in **paper II** are not in conflict as the biomass of dead noble crayfish after an entire population has been wiped out by the plague is much higher than that of the 15 specimens of *Procambarus clarkii* used by Curtis and Larson (*2020*). Furthermore, the current in the stream most likely facilitated downstream transport of eDNA away from the sample site, whereas our study was carried out in a large waterbody with low current. However, our study clearly demonstrates that live crayfish emit less eDNA than dead, decaying crayfish. It might therefore be difficult to interpret if high levels of eDNA from crayfish are a sign of high population density or rather a mass mortality event.

In the aquarium experiment conducted in **paper V**, we found no consistent number of eDNA copies emitted from individual crayfish. In tanks with cold and clean water, the increase in eDNA copies was 1000-fold with a 10-fold increase in crayfish density. This shows that eDNA quantities certainly will rise with density in the absence of factors that decrease eDNA detectability, but not necessarily in a linear fashion. In our case, we suspect aggressive behaviour leading to at least one event with loss of claws, might explain the high increase. However, for all other observations there was hardly any difference between the eDNA content in the water for 2 and 20 crayfish kept together. We found that a limited set of controlled factors can considerably change the detectable amount of eDNA, while the physical presence of the target organisms remains the same. Here, the most prominent example is the near failure of positive eDNA results for *A. astaci* at high temperature compared to low – even though the infection prevalence in the experimental crayfish population was high in both experiments. While we expected the eDNA concentrations of crayfish and *A. astaci* to increase in a linear fashion with increased crayfish density, we conclude that this increase can be concealed by other factors, both environmental and biological (see below).

We also analysed water samples that were taken from two lakes with varying signal crayfish density and *A. astaci* prevalence and compared eDNA quantities with CPUE data in **paper V**. We observed little correspondence between CPUE data and crayfish eDNA quantities. Our findings were consistent with those of Johnsen *et al*. (*2020*) who compared CPUE data of noble crayfish with eDNA concentrations and other studies that report no clear or only weak correlations between crayfish density and eDNA concentration (*Dougherty et al. 2016, Cai et al. 2017, Larson et al. 2017, Rice et al. 2018*). However, there is a clear correlation between crayfish density and eDNA detection probability (*Johnsen et al. 2020*). Thus, as observed for *G. salaris*, detection frequency and probability of positive detection might be a better indicator of crayfish population density or parasite intensity of *G. salaris*.

It seems that in the current state of development of the eDNA methodology, and also more generally due to the multitude of influencing factors, eDNA quantities alone cannot serve as a suitable proxy for host density or pathogen intensity. Combined with good knowledge of the organisms and the habitat, fluctuating eDNA quantities might yield meaningful snapshots of a situation, such as an outbreak and mortality event (**paper II**). However, the analyses of the two particular host-pathogen complexes investigated in this thesis show that the possibility of using eDNA copy numbers as a proxy for host density or pathogen intensity is dependent on the quantity of eDNA shed by the respective organism, which in turn is not only influenced by its biology but also external/environmental factors (see below).

Environmental factors and organism biology - influence on eDNA emission and detectability

In order to potentially increase detection possibilities and optimise sampling strategies, we attempted to explore how environmental factors and organism biology influence the emission and detectability of host-pathogen eDNA. This is an overreaching topic in all papers in the study, but still difficult to measure even under experimental, controlled conditions. In the experiment of **paper V,** we heavily influenced the conditions within the tanks to determine to what degree certain environmental factors impact the detection of eDNA of two dissimilar target organisms. While the plan was to test for life-cycles events such as moulting, reproduction and death, these experiments failed (see *Laurendz 2017*). Instead, we focussed on simple controlled factors such as density, temperature and food availability. Crayfish tanks were filled with water prior to the experiment with no water exchange for the duration of the experiment. The long period of time between placing crayfish in the tanks and sampling could have led to a state of equilibrium where eDNA is emitted from crayfish at a similar rate to its degradation while *A. astaci* eDNA persists and accumulates. A study by Harper *et al*. (*2018*) highlighted the short-lived nature of crayfish eDNA where tanks contained either one or three crayfish and seven days after the crayfish had been removed their eDNA was detected only in the tanks containing three crayfish. In our study, detectable eDNA quantities of both targets increased far more than in a linear fashion with increased crayfish density in cold, clear water, while an increase of temperature significantly reduced the concentration of detectable eDNA. The presence of food decreased the detectability of crayfish eDNA, presumably through increased microbial-induced eDNA degradation (*Barnes et al. 2014, Barnes and Turner 2016, Salter 2018, Saito and Doi 2021*). For *A. astaci*, food did not affect the detectability, but high water temperature substantially reduced the amount of detected eDNA.

The higher temperatures were close to the temperature limit of *A. astaci,* which has its optimum temperature for sporulation at lower temperatures (*Diéguez-Uribeondo et al. 1995, Strand 2013*). Furthermore, the higher water temperatures may have, in fact, restricted movement of signal crayfish rather than increased it as this species originates from temperate regions in northern America (*Hobbs 1974*). Water temperature of 10 °C is a more accurate representation of natural waters in Europe. The influence of food was particularly interesting. While we detected an increase of *A. astaci* eDNA copies per litre with an increased crayfish density both when food was present and when it was absent, we detected an increase of signal crayfish eDNA copies per litre only when food was absent. We observed a marked increase of turbidity in the tanks with food, which could originate from higher (digestive) activity of the crayfish and leading to higher microbial activity within the tanks. We assume this may have led to a faster degradation of crayfish eDNA than of *A. astaci* eDNA. The zoospores of *A. astaci,* the main source of *A. astaci* eDNA, are living unicellular organisms, capable of encystation (*Cerenius and Söderhäll 1984*) while the most likely source of crayfish eDNA is shed or abraded epithelial cells rather than propagules such as sperm-cells.

Contrary to crustaceans, fish shed substantially more eDNA (*Merkes et al. 2014, Klymus et al. 2015, Forsström and Vasemägi 2016, Takeuchi et al. 2019, Crane et al. 2021*) as they are covered by a mucus layer rather than a hard exoskeleton. Furthermore, during spawning, when the male fertilises the eggs laid by the female, large quantities of eDNA are also released directly into the water as opposed to the crayfish mating procedure (*Stebbing et al. 2003*). As crayfish shed little eDNA compared to other organisms such as fish, monitoring should be conducted during a period of high crayfish activity to increase detection probability. Crayfish activity is highest during the summer months due to elevated water temperatures (*Flint 1977, Rusch and Füreder 2015*) whereas crayfish display very little activity during the winter months (*Wittwer et al. 2018*). Mating, spawning and moulting occur during the period of high crayfish activity, all of which are life-cycle events where higher amounts of eDNA than normal are expected to be released into the environment. In an experimental setup not included in **paper V**, eDNA emission during moulting was attempted to be monitored. Here, Laurendz (*2017*) observed a clear trend that moulting events increased the amount of detectable eDNA although a low sample number only allowed a qualitative evaluation. For other species, spawning events increased the amount of detectable eDNA (*Bayer et al. 2019, Takeuchi et al. 2019, Wacker et al. 2019*). Adult crayfish only moult once or twice per year (*Westman and Savolainen 2002*) and moulting is induced by a rise in water temperatures (*Westin and Gydemo 1986, Aiken and Waddy 1992, Kozák et al. 2015*). Conversely, the mating period is induced by a decrease in

water temperatures (*Abrahamsson 1971, Westin and Gydemo 1986, Kozák et al. 2015*) towards the end of the activity period. For *A. astaci*, Wittwer *et al.* (*2018*) identified a period of seven months from April to October where water temperatures ranged between 12 °C and 16 °C, in which *A. astaci* eDNA concentrations were high due to increased spore production. We observed during monthly sampling that at very low population density (CPUE 0.12), signal crayfish eDNA was detected only in July and October (**paper II**), coinciding with the presumed period of moulting (summer) and ovigerous females (late autumn) (*Dunn et al. 2017*).

For Gyrodactylids, it has been shown that up to 30 generations of asexual reproduction are possible without the need for a sexual partner (*Braun 1966, Harris 1989*), but Harris *et al*. (*1994*) found whorls of inseminated spermatozoa within the seminal receptacle of *G. salaris* from which they inferred sexual reproduction. Moreover, copulation has been observed in Gyrodactylids (*Braun 1966*). Nonetheless, it is unlikely that sexual production of *G. salaris* will yield substantial contributions to eDNA shed into the environment when considering that the entire organism consists of little more than 1,000 cells (*Bakke et al. 2007*). Due to the small size of the organism it is more likely that specimens that have become detached from the host or parts of them constitute the main source of detectable eDNA of *G. salaris* rather than cells shed through metabolic or reproductive processes. This is reflected by our observations (**paper IV**) where the prefilters prevented the capture of whole parasites and detection was consistent only above a threshold of parasite intensity. Results from field samples – without prefilters – by Fossøy *et al*. (*2019*) and Hansen *et al*. (*2021b*), where the observed pattern of eDNA concentrations resembled the parasite abundance observed by conventional methods, support rather than contradict this assumption: under field conditions, Gyrodactylids will become separated from the host at a constant rate and thus also be captured on filters at a constant rate, both of which will increase with increasing parasite intensity. Hence the organism biology and the thus derived capability to shed eDNA - or the lack thereof - is of paramount importance to the detectability of organisms when using the eDNA methodology.

Experiments by Strand *et al*. (*2012*) revealed that in the absence of death or moulting events, latent carrier crayfish (signal crayfish) released moderate amounts of spores at a continuous rate. However, in susceptible noble crayfish a clear pattern of increased *A. astaci* sporulation has been observed, coinciding with progressing infection (*Makkonen et al. 2013*). During a crayfish plague outbreak, we observed similar patterns of increasing *A. astaci* eDNA concentrations (**paper II**). This highlights the importance of taking biological events and patterns into account to improve sampling strategy. However, water temperatures are also

important to consider. Our results regarding the failure of detection *A. astaci* in 20° C tanks with infected signal crayfish might indicate that water temperatures near 20° C and above might camouflage the presence of *A. astaci* in signal crayfish populations.

While crayfish plague outbreaks are hard to predict, the reproductive cycle of aquatic animals is well studied. Reproductive events have shown to increase eDNA detection for fish (*Tillotson et al. 2018, Bracken et al. 2019, Takeuchi et al. 2019*), great crested newt (*Buxton et al. 2017*) and freshwater pearl mussel (*Wacker et al. 2019*) or sea scallops (*Bayer et al. 2019*) and crustaceans (*Dunn et al. 2017, Crane et al. 2021*). Another stage of the life cycle that is of importance to eDNA detection is death. Tillotson *et al*. (*2018*) found substantial amounts of eDNA being released by dead fish which correlated with our observations in **paper II** where we attributed a spike in eDNA concentrations of noble crayfish during an ongoing crayfish plague outbreak to the decay of dead noble crayfish. Studies by Dunn *et al*. (*2017*) and Crane *et al*. (*2021*) examined the relationship between eDNA concentration and biomass of crustaceans. Dunn *et al*. (*2017*) were able to detect eDNA of *P. leniusculus* only when female crayfish specimens were ovigerous, and also Crane *et al*. (*2021*) observed significantly higher eDNA detection rates in tanks containing ovigerous female European green crabs (*Carcinus maenas*) compared to tanks containing non-ovigerous hard- and soft-shelled crabs. Thus, the late autumn period with colder water and ovigerous crayfish stands out as a promising period for eDNA monitoring of the *A. astaci* – signal crayfish complex.

Under natural conditions, crayfish dwell at the bottom of the water column and can also burrow into the sediments (*Hager 1996*). Combined with the low emission rate of eDNA compared to other organisms, this further decreases the detectability of this group of organisms as the emitted eDNA might be within the sediments while the crayfish stay buried. This might explain why an increasing body of studies does not find a correlation between crayfish density estimates (CPUE) and eDNA quantities in the water (*Dougherty et al. 2016, Cai et al. 2017, Rice et al. 2018, Johnsen et al. 2020, Troth et al. 2020, Chucholl et al. 2021;* **paper V**).

While all organisms shed eDNA into their surroundings, this project demonstrates that this does not happen at a uniform rate or manner, and that the main source of eDNA also differs. We show that the amount of detectable eDNA can fluctuate in response to environmental or biological influences while the physical presence of the target organisms remains the same.

During the period of this thesis we experienced that the eDNA methodology follows the dynamics of the "Gartner hype cycle" (*Blosch and Fenn 2018*) in which a technological trigger leads to a peak of inflated expectations, followed by a "trough of disillusionment" with a subsequent "slope of enlightenment" and finally a plateau of productivity. Initially, high expectations were held regarding the potential of the eDNA methodology for wildlife biology and biodiversity monitoring including the possibility of using eDNA to estimate relative abundance from relatively small water volumes (*Lodge et al. 2012, Bohmann et al. 2014*). While the expectations were met to some extent for fish, other studies revealed that eDNA was both subject to certain limitations (*Tréguier et al. 2014, Harper et al. 2019, Stewart 2019*), and that detection success can be constrained by both sample volume (*Strand et al. 2014, Fossøy et al. 2019, Troth et al. 2020*) and sampling intensity (*Schmidt et al. 2013, Johnsen et al. 2020, Sieber et al. 2020*). Several studies point out the potential pitfalls of the eDNA methodology while also adding valuable suggestions on how to overcome them (*Goldberg et al. 2016, Sepulveda et al. 2020b, Thalinger et al. 2021*) thus forming the "slope of enlightenment" which will lead to the "plateau of productivity" where the eDNA approach is commonly used, with its limitations and mostly for presence-absence purposes, as an animal-welfare friendly and low-cost alternative or supplement to conventional methods (*Foote et al. 2012, Mächler et al. 2014, Bass et al. 2015, Smart et al. 2015, Vrålstad et al. 2017, Wittwer et al. 2018*).

The differences in eDNA emission or production within and between the two host-pathogen models will certainly require special considerations for monitoring strategies. There are both potentials and challenges of eDNA monitoring for these dissimilar target organisms related to biology, environmental factors and sampling effort. For the host-parasite complex Atlantic salmon and *G. salaris*, we detected far more eDNA of fish hosts (Atlantic salmon and rainbow trout) than of the parasite, as expected. However, due to experimental constraints and the fish requirement for constant flow-through in the aquarium experiment, the data we obtained for *G. salaris* detectability at low parasite intensities reflects a snapshot of eDNA emission with continuous water replacement, opposed to the week of accumulation in the signal crayfish – *A. astaci* study. With less dilution and more sampling effort, the detectability at lower parasite intensities might change drastically in favour of earlier detection. Here, an increased number of samples may theoretically have given us a higher resolution of the "big picture" and enabled a closer approximation of the median values of eDNA copies of *G. salaris*. However, this was not technically feasible for logistical and financial reasons as well as due to ethical considerations where the use of a high number of juvenile salmon would have contravened the

three Rs (Replacement, Reduction and Refinement) (*Russell and Burch 1959, NC3Rs 2021*), the guiding principles for more ethical use of live animals in experiments. This, combined with more suited DNA extraction methods, urgently needs further investigations for clarifying the eDNA methodology potential for early detection of *G. salaris*. Although the results we obtained indicate that eDNA monitoring may be unsuitable for early detection of *G. salaris*, a recent report showed that results from eDNA analyses and conventional methods corresponded well (*Hansen et al. 2021b*). Data in this report was obtained using the same DNA extraction method as used by Fossøy *et al*. (*2019*) which may be better suited than the extraction method used in this thesis. The use of pre-filters made from plankton mesh (**paper IV**) was included for experimental purposes, and we do not recommend this for monitoring since capturing recently detached parasites suspended in the water column will increase detection likelihood.

For successful monitoring of the host-pathogen complex crayfish and *A. astac*i, several considerations should be taken into account, including water temperature and crayfish biology. The higher detectability of *A. astaci* and crayfish eDNA at lower water temperatures, suggests that the detection success might increase when sampling is conducted when water temperatures start to decrease. In the light of the increased emission of eDNA from ovigerous female crayfish (*Dunn et al. 2017*) and general increased activity of crayfish in the reproduction period where they to a lesser extent stay buried (*Hager 1996*), the late autumn period with colder water and crayfish reproduction season stands out as a promising period for eDNA monitoring of the *A. astaci* – signal crayfish complex. However, for the general surveillance of indigenous crayfish and crayfish plague, the discovery of *A. astaci* introductions and spread, potentially leading to outbreaks, depends on more frequent sampling events throughout the year. This will, however, increase the monitoring costs (*Johnsen et al. 2020*) but also increase the probability to unveil an emerging outbreak while it is still possible to minimise the resulting damage.

Any sampling strategy should take the sample numbers into account and evaluate the need for increased numbers of samples for an increased detection probability depending on the target organism. Ideally, a pilot study combined with occupancy modelling should precede any large scale monitoring program to optimise detection. Furthermore, we highly recommend analysing samples for both targets when monitoring for host-pathogen complexes even if only one of the targets is of direct interest. For American crayfish and *A. astaci*, this will further increase probability of detection as the detection of one indicates the presence of the other. For *G. salaris* monitoring, Atlantic salmon eDNA serves as a control for the actual relevance of the sample; Atlantic salmon alone predict the absence of *G. salaris* in that population while a sample

negative for both targets implies failure to sample at a relevant site for Atlantic salmon. For *A. astaci* monitoring focussing on crayfish plague outbreaks in indigenous crayfish populations, the sole presence of crayfish eDNA indicates an uninfected crayfish population, while the combined detection of *A. astaci* with indigenous crayfish implies an emerging or ongoing outbreak situation. In order to further examine the eDNA dynamics of the host-pathogen complexes studied in this thesis and based on the experience gained from the conducted experiments, it could prove insightful to conduct further, more elaborate experiments. An experiment, similar to the one conducted in **paper V** with signal crayfish and *A. astaci* but with higher sampling frequency and also one with flowing water might shed further light. Furthermore, Atlantic salmon, in various densities and infected with *G. salaris* could be held in systems or half-pipes mimicking streams. Sampling at different distances downstream might possibly help gain better understanding of the eDNA dynamics of this host-pathogen complex. Based on our observations and the results of other studies where assays targeting nuclear markers outperform those targeting mitochondrial markers regarding sensitivity (*Minamoto et al. 2017, Dysthe et al. 2018, Moushomi et al. 2019*) (**paper IV**), it may be beneficial to target nuclear markers in organisms that shed only little eDNA such as crayfish. Currently, all available assays for crayfish target mitochondrial markers and the commonly used barcode for animals is the mitochondrial *COI* sequence, therefore reference libraries may be lacking.

The use of the eDNA methodology and early detection of *A. astaci* in freshwater ecosystems can substantially aid the conservation of native European crayfish which face a growing number of threats (*Edsman et al. 2010, Kouba et al. 2014, Kozák 2015*). Suggested conservation measures include the restoration of aquatic habitats (*Taugbøl and Skurdal 1999*) and the creation of ark sites (*Peay 2009*). Screening these waterbodies for *A. astaci* eDNA and eDNA of non-native crayfish species prior to stocking efforts as well as regular monitoring after introduction, as is already practised in some countries (*Johnsen et al. 2019, Swords et al. 2020*), could help ensure successful conservation. The spread of *G. salaris* has been linked to movement of susceptible fish species across Europe (*Peeler et al. 2006, Grano-Maldonado et al. 2011, Paladini et al. 2021*). A screening of water samples from the transport tanks prior to movement may help determine the presence of *G. salaris*. As fish in these tanks are kept in high densities, which leads to a concentration of eDNA, detection may be possible even though current methods lack the sensitivity to detect low parasite intensities.

For monitoring, interesting technologies have emerged like an integrated backpack sampler (*Thomas et al. 2018*) or even sampling drones (*Benson et al. 2019*). There is a shift towards

more mobile and portable devices for both sampling and analysis. An example is handheld pumps (e.g. **paper III** or (*Cantera et al. 2019*)) and handheld PCR thermocyclers (*Hole and Nfon 2019*) with associated field-extraction kits. For ease-of-use, and to reduce sampling cost and particularly sampling time, passive sampling (*Bessey et al. 2021, Verdier et al. 2021*) or automated robotic sampling (*Yamahara et al. 2019, Hansen et al. 2020, Sepulveda et al. 2020a*) are promising approaches. A different approach is citizen science projects where sampling with single-use equipment is outsourced to the public (*Biggs et al. 2015, Miralles et al. 2016, Larson et al. 2020*), a concept which could be extended to any number of target species.

The past decade has seen an exponential increase of studies utilizing eDNA in many creative ways. As a relatively new approach, the eDNA methodology undoubtedly still has certain teething problems – some of which became apparent during this thesis. Nonetheless, the potential of this technology has been highlighted and eDNA monitoring will undoubtedly contribute to fast, efficient and animal-welfare friendly solutions of (future) challenges.

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Paper I

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Parasites & Vectors

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Catching the fish with the worm: a case study on eDNA detection of the monogenean parasite *Gyrodactylus salaris* and two of its hosts, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*)

Johannes C. Rusch[1,2], Haakon Hansen[1*], David A. Strand[1], Turhan Markussen[3], Sigurd Hytterød[1] and Trude Vrålstad[1]

Abstract

Background: Environmental DNA (eDNA) monitoring is growing increasingly popular in aquatic systems as a valuable complementary method to conventional monitoring. However, such tools have not yet been extensively applied for metazoan fish parasite monitoring. The fish ectoparasite *Gyrodactylus salaris*, introduced into Norway in 1975, has caused severe damage to Atlantic salmon populations and fisheries. Successful eradication of the parasite has been carried out in several river systems in Norway, and Atlantic salmon remain infected in only seven rivers, including three in the Drammen region. In this particular infection region, a prerequisite for treatment is to establish whether *G. salaris* is also present on rainbow trout upstream of the salmon migration barrier. Here, we developed and tested eDNA approaches to complement conventional surveillance methods.

Methods: Water samples (2 × 5 l) were filtered on-site through glass fibre filters from nine locations in the Drammen watercourse, and DNA was extracted with a CTAB protocol. We developed a qPCR assay for *G. salaris* targeting the nuclear ribosomal ITS1 region, and we implemented published assays targeting the mitochondrial cytochrome-b and NADH-regions for Atlantic salmon and rainbow trout, respectively. All assays were transferred successfully to droplet digital PCR (ddPCR).

Results: All qPCR/ddPCR assays performed well both on tissue samples and on field samples, demonstrating the applicability of eDNA detection for *G. salaris*, rainbow trout and Atlantic salmon in natural water systems. With ddPCR we eliminated a low cross-amplification of *Gyrodactylus derjavinoides* observed using qPCR, thus increasing specificity and sensitivity substantially. Duplex ddPCR for *G. salaris* and Atlantic salmon was successfully implemented and can be used as a method in future surveillance programs. The presence of *G. salaris* eDNA in the infected River Lierelva was documented, while not elsewhere. Rainbow trout eDNA was only detected at localities where the positives could be attributed to eDNA release from upstream land-based rainbow trout farms. Electrofishing supported the absence of rainbow trout in all of the localities.

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* Correspondence: haakon.hansen@vetinst.no
[1]Norwegian Veterinary Institute, P.O. Box 750, Sentrum, NO-0106 Oslo, Norway
Full list of author information is available at the end of the article

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Conclusions: We provide a reliable field and laboratory protocol for eDNA detection of *G. salaris*, Atlantic salmon and rainbow trout, that can complement conventional surveillance programs and substantially reduce the sacrifice of live fish. We also show that ddPCR outperforms qPCR with respect to the specific detection of *G. salaris*.

Keywords: Environmental DNA, Multiplex PCR, Droplet digital PCR (ddPCR), Internal transcribed spacer (ITS), Mitochondrial DNA (mtDNA), Invasive species

Background

Gyrodactylus salaris Malmberg, 1957 (Monogenea) is an ectoparasite first described on the skin of Atlantic salmon *Salmo salar* (L. 1758), where it attaches itself to the host with a haptor, a specialized attachment organ consisting of a large disc with 16 peripheral articulated marginal hooks and a single pair of ventrally orientated hamuli [1]. This ~500 μm long parasite [2] has also been found on other salmonids such as rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) [3], brown trout *Salmo trutta* (L., 1758) and Arctic charr *Salvelinus alpinus* (Linnaeus, 1758) [4]. While most species and populations of fish which act as hosts, including Baltic populations of Atlantic salmon, do not experience serious consequences of a *G. salaris* infection [1, 5], Atlantic populations of salmon are highly susceptible to *G. salaris* resulting in high mortality rates in mainly Norwegian populations (see below). Rainbow trout is less susceptible, and can sustain infections for long periods, often at low intensities making it an important host when considering spreading between fish farms in Europe [6].

In 1975, *G. salaris* was detected in Norway for the first time [7–9]. The parasite has since caused severe damage to several Atlantic salmon populations [1]. Altogether, fish in 50 rivers in Norway have been infected by *G. salaris* and extensive eradication programs, mostly using pesticides such as rotenone, have been carried out in several of these watercourses [10] since 1981 [11]. Over the last 15 years [12], the eradication programs have been highly successful and to date the parasite is present only in seven rivers [10]. To document the absence of *G. salaris* in Norwegian river systems and to detect new infections at an early stage, large-scale national surveillance programs are carried out every year [10, 13]. Present surveillance is based on the catching and killing of numerous Atlantic salmon juveniles in rivers and farms, as well as rainbow trout reared in farms, for morphological screening for the presence or absence of *G. salaris*. In 2016 alone, 6981 fish were killed and examined [10, 13].

One of the remaining regions where *G. salaris* is still present is the Drammen region (Buskerud and Vestfold County) in southern Norway, consisting of the rivers Drammenselva, Lierelva and Sandeelva (hereafter referred to by their Norwegian names). The infection region including a control area is described in the Norwegian legislation [14]. A strategy to implement treatment of this region has not yet been conclusively devised by the Norwegian authorities, as this watercourse in many aspects is more complicated than previously treated systems. This results from three basic factors. First, rainbow trout in the system upstream of the current migration barriers for salmon have a history of infection with *G. salaris* [8]. Secondly, Drammenselva contains a much higher fish species diversity than other treated rivers, which mainly contain salmonids. Thirdly, this river discharges into a large estuary with surface water containing low salinity (< 2%) where *G. salaris* can survive for longer periods [15]. In order to decide on measures regarding treatment of this water system, exact knowledge of the status of infections with *G. salaris* in the area is a prerequisite. Rainbow trout farms in the northern parts of the Drammen watercourse were infected with *G. salaris* in the mid-1980s and later there have been both documented [16, 17] and anecdotal reports of free-living rainbow trout in the system. There is thus a possibility that free-living rainbow trout are still present in the system and these might have sustained the introduced *G. salaris* infection from the 1980s. Therefore, a surveillance program [18, 19] has been established to detect any possible presence of *G. salaris* on free-living populations of rainbow trout upstream of the anadromous parts of the Drammenselva catchment.

Standard surveillance for fish parasites, including the surveillance programs for *G. salaris* in Norway, involves capture and euthanasia of fish, prior to manual examination for the presence of parasites. This is both costly and labour-intensive, and results in the sacrifice of a large number of usually infection-free healthy fish. In recent years, capturing, amplifying and detecting species-specific DNA fragments of several species in water samples has been established as an accurate low-cost alternative or a complement to traditional monitoring [20–23]. This approach, harnessing so-called environmental DNA (eDNA), makes use of the knowledge that all organisms shed cells into their surroundings (excretion, mucus layers, abrasions of epithelial tissue, gametes) [24, 25]. For eDNA monitoring of natural waters, the eDNA content represents to a large extent a snap-shot of the present living species, with a time lag of only some weeks after a species has disappeared from the system until eDNA is no longer detectable [26]. Results are

delivered relatively fast and efficiently [27], often at lower agent-prevalence than through traditional methods [28].

To complement conventional surveillance methods for *G. salaris*, we aimed at developing an eDNA approach for targeted detection of the parasite-host combination in water samples. We applied this method in a case-study, where eDNA detection by means of species specific quantitative PCR (qPCR) and droplet digital PCR (ddPCR) was used as a supplement to standard surveillance methods for *G. salaris*, Atlantic salmon and rainbow trout in the Drammen infection region, Norway.

Methods

Description of the study area

One part of this study was conducted in the northern part of the Drammenselva watercourse (Oppland County) where a presence of wild rainbow trout populations is possible and the status of *G. salaris* is unknown. The other part of the study was conducted in Lierelva (Buskerud County), a small river in the Drammen infection region where Atlantic salmonhasbeen infected with *G. salaris* since 1987 [1]. Drammenselva drains from the Jotunheimen Mountains in the north, down to Drammensfjorden (Buskerud and Vestfold Counties) which connects the watercourse with the Atlantic Ocean (Fig. 1). The infection region in Drammen incorporates three of the remaining seven rivers in Norway where *G. salaris* is still present. These are: Drammenselva, Lierelva (both Buskerud County) and Sandeelva (Vestfold County) (Fig. 1), in all of which Atlantic salmon is present. Lierelva and Sandeelva are smaller rivers with catchment sizes of 309.6 and 193.4 km^2, respectively, while Drammenselva drains from a much larger area (17,110.8 km^2). In the northern part of the Drammen watercourse (see Fig. 1), several rainbow trout producers can be found. Fish in farms in this area were infected by *G. salaris* in the mid-1980s and there were many reports of escaped fish from the farms [14]. However, the fish populations in the farms were eradicated and all these farms were declared

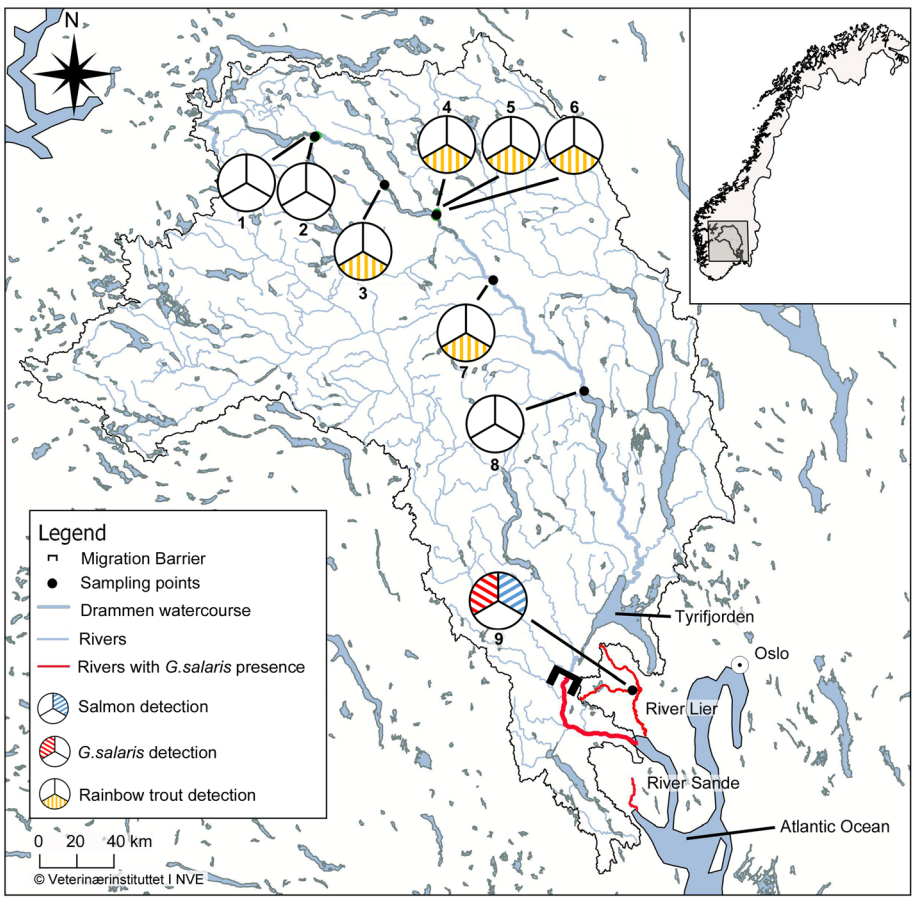

Fig. 1 Map of the Drammen watercourse region with all sampling locations and its location within Norway. Green points represent localities sampled. The thick blue line represents the Drammen watersystem, the thin blue lines represent the main rivers, the red lines indicate rivers where *G. salaris* is present and the black lines outline the Drammenselva drainage basin. The numbers refer to the sampling sites in Table 1. Pie charts: blue colour indicates detection of Atlantic salmon, red indicates detection of *Gyrodactylus salaris* and yellow indicates detection of rainbow trout. Rivers flow north to south

free from *G. salaris* in 1987 [29]. In 1986, *G. salaris* was also diagnosed from farmed rainbow trout and salmon in the Lake Tyrifjorden which is a part of the Drammen watercourse [8, 30]. The fish populations in these farms were also eradicated, but a short time later, the parasite was detected on salmon juveniles from Drammenselva and Lierelva [30].

Sample locations

The sampling sites included eight localities in the northern part of the Drammenselva watercourse, (Fig. 1, Table 1). These sampling sites were chosen as part of a monitoring program [19] and with the intention of both declaring this region free from *G. salaris* and mapping the presence of free-living rainbow trout. One of these eight sampling sites was a fish pond at a local trout farm that served as a rainbow trout positive field control sample. The ninth sample was chosen as a positive field control sample for only *G. salaris* and Atlantic salmon and collected from a stretch in Lierelva (Fig. 1), a river with a confirmed presence of Atlantic salmon infected with *G. salaris*.

Within the area where rainbow trout farms can be found, the locations of sample nos. 4 and 5 were chosen based on information obtained from the local authorities prior to the field work. These samples were taken in streams flowing into the main watercourse in order to avoid positive detections due to outlet water from farms situated upstream in the main watercourse. For an indication of the sensitivity of the rainbow trout eDNA assay for detection in the field, three samples (nos. 6, 7 and 8) were taken from the main watercourse at different distances from the rainbow trout farms. Samples 1 and 2 were taken upstream of the area containing rainbow trout farms.

Electrofishing and *Gyrodactylus* counts

Electrofishing was carried out in rivers and tributaries in the Drammen watercourse to reveal the possible presence of rainbow trout, using this standard surveillance method. The area examined was chosen on site according to local conditions (stream size, depth, water flow). Electrofishing was also conducted in Lierelva to collect salmon juveniles for estimation of the infection prevalence and intensity of *G. salaris* in the same locality as water samples were taken. Fish captured for further examination were euthanised following the strict codes of practice in force in Europe, preserved intact in 96% ethanol and later examined for the presence of *Gyrodactylus* spp. using a stereo microscope (Leica MZ 7.5, Leica microsystems, St. Gallen, Switzerland).

Water filtering for eDNA sampling

At each sampling location, duplicate water samples of 5 L (2 × 5 l) were collected and filtered on site on to glass fibre filters (47 mm AP25 Millipore, 2 μm pore size, Millipore, Billerica, USA) using a portable peristaltic pump (Masterflex E/S portable sampler, Masterflex, Gelsenkirchen, Germany), tygon tubing (Masterflex) and an in-line filter holder (Millipore) according to Strand et al. [31]. At Lierelva, four samples were taken instead of two as this river was intended as a positive field control for *G. salaris* and Atlantic salmon. Filters were placed in separate 15 ml Falcon tubes containing cetyl trimethyl ammonium bromide (CTAB) buffer and stored on ice directly after filtration. Upon arrival at the laboratory the samples were stored at -20 °C until further analysis. As a safety precaution and part of the filtering protocol, the entire equipment was disinfected with a 10% bleach solution after use at each location. Thus, any residual eDNA was broken down and contamination was prevented. Before further sampling, the tubes were rinsed with sodium thiosulphate to neutralise the bleach solution, and then flushed with ambient river water directly before sampling.

DNA extraction

DNA was extracted from the filters according to a CTAB protocol described in Strand et al. [31], with the exception that the CTAB buffer contained no added 1% 2-mercapto-ethanol. During extraction each filter was

Table 1 List of sampling sites including location, sampling date and amount of water filtered

Site no.	Site name	Water filtered (l)	Coordinates	Date
1	Storåne at Ala camping	5 (×2)	61.1473N, 8.7121E	26.06.2017
2	Storåne at Tørpegårdsvegen/bru	5 (×2)	61.1522N, 8.7250E	26.06.2017
3	Trout farm	5 (×2)	61.0379N, 9.0466E	14.11.2016
4	Leireelvi at Leira/Garlivegen	5 (×2)	60.9742N, 9.2936E	26.06.2017
5	Leireelvi at Leira camping	5 (×2)	60.9680N, 9.2884E	26.06.2017
6	Lake Strondafjorden at Faslefoss	5 (×2)	60.9671N, 9.2889E	26.06.2017
7	River Begna at Bagn	5 (×2)	60.8198N, 9.5612E	26.06.2017
8	River Begna at Nes	5 (×2)	60.5628N, 9.9929E	26.06.2017
9	Lierelva at Sjåstad	5 (×4)	59.8580N, 10.2213E	31.08.2017

split into two subsamples (A and B) due to volume restrictions imposed by centrifuge size and extracted separately. An environmental control and a blank extraction control were included as a precaution to detect any possible contamination during DNA extraction. The blank extraction control consisted of a Falcon tube containing the CTAB buffer but no filter, which was then processed in the same way as all other tubes containing buffer and filters. The environmental control used in this study consisted of an Eppendorf tube containing 200 µl PCR-grade water. This tube remained open in the fume hood throughout the entire process of extraction.

PCR-based assays for eDNA detection of *G. salaris*, rainbow trout and Atlantic salmon

A quantitative PCR assay (qPCR) using species-specific primers and a minor groove binder (MGB) probe targeting the *G. salaris* internal transcribed spacer region 1 (ITS1) was developed. The ITS1 sequence published as GenBank accession no. DQ898302 was used as template and the specificity of the designed primers and probe was checked against closely related species and other species that might be present in Norwegian watercourses: *G. salmonis* Yin & Sproston, 1948 (GQ368233), *G. truttae* Gläser, 1974 (AJ132260), *G. lucii* Kulakovskaya, 1952 (EU304811), *G. arcuatus* Bychowsky, 1933 (JN703797) and *G. derjavinoides* Malmberg, Collins, Cunningham & Jalali, 2007 (EU304810). Multiple sequences were aligned using AlignX (Vector NTI Advance 11.5, Invitrogen, Carlsbad, USA). The design of primers and probe was performed manually, targeting ITS1 sequence regions displaying the highest sequence diversity between *G. salaris* and the species listed above. The final primer and probe sequences (Table 2) partly overlap with those previously published for this parasite [32] and their specificity was confirmed through matching them against the database of the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) nucleotide database using the Basic Local Alignment Search Tool (BLASTn). The aim of the new qPCR assay was to attempt to obtain the best possible sensitivity and specificity for eDNA applications. Similar to the assay Collins et al. [32] designed, the newly designed assay is not able to distinguish between *G. salaris* and *G. thymalli* Žitňan, 1960 as these two species have indistinguishable ITS sequences [33].

The assays used for eDNA-detection of Atlantic salmon and rainbow trout (Table 2) follow Matejusova et al. [34] and Wilcox et al. [35], respectively. These were successfully tested on DNA extracts from tissue of Atlantic salmon and rainbow trout before use in the current study (data not shown). The ddPCR assay for *G. salaris*, rainbow trout and Atlantic salmon applied the same primers and probes as the qPCR.

Evaluation of qPCR and ddPCR assay specificity

The specificity of the assay was tested on DNA extracts of *G. salaris* collected from three different locations in Norway in addition to DNA extracts from the following other species present in the collection at the NVI: *G. thymalli*, *G. salmonis*, *G. arcuatus*, *G. lucii* and *G. derjavinoides*. Species identification of these samples had been done previously by sequencing of ITS (results not shown). We also ran the same samples with the previously published assay [32] to compare the specificity and sensitivity of the assays. ddPCR applies the same primers and probes as qPCR and the specificity was tested on *G. derjavinoides* due to the low level of cross amplification shown in a previously published assay [32]. The ddPCR assay was also tested on isolates of *G. salaris* obtained from fish from Lierelva to determine optimal annealing temperature.

Table 2 Primers and probes for *Gyrodactylus salaris*, rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) used in the present study. The probes used are TaqMan MGB probes with either Fam or Hex reporter dyes

Target species/gene	Name	Primer/probe	Sequence (5'-3')	Reference
G. salaris/ITS	G.sal208F	Forward	GGTGGTGGCGCACCTATTC	Present study
	G.sal149R	Reverse	ACGATCGTCACTCGGAATCGAT	Present study
	G.sal188P	Probe	(FAM)CAAGCAGAACTGGTTAAT(MGBNFQ)	Present study
G. salaris/ITS	F	Forward	CGATCGTCACTCGGAATCG	Collins et al. [32]
	R	Reverse	GGTGGCGCACCTATTCTACA	Collins et al.[32]
	Gsal2	Probe	(FAM)TCTTATTAACCAGTTCTGC(MGBNFQ)	Collins et al. [32]
O. mykiss/cytb	RBTF	Forward	AGTCTCTCCCTGTATATCGTC	Wilcox et al. [35]
	RBTR	Reverse	GATTTAGTTCATGAAGTTGCGTGAGTA	Wilcox et al. [35]
	RBTP	Probe	(FAM)CCAACAACTCTTTAACCATC(MGBNFQ)	Wilcox et al. [35]
S. salar/cytb	Salmonid Cyt B FOR	Forward	CGGAGCATCTTTCTTCTTTATCTGT	Matejusova et al. [34]
	S. salar REV	Reverse	ACTCCGATATTTCAGGTTTCTTTATATAGA	Matejusova et al. [34]
	S. salar Cyt B Probe	Probe	(HEX)CCAACAACTCTTTAACCATC-(MGBNFQ)	Matejusova et al. [34]

qPCR and ddPCR protocols for *G. salaris* eDNA detection

All qPCR analyses were carried out on an Mx3005P qPCR system (Stratagene, San Diego, USA). Droplet digital PCR was performed on a QX200 AutoDG Droplet Digital PCR System (Bio-Rad, Hercules, USA).

For qPCR detection of *G. salaris*, three qPCR replicates were run for each eDNA extract in the following 25 μl reactions: 1.25 μl of PCR-grade water, 12.5 μl of ExTaq mastermix (Takara Biotechnology, Dalian, China), 1.5 μl of each 10 μM primer (forward and reverse), 0.75 μl of 10 μM probe, 0.5 μl of Rox II reference dye and 5 μl of eDNA template. The qPCR cycling conditions were as follows: an initial denaturation at 95 °C for 15 min; 45 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 45 s, and extension at 72 °C for 1 min; followed by a final elongation phase at 72 °C for 10 min.

The following 22 μl reactions were run for each eDNA extract on ddPCR: 11 μl ddPCR Supermix for probes - no dUTP (Bio-Rad), 1.98 μl of each 10 μM primer, 0.55 μl of 10 μM probe, 0.49 μl PCR-grade water and 1 μl of restriction-enzyme mix consisting of 0.2 μl HindIII, 0.1 μl buffer (10×) and 0.7 μl PCR-grade water and 5 μl of DNA sample. The optimal primer-probe concentration was determined to be 900:250 and the optimal annealing temperature of 58 °C was confirmed through amplification tests along a temperature gradient. Here, we used the *Hind*III restriction enzyme to fragment the repetitive multi-copy ITS regions within the nuclear ribosomal DNA in order to ensure that the targeted DNA copies were distributed among different droplets for accurate quantification.

To allow for sufficient time for the restriction enzymes to digest, the plate was sealed using Microseal 'B' plate sealing film (Bio-Rad), wrapped in tin foil and left on the bench for 20 min. Droplet generation in the QX200 AutoDG Droplet Digital PCR System (Bio-Rad) creates an emulsion with 20 μl of the 22 μl originally pipetted into each well, resulting in a 10% loss of template and mastermix. After generation of the droplets, the new plate was immediately transferred to a TM100 thermocycler (Bio-Rad) and the QX200 Droplet Digital PCR system (Bio-Rad) with the following cycling conditions: An initial denaturation at 95 °C for 10 min; 45 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 60 s; followed by a final elongation phase at 58 °C for 10 min. The threshold for a positive sample was set at three positive droplets per well according to Dobnik et al. [36]. To ensure the validity of each run, positive and blank PCR controls containing *G. salaris* DNA and distilled water, respectively, were run on each plate for both qPCR and ddPCR.

To be able to detect *G. salaris* and Atlantic salmon simultaneously in future surveillance programmes in Norwegian rivers, we also tested a duplex method using the same primers and probes as for the singleplex reactions. This duplex method was set up by running the following 22 μl reactions for each eDNA extract in duplicates: 11 μl ddPCR Supermix for probes - no dUTP (Bio-Rad), 0.99 μl of 20 μM of Salmonid Cyt B FOR and S. salar REV primers, 0.55 μl of 10 μM S. salar Cyt B Probe, 0.99 μl of 20 μM of G.sal208F and G.sal149R primers, 0.275 μl of 20 μM G.sal188P probe, 0.215 μl PCR-grade water and 1 μl of restriction-enzyme mix consisting of 0.2 μl *Hind*III, 0.1 μl buffer (10×), 0.7 μl PCR-grade water and 5 μl of DNA sample. The optimal primer-probe concentration for both assays had been determined to be 900:250. The same cycling conditions were used as in the *G. salaris* singleplex reaction.

For qPCR detection of *O. mykiss*, three qPCR replicates were run for each eDNA extract in the following 12 μl reactions: 2.35 μl of PCR-grade water, 6.25 μl of ExTaq mastermix (Takara), 0.3 μl of 10 μM RBTF forward primer and 0.6 μl of 10 μM RBTR reverse primer, 0.25 μl of 10 μM RBTP probe, 0.25 μl of Rox II reference dye and 2 μl of DNA template. The qPCR (Stratagene) cycling conditions were as follows: an initial denaturation at 95 °C for 1 min; 45 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 45 s and extension at 72 °C for 1 min; followed by a final elongation phase at 72 °C for 10 min. We used a cut-off at Cq 41 for the rainbow trout-assay, similar to the suggestion for eDNA qPCR detection cut-off in Agersnap et al. [37].

For the singleplex ddPCR detection of rainbow trout, the following 22 μl reactions for each eDNA extract were run in duplicates: 11 μl ddPCR Supermix for Probes - no dUTP (Bio-Rad), 0.99 μl of RBTF 10 μM forward primer, 1.98 μl of 10 μM RBTR reverse primer, 0.55 μl of 10 μM RBTP probe, 2.48 μl PCR-grade water and 5 μl of DNA template. The optimal primer-probe concentration for both assays had been determined to be 450:900:250 for forward primer, reverse primer and probe, respectively, which follows the suggestions in Wilcox et al. [35]. The same cycling conditions were used as in all other ddPCR reactions.

Calculation of eDNA copies

The number of eDNA copies (for each target species) per litre of water for each sample is calculated according to the following formula, also used by Agersnap et al. [37]:

$$C_L = \frac{C_{rdd} * \left(\frac{V_e}{V_r}\right)}{V_w}$$

where C_L is the number of target-eDNA copies per litre of filtered water, C_{rdd} is the ddPCR calculation of eDNA

copy numbers per reaction volume (20 μl), adjusted for a 10% loss during droplet generation, V_e is the total elution volume after extraction, V_r is the volume of eluted extract used in the ddPCR reaction, V_w is the volume of filtered water. The copy numbers of both subsamples (A and B) were added together, thus revealing the number of eDNA copies per litre of any given sample. Calculation of eDNA copy numbers per reaction volume was performed by the QuantaSoft software (v.1.7.4, Bio-Rad) and was estimated using the ratio between positive and negative droplets within a sample, using Poisson statistics.

Results

qPCR assay optimisation and specificity tests

The current assay proved slightly more sensitive (by ~0.5 Cq) towards *G. salaris* than the assay in Collins et al. [31]. The PCR efficiency ([E = $^{10-1/slope}$] -1) × 100 calculated from triplicates of non-diluted and three 10-fold dilutions of a DNA extract originating from a single parasite, was 100 % (Cq = 20.5 to 30.6, slope = 3.312) (not shown). The qPCR assay for *G. salaris* yielded negative qPCR results for all other species except *G. salaris* (and *G. thymalli* as previously explained), except for a low level of cross-reaction towards the tested specimen of *G. derjavinoides* (Cq = 35.6).

Optimisation of ddPCR assay and specificity tests

Both the qPCR assay (primers and probes) for *G. salaris* developed in this study and the assays for rainbow trout and Atlantic salmon [32, 34] were transferable to the ddPCR platform without further optimisation, using an annealing temperature of 58 °C. Unlike the qPCR assay however, the ddPCR assay showed no signs of cross amplification of *G. derjavinoides*.

eDNA monitoring of *G. salaris*, Atlantic salmon and rainbow trout

The positive control field samples for *G. salaris* taken from Lierelva all yielded positive results in qPCR with Cq-values ranging from 24.76 to 35.86, and in ddPCR with eDNA copies/l ranging from 371,440 to 560, respectively. For Atlantic salmon, the eDNA copy numbers ranged from 10,160 (sample 9/2) to 7520 (sample 9/4) (Table 3) at an average of 8948 copies (± SD = 945).

The two positive control field samples for rainbow trout obtained at the trout farm in 2016 tested positive for rainbow trout (Cq 17.48 and Cq 17.50; 8,800,000 eDNA copies/l and 7,848,000 eDNA copies/l, respectively) (see Table 3). Of the other 18 water samples that were collected at the eight sampling points in June and August 2017, five were positive for rainbow trout. Positive samples for rainbow trout were obtained from locations 3, 4, 5, 6 and 7 (see Table 3). One of the five positive sampling sites (no. 6) was at the outlet of the lake into which all the rainbow trout farms drain, while another (no. 7) was found in the main river 25 km downstream of the outlet. According to new information from local authorities we received upon enquiry after our analyses detected rainbow trout DNA in samples 4 and 5, these locations were indeed also situated roughly 400 and 1200 m, respectively, downstream of a trout farm (see Table 3). None of the field samples in the northern part of the Drammenselva watercourse yielded a positive result when tested against *G. salaris*, neither did the rainbow trout positive control at the trout farm. All extraction blank controls and environmental blank controls were negative, both in qPCR and ddPCR.

Conventional monitoring methods

At location 1, electrofishing of an area of roughly 300 m^2 yielded seven juvenile brown trout. Two juvenile brown trout were caught at location 2 after electrofishing an area of *c.*200 m^2. At location 3, electrofishing was carried out in selected pot-holes along a stretch of 150 m. A high density of brown trout with sizes ranging from juveniles up to 500 g adults was observed. At the fourth location, electrofishing was carried out along a stretch of 200 m. Several minnows *Phoxinus phoxinus* (L., 1758) were observed and many brown trout (juveniles to 300 g) were captured in the stream while electrofishing. No electrofishing was carried out at locations 5, 6 and 7 as none of these locations were suitable for electrofishing. A total of 21 Atlantic salmon with a length of 9.6 cm (± SD 3.6 cm) were caught in Lierelva. The parasite prevalence and intensity on these fish was determined to be 85.7% and 83 parasites (± SD 63), respectively. Throughout the entire electrofishing, no rainbow trout were caught.

Discussion

In the present study, eDNA monitoring is used for the first time to detect the monogenean parasite *G. salaris* along with two of its hosts, Atlantic salmon and rainbow trout. Detections were successfully obtained both in all singleplex reactions (qPCR and ddPCR) and in a duplex reaction (ddPCR) targeting both *G. salaris* and Atlantic salmon. The prevalence in susceptible Atlantic salmon populations most often reaches 100 % [11]. In general, the infection grows exponentially on non-responding hosts and may reach several thousand individuals per fish [5]. In our study, the *G. salaris* infected Atlantic salmon individuals caught in Lierelva were only moderately infected (prevalence of 85.7%, mean parasite abundance of 83 parasites). Here *G. salaris* eDNA was detected in amounts ranging from 500 to > 350,000 copies per litre of water in the same river stretch. These

Table 3 Overview of results from qPCR and ddPCR analyses for *Gyrodactylus salaris* (ITS), *Oncorhynchus mykiss* (CytB) and *Salmo salar* (CytB) at each sampling site. List of sampling sites including amount of water filtered, number of samples per site (each sample constitutes of one filter), the Cq value (from qPCR) and number of eDNA copies per litre (ddPCR) from all filters taken at each point, respectively. eDNA copies per litre are abbreviated as eDNA/l. No detection is indicated with a minus (-) for qPCR and a zero for ddPCR and those samples where analysis was not applicable are indicated with NT

Site no.	Site name	Sample	Volume (l)	*Gyrodactylus salaris*		*Oncorhynchus mykiss*		*Salmo salar*	
				qPCR[a]	ddPCR[b]	qPCR[a]	ddPCR[a]	qPCR	ddPCR[b, a]
1	Storåne at Ala camping	1	1	-	-	-	0	-	0
		2	1	-	-	-	0	-	0
2	Storåne at Tørpegårdsvegen/bru	1	1	-	-	-	0	-	0
		2	1	-	-	-	0	-	0
3	Trout farm	1	1	-	-	17.48	7,848,000	-	0
		2	1	-	-	17.50	8,800,000	-	0
4	Leireelvi at Leira/Garlivegen	1	1	-	-	29.62	1624	-	0
		2	1	-	-	29.09	3816	-	0
5	Leireelvi at Leira camping	1	1	-	-	30.05	2240	-	0
		2	1	-	-	30.02	2124	-	0
6	Lake Strondafjorden at Faslefoss	1	1	-	-	32.3	560	-	0
		2	1	-	-	31.68	576	-	0
7	River Begna at Bagn	1	1	-	-	> cut-off[c]	0	-	0
		2	1	-	-	36.91	22	-	0
8	River Begna at Nes	1	1	-	-	-	0	-	0
		2	1	-	-	-	0	-	0
9	River Lierelva at Sjåstad	1	1	34.52	560	-	NT	NT	9200
		2	1	33.56	840	-	NT	NT	10,160
		3	1	33.94	864	-	NT	NT	7520
		4	1	24.89	371,440	-	NT	NT	8912

[a]Run as singleplex
[b]Run as duplex
[c]Cut-off value was set at Cq 41

results strongly indicate that eDNA analysis of samples obtained by water filtering can indeed be used for monitoring the occurrence of *G. salaris* in freshwater ecosystems containing natural Atlantic salmon populations.

Environmental DNA-detection is a promising tool that can be used to supplement or even replace classical surveillance where it produces fast and robust results. This is reflected in the ever growing number of assays being developed to monitor parasites which infect fish. These include both ectoparasites like *Amyloodinium ocellatum* Brown, 1931 [38], *Chilodonella hexasticha* Kiernik, 1909 [39] or *Neobenedenia girellae* Hargis, 1955 [40] and endoparasites such as *Opisthorchis viverrini* Poirier, 1886 [41], *Ichthyophonus* spp. [42] and myxozoans [43, 44]. Unlike traditional monitoring, there is no need to kill large numbers of fish or to carry out time-consuming manual examinations. Thus, the eDNA monitoring method has far-reaching potential as it reduces the time and cost of sampling and improves fish welfare. A further advantage of this method is the simultaneous detection of parasite and host. Using the protocol for filtration, DNA-extraction and the analysis we describe here, it is not only possible to detect the parasite *G. salaris* but also two of its hosts within on single sample. With the use of other assays, the presence of virtually any aquatic host-pathogen complex can be detected and monitored, provided that the filter size is appropriate to capture eDNA from the target organism.

The aim of the *G. salaris* qPCR assay designed in the present study was to achieve an optimal combination of both specificity and sensitivity, and the assay was chosen over the one previously published by Collins et al. [32] due to its slightly higher sensitivity. Both the qPCR assay presented in this paper and the qPCR assay designed by Collins et al. [32] display a low-level amplification of *Gyrodactylus derjavinoides*. However, this issue was not observed when applying the newly designed primers and probe in ddPCR. Any assay for *Gyrodactylus salaris* targeting the ITS1 region will yield positive results for *G. thymalli* since these two species have nearly identical

sequences [33] and it is therefore impossible to differentiate between them in this way. This does not affect the monitoring of *G. salaris* in systems uninhabited by grayling, the host for *G. thymalli*. In systems where grayling occur, negative samples would still indicate the absence of the parasite. A positive detection would certainly require additional examination and attention. Here, one option would be to design assays targeting the more variable mitochondrial cytochrome oxidase gene (see, e.g. Meinilä et al. [45], Hansen et al. [46]).

In the present eDNA study, as well as for most other applications, the low level of cross-reaction against *G. derjavinoides* when using qPCR poses no problem. If a population of fish were infected with a high number of *G. derjavinoides* and a low number of *G. salaris*, analysis with qPCR could yield ambiguous results. We therefore recommend the use of ddPCR analysis since this method bypasses the problem of cross-amplification. Alternatively, sampling by electrofishing followed by manual examination and standard species identification could be carried out in this particular case.

We detected rainbow trout eDNA at four locations in the northern part of the Drammen watercourse in addition to the sample taken at the trout farm (sample no. 3). We observed an apparent decline in eDNA concentration with increasing distance from the source (area with trout farms, sample nos. 6 and 7). This corresponds with data from studies that examine the dilution effects of eDNA in river ecosystems [47, 48]. However, the number and the distribution of sampling points in this study were not comprehensive enough to examine a gradient thoroughly. Extensive electrofishing at each sampling point produced no evidence for the presence of rainbow trout in the streams. We therefore attribute all positive samples to eDNA discharge/emission from trout farms and assume the areas and streams of the northern part of the Drammenselva watercourse that were tested to be free from wild populations of rainbow trout. The occurrence of these positive samples reveals one of the pitfalls of the eDNA methodology, as it simply points out the presence of eDNA from the targeted organism without verifying the actual presence of the organism within the examined body of water [20, 49, 50]. It does, however, also highlight the sensitivity of this method.

One of the four filter samples taken at Lierelva, the river with a known presence of *G. salaris*, displayed a significantly higher signal than the other three filters, even though the very same location was sampled. These results were observed in qPCR, and both the singleplex and multiplex ddPCR reactions. We presume that this is due to one or more whole specimens of *G. salaris* being picked up on this particular filter. The signal difference in qPCR is roughly ten cycles which would suggest a 1000-fold higher amount of eDNA in sample 9/4. This calculation is also reflected in the ddPCR results where an increase from 560 copies/l to 371,440 copies/l was observed. This assumption is substantiated by the fact that Gyrodactylids are reported to consist of roughly 1000 cells [1]. The possibility that one sometimes might catch a whole parasite specimen in the filter does not pose a problem for a simple proof of presence detection, but in fact increases the certainty of the results. However, while some studies have demonstrated a correlation between biomass and eDNA concentration [51], quantification of parasites and establishing an agent-level would, in this case, result in an overestimation of parasite numbers. The use of a pre-filter such as fitting a plankton net in front of the filter with a mesh size small enough to prevent an entire specimen to pass on to the filter may solve this problem of overestimation. In comparison to the results for *G. salaris*, the copy number for Atlantic salmon eDNA was fairly constant in all four samples at an average of 8948 copies (± SD = 945) as displayed in Fig. 2. This indicates a constant emission rate of eDNA into the water by Atlantic salmon which has also been observed in other studies [52].

Comparison of qPCR/ddPCR monitoring

Quantitative real-time PCR (qPCR) offers the possibility to measure the rate of generation of the amplified product after each cycle, thus making it possible to calculate the amount of copies in the original sample. Previous studies have demonstrated that quantification of biomass and calculation of population size through using qPCR is possible [22, 53]. ddPCR, which now allows the user to operate on a nanolitre rather than on a microlitre scale, enables even more precise detection and absolute quantification of target molecules while simultaneously removing the need for standard curves [51, 54]. Our results demonstrate this precision by detecting both rainbow trout and *G. salaris* at very low copy levels with 22 eDNA copies/l and 560 eDNA copies/l, respectively. Furthermore, this technology has been proven to perform better on inhibition prone samples than the predecessor qPCR [55]. This is a particular advantage when analysing environmental samples which often tend to include PCR inhibitors [56–58]. Our study also shows that ddPCR seems to surpass qPCR regarding specificity, as there was no cross-amplification of *G. derjavinoides* in the *G. salaris* assay although the same primer-probe combinations were used. We speculate that this is due to the lower copy numbers of both target and non-target DNA per reaction (droplets) in the ddPCR system. Ideally, one droplet contains only one copy of the target DNA and only a few non-target copies, thus reducing the possibility of unspecific amplification.

For a more precise monitoring of *G. salaris* and its hosts, further research and development is needed in

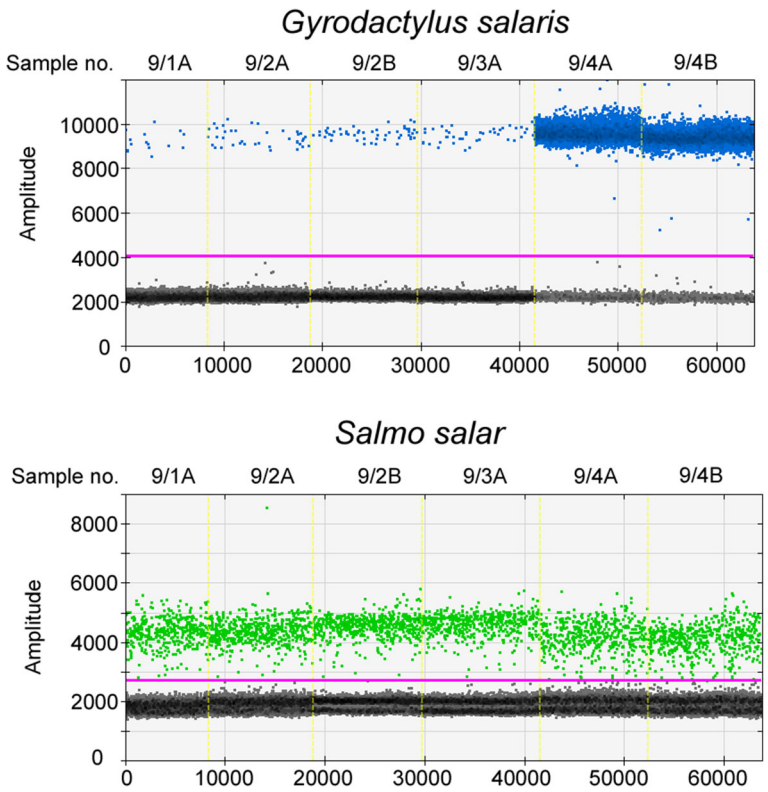

Fig. 2 Visual output from the duplex ddPCR for *G. salaris* in Channel 1 (blue) and Atlantic salmon in Channel 2 (green) on the samples taken at Lierelva. Wells containing samples 9/1B and 9/3B are not displayed and were excluded due to insufficient droplet generation. Each blue and green point represents a positive amplification of respective DNA template. The horizontal purple line represents the threshold and the black points represent negative droplets. The eDNA copy number for *G. salaris* is markedly higher in two of the wells containing samples 9/4A and 9/4B. However, the copy number of Atlantic salmon eDNA remains relatively stable in all four samples

order to improve the specificity of the *G. salaris* assay to distinguish from *G. thymalli*, as well as to determine when it is no longer possible to obtain a positive eDNA result (limit of detection) when the parasite load per fish drops.

Conclusions

We have successfully designed and implemented a method for eDNA detection of an aquatic host-parasite system, specifically *G. salaris* and its two hosts Atlantic salmon and rainbow trout. Thus, we demonstrate for the first time that eDNA monitoring can be used for the detection of *G. salaris* and its host Atlantic salmon in natural freshwater systems with a moderately infected salmon population. Furthermore, we have determined the assay we designed to be species-specific and demonstrated the usefulness of eDNA methodology when examining a river system for the presence of *G. salaris*. Within the paper we present a protocol, both field and laboratory, on how to conduct eDNA monitoring of *G. salaris* and Atlantic salmon successfully, using a duplex ddPCR. We show that ddPCR appears to be a better tool than qPCR when screening samples for *G.salaris*. Further studies are needed to determine the limit of detection regarding eDNA and to compare the eDNA signal against fish parasite load in experimental and natural settings.

Abbrevations

BLASTn: Basic Local Alignment Search Tool; Cq-Value: quantification cycle; CTAB: cetyl trimethyl ammonium bromide; ddPCR: droplet digital PCR; eDNA: environmental DNA; ITS1: internal transcribed spacer 1; MGB: minor groove binder; mtDNA: mitochondrial DNA; NADH: nicotinamide adenine dinucleotide dehydrogenase; qPCR: quantitative PCR

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

HH, JR, SH and TV planned the study. SH was in charge of the field work and performed the electrofishing and JR and HH participated in the field work. JR

carried out water filtering, qPCR analyses, ddPCR analyses and optimisation of ddPCR assays. DS took part in the design and optimisation of ddPCR assays. TM and HH designed the qPCR assay for *G. salaris* and TM optimized the qPCR assay. JR and HH wrote the first draft of the manuscript and all authors contributed in the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

No approval from Institutional Animal Care and Use Committee (IACUC) or ethics committee was necessary. No experiments that involved fish were performed. All fish were euthanised following the strict codes of practice in force in Europe.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details

[1]Norwegian Veterinary Institute, P.O. Box 750, Sentrum, NO-0106 Oslo, Norway. [2]Department of Biosciences, University of Oslo, P.O. Box 1066, Blindern, NO-0316 Oslo, Norway. [3]Faculty of Veterinary Medicine, Norwegian University of Life Sciences, P.O. Box 8146, Dep, NO-0033 Oslo, Norway.

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Paper II

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RESEARCH ARTICLE

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

David A. Strand[1,2] | Stein Ivar Johnsen[3] | Johannes C. Rusch[1,4] | Sune Agersnap[5,6] | William Brenner Larsen[5] | Steen Wilhelm Knudsen[5] | Peter Rask Møller[5] | Trude Vrålstad[1]

[1]Norwegian Veterinary Institute, Oslo, Norway; [2]Norwegian Institute for Water Research, Oslo, Norway; [3]Norwegian Institute for Nature Research, Lillehammer, Norway; [4]Department of Biosciences, University of Oslo, Oslo, Norway; [5]Natural History Museum of Denmark, University of Copenhagen, Copenhagen, Denmark and [6]Department of Bioscience, Aarhus University, Aarhus, Denmark

Correspondence
David A. Strand
Email: david.strand@vetinst.no

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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

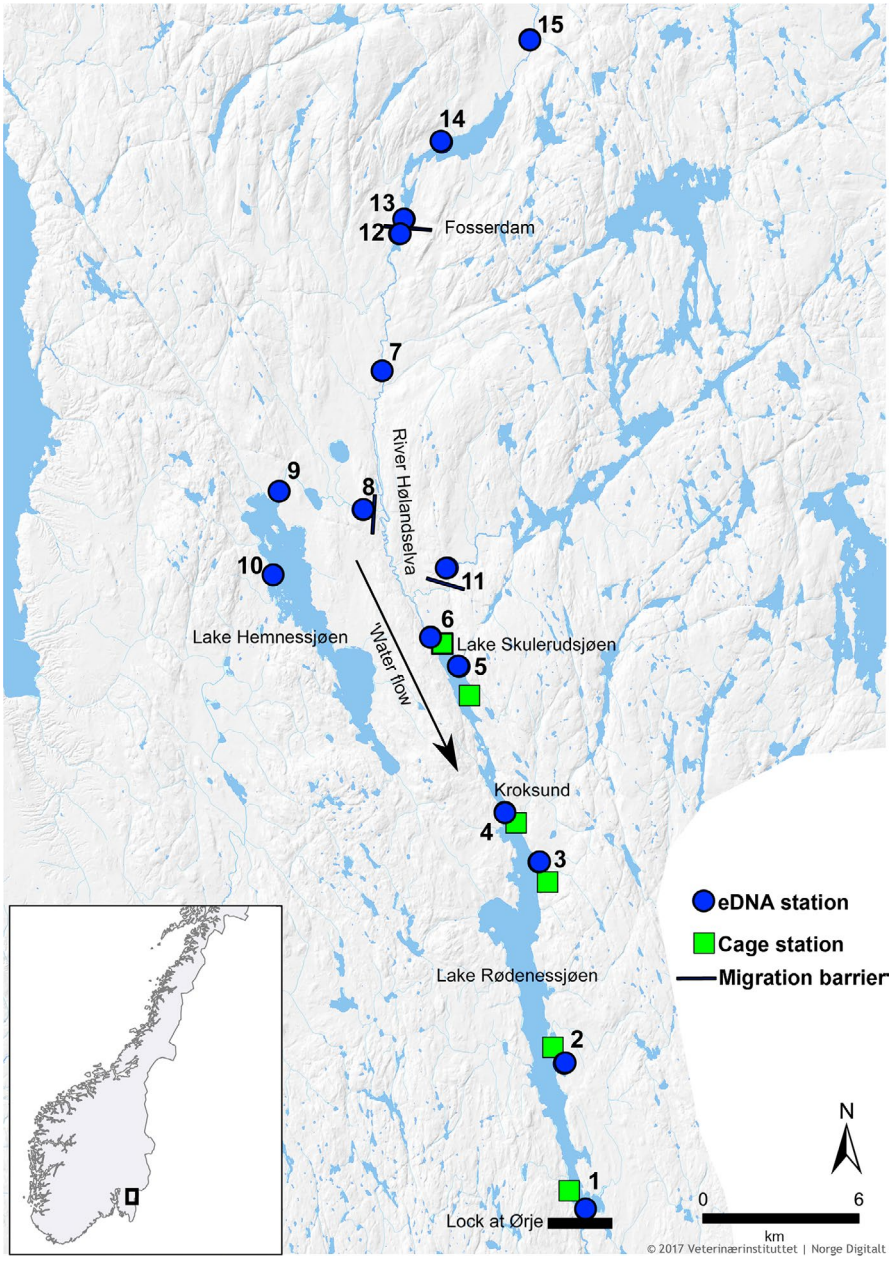

FIGURE 1 The study site includes parts of the large Halden watercourse in Norway with names for involved lakes, channels and rivers. Cage stations (green squares) and environmental DNA (eDNA) stations (blue circles) were established successively from 2014 to 2016 in a south–north direction, starting 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

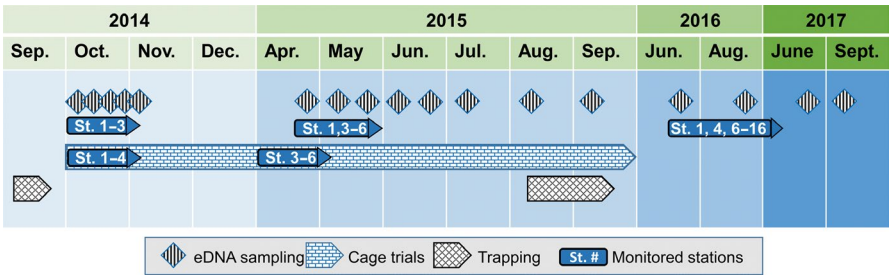

FIGURE 2 Timeline of the sampling methods and sampling frequency/effort. Involved stations (environmental DNA [eDNA] and cage) are indicated for different periods. eDNA was sampled at 10-day intervals in 2014 and at 2- to 4-week intervals in 2015. Cages with live noble crayfish were checked twice a week 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

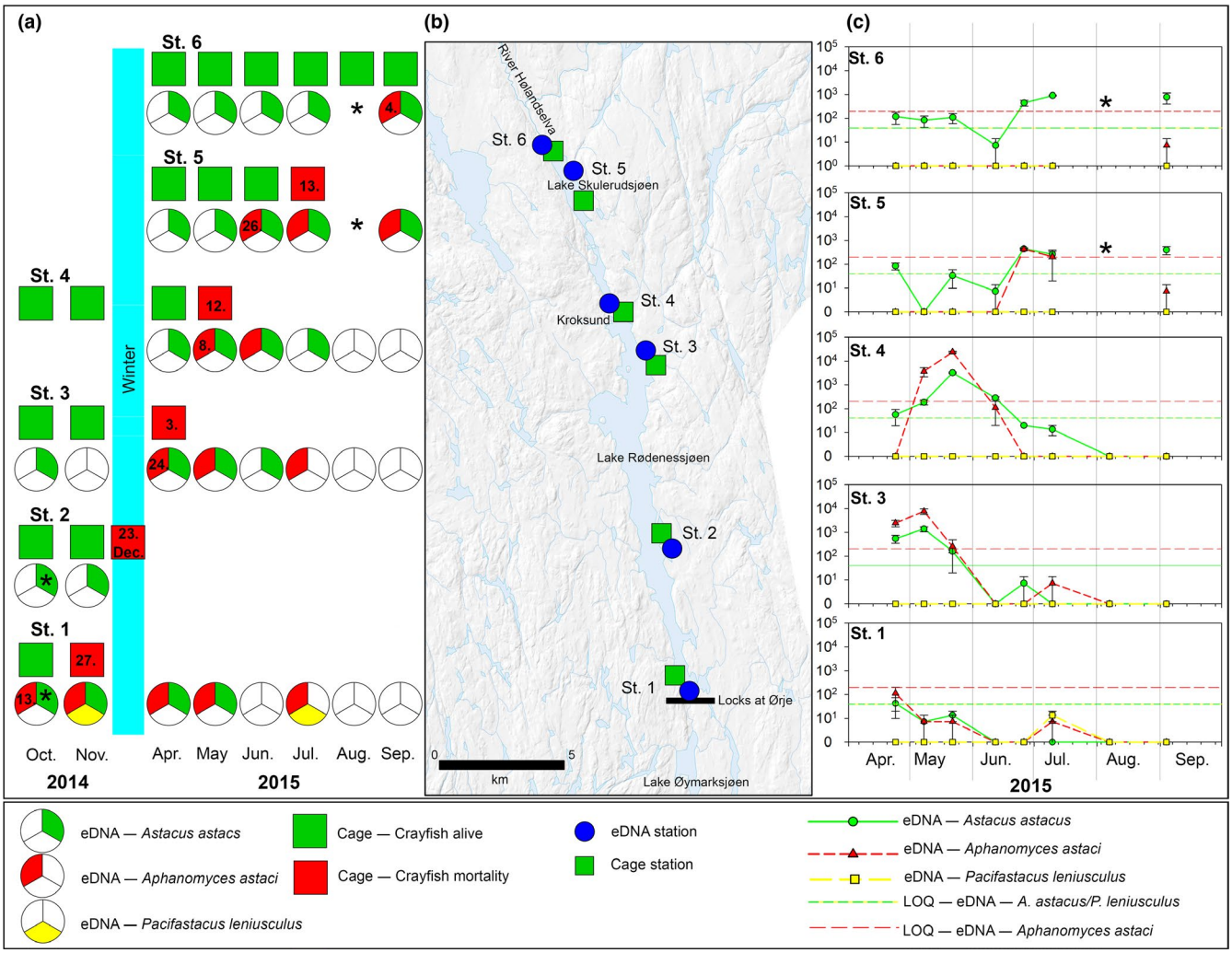

FIGURE 3 Comparison of environmental DNA (eDNA) presence/absence of *Astacus astacus*, *Aphanomyces astaci* and *Pacifastacus leniusculus* and mortality of caged *Astacus astacus* (a) at the cage and eDNA stations 1–6 in the Halden watercourse (b), with details for the eDNA concentration dynamics in 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

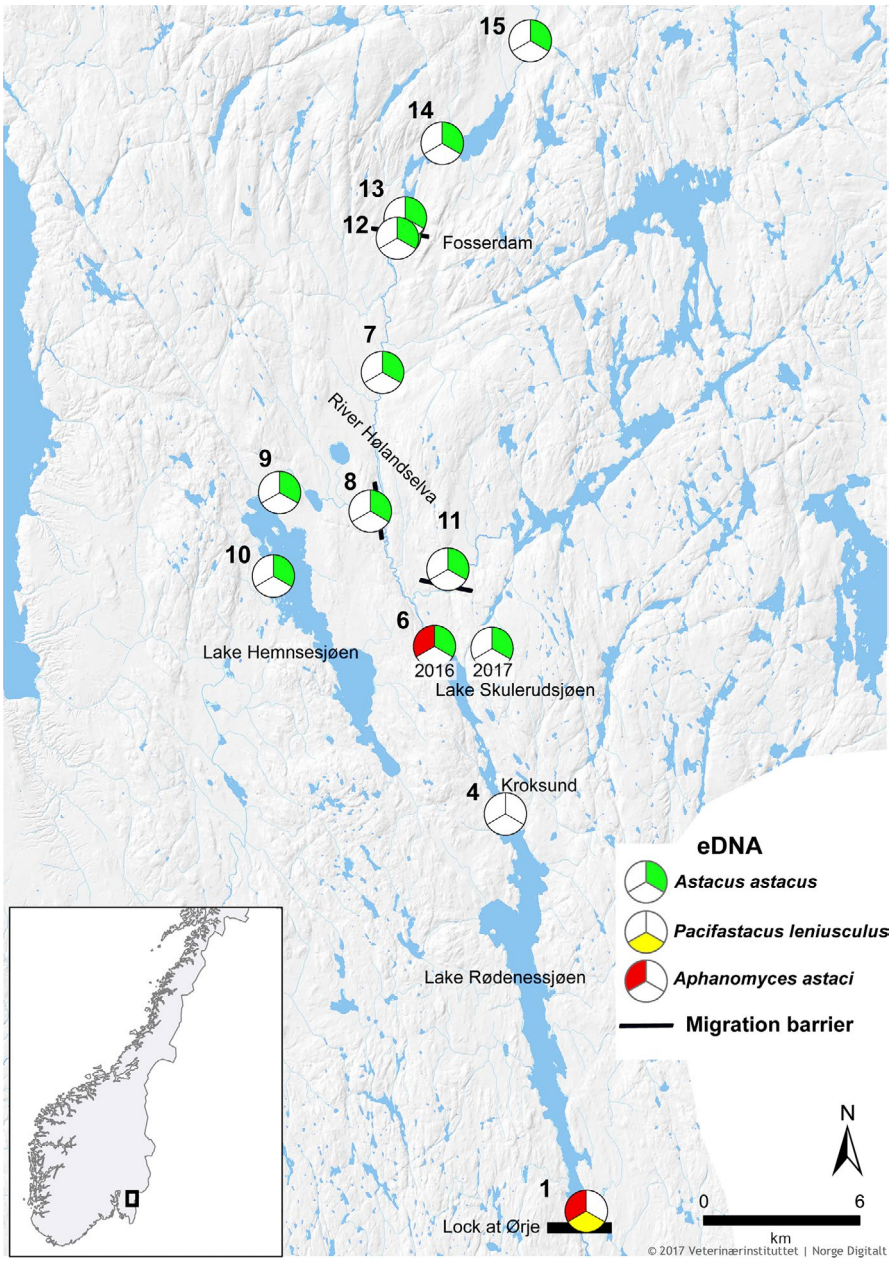

FIGURE 4 Triangular split circles indicate detection of eDNA from *Astacus astacus* (green), *Pacifastacus leniusculus* (yellow) and *Aphanomyces astaci* (red) per station in 2016 and 2017; these are not to be interpreted as pie charts. No detection is indicated with no colouring. Stations 1, 4, 6–7 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).

ORCID

David A. Strand https://orcid.org/0000-0001-8804-7994
Johannes C. Rusch https://orcid.org/0000-0003-3935-3009
Sune Agersnap https://orcid.org/0000-0001-9193-8069
Steen Wilhelm Knudsen https://orcid.org/0000-0003-0428-9940
Peter Rask Møller https://orcid.org/0000-0002-0177-0977
Trude Vrålstad https://orcid.org/0000-0002-5859-8039

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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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Table S1 – eDNA results for 2014 showing frequency of positive detection (number of positive sample/total samples) and average eDNA copies/L ± Standard deviation.

	Target species	03.10.2014		10.10.2014		20.10.2014		30.10.2014		11.11.2014	
		Freq.	eDNA	Freq.	eDNA	Freq.	eDNA	Freq.	eDNA	Freq.	eDNA
Station 1	Astast	3/6*	86±140	6/9	102±97	3/9	<LOQ	2/9	<LOQ	3/9	<LOQ
	Paclen	0/9	0	3/9	<LOQ	2/9	<LOQ	0/9	0	0/9	0
	Aphast	5/9	<LOQ	4/9	<LOQ	0/9	0	3/9	<LOQ	3/9	<LOQ
Station 2	Astast	-	-	2/3	<LOQ	NA*	NA*	3/3	<LOQ	2/3	<LOQ
	Paclen	-	-	0/3	0	NA*	NA*	0/3	0	0/3	0
	Aphast	-	-	0/3	0	NA*	NA*	0/3	0	0/3	0
Station 3	Astast	0/3	0	0/3	0	0/3	0	2/3	363±611	0/3	0
	Paclen	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0
	Aphast	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0
Station 4	Astast	-	-	-	-	-	-	-	-	-	-
	Paclen	-	-	-	-	-	-	-	-	-	-
	Aphast	-	-	-	-	-	-	-	-	-	-
Station 5	Astast	-	-	-	-	-	-	-	-	-	-
	Paclen	-	-	-	-	-	-	-	-	-	-
	Aphast	-	-	-	-	-	-	-	-	-	-
Station 6	Astast	-	-	-	-	-	-	-	-	-	-
	Paclen	-	-	-	-	-	-	-	-	-	-
	Aphast	-	-	-	-	-	-	-	-	-	-

- Samples not colleceted

* Samples excluded due to amplification in environmental or extraction control

Supplementary table 3 - eDNA detection frequency (number of positive sample/total samples) in 2016 and 2017 for the extended sampling upstream River Hølandselva.

		June 2016	Aug. 2016	June 2017	Sept. 2017
		Freq.	Freq.	Freq.	Freq.
Station 1	Astast	0/4	0/4	0/4	0/4
	Paclen	0/4	1/4	3/4	1/4
	Aphast	1/4	3/4	2/4	0/4
Station 4	Astast	0/2	0/2	0/2	0/2
	Paclen	0/2	0/2	0/2	0/2
	Aphast	0/2	0/2	0/2	0/2
Station 6	Astast	2/2	2/2	1/2	3/3
	Paclen	0/2	0/2	0/2	0/3
	Aphast	1/2	2/2	0/2	0/3
Station 7	Astast	2/2	1/2	3/3	3/3
	Paclen	0/2	0/2	0/3	0/3
	Aphast	0/2	0/2	0/3	0/3
Station 8	Astast	2/2	2/2	2/2	2/3
	Paclen	0/2	0/2	0/2	0/3
	Aphast	0/2	0/2	0/2	0/3
Station 9	Astast	2/2	2/2	2/2	2/2
	Paclen	0/2	0/2	0/2	0/2
	Aphast	0/2	0/2	0/2	0/2
Station 10	Astast	1/2	3/3	1/2	2/2
	Paclen	0/2	0/3	0/2	0/2
	Aphast	0/2	0/3	0/2	0/2
Station 11	Astast	2/2	2/2	2/2	2/2
	Paclen	0/2	0/2	0/2	0/2
	Aphast	0/2	0/2	0/2	0/2
Station 12	Astast	0/4	2/2	1/2	2/2
	Paclen	0/4	0/2	0/2	0/2
	Aphast	0/4	0/2	0/2	0/2
Station 13	Astast	2/2	2/2	2/2	0/2
	Paclen	0/2	0/2	0/2	0/2
	Aphast	0/2	0/2	0/2	0/2
Station 14	Astast	0/2	1/2	1/2	1/2
	Paclen	0/2	0/2	0/2	0/2
	Aphast	0/2	0/2	0/2	0/2
Station 15	Astast	0/2	1/2	3/3	1/2
	Paclen	0/2	0/2	0/3	0/2
	Aphast	0/2	0/2	0/3	0/2

Table S2 - eDNA results for 2015 showing frequency of positive detection (number of positive sample/total samples) and average eDNA copies/L ± Standard deviation.

	Target species	24.04.2015		08.05.2015		22.05.2015		12.06.2015		26.06.2015		10.07.2015		07.08.2015		04.09.2015	
		Freq.	eDNA	Freq.	eDNA	Freq.	eDNA	Freq.	eDNA	Freq.	eDNA	Freq.	eDNA	Freq.	eDNA	Freq.	eDNA
Station 1	Astast	2/3	<LOQ	1/3	<LOQ	2/3	<LOQ	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0
	Paclen	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	2/3	<LOQ	0/3	0	0/3	0
	Aphast	3/3	<LOQ	1/3	<LOQ	1/3	<LOQ	0/3	0	0/3	0	1/3	<LOQ	0/3	0	0/3	0
Station 2	Astast	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Paclen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Aphast	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Station 3	Astast	3/3	841±296	3/3	1391±575	3/3	162±35	0/3	0	1/3	<LOQ	0/3	0	0/3	0	0/3	0
	Paclen	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0
	Aphast	3/3	2482±1318	3/3	7630±3459	3/3	255±407	0/3	0	0/3	0	1/3	<LOQ	0/3	0	0/3	0
Station 4	Astast	2/3	<LOQ	3/3	187±73	3/3	3236±444	3/3	283±58	3/3	<LOQ	2/3	<LOQ	0/3	0	0/3	0
	Paclen	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0
	Aphast	0/3	0	3/3	3726±2649	3/3	24000±2749	3/3	<LOQ	0/3	0	0/3	0	0/3	0	0/3	0
Station 5	Astast	3/3	<LOQ	0/3	0	2/3	<LOQ	1/3	<LOQ	3/3	444±82	3/3	261±157	NA*	NA*	3/3	400±267
	Paclen	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	NA*	NA*	0/3	0
	Aphast	0/3	0	0/3	0	0/3	0	0/3	0	3/3	431±57	3/3	207±324	NA*	NA*	1/3	<LOQ
Station 6	Astast	3/3	118±109	2/3	82±71	3/3	108±87	1/3	<LOQ	3/3	444±205	3/3	902±80	NA*	NA*	3/3	776±669
	Paclen	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	NA*	NA*	0/3	0
	Aphast	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	NA*	NA*	1/3	<LOQ

- Samples not colleceted

* Samples excluded due to amplification in environmental or extraction control

Table S3 - eDNA detection frequency (number of positive sample/total samples) in 2016 and 2017 for the extended sampling upstream River Hølandselva.

		June 2016	Aug. 2016	June 2017	Sept. 2017
		Freq.	Freq.	Freq.	Freq.
Station 1	Astast	0/4	0/4	0/4	0/4
	Paclen	0/4	1/4	3/4	1/4
	Aphast	1/4	3/4	2/4	0/4
Station 4	Astast	0/2	0/2	0/2	0/2
	Paclen	0/2	0/2	0/2	0/2
	Aphast	0/2	0/2	0/2	0/2
Station 6	Astast	2/2	2/2	1/2	3/3
	Paclen	0/2	0/2	0/2	0/3
	Aphast	1/2	2/2	0/2	0/3
Station 7	Astast	2/2	1/2	3/3	3/3
	Paclen	0/2	0/2	0/3	0/3
	Aphast	0/2	0/2	0/3	0/3
Station 8	Astast	2/2	2/2	2/2	2/3
	Paclen	0/2	0/2	0/2	0/3
	Aphast	0/2	0/2	0/2	0/3
Station 9	Astast	2/2	2/2	2/2	2/2
	Paclen	0/2	0/2	0/2	0/2
	Aphast	0/2	0/2	0/2	0/2
Station 10	Astast	1/2	3/3	1/2	2/2
	Paclen	0/2	0/3	0/2	0/2
	Aphast	0/2	0/3	0/2	0/2
Station 11	Astast	2/2	2/2	2/2	2/2
	Paclen	0/2	0/2	0/2	0/2
	Aphast	0/2	0/2	0/2	0/2
Station 12	Astast	0/4	2/2	1/2	2/2
	Paclen	0/4	0/2	0/2	0/2
	Aphast	0/4	0/2	0/2	0/2
Station 13	Astast	2/2	2/2	2/2	0/2
	Paclen	0/2	0/2	0/2	0/2
	Aphast	0/2	0/2	0/2	0/2
Station 14	Astast	0/2	1/2	1/2	1/2
	Paclen	0/2	0/2	0/2	0/2
	Aphast	0/2	0/2	0/2	0/2
Station 15	Astast	0/2	1/2	3/3	1/2
	Paclen	0/2	0/2	0/3	0/2
	Aphast	0/2	0/2	0/3	0/2

Appendix S1. Full protocol for DNA extraction from fibreglass filters

1. Transfer filter with spores / filtrate to sterile 15 ml falcon tube

2. Freeze dry the filter to remove excess water

3. Add 4 ml CTAB buffer (20 g l^{-1} CTAB, 1.4 M NaCl, 0.1 M Tris-HCl, 20 mM Na_2EDTA) and homogenize the filter inside the tube with a sterile pestle.

4. Freeze samples at -80 °C for at least 30 minutes (to rupture cells). Subsequently thaw samples in 65 °C water-bath for 15 minutes.

5. Add 40 µl proteinase K solution (20 mg / ml), vortex and incubate at 65 ° C for 60 minutes. (Isolation can be paused by freezing samples at -80 ° C and continued after thawing the sample at 65 ° C for 15 minutes).

6. Add 4 ml chloroform and mix gently with the pipette tip.

7. Centrifuge samples for 15 minutes at max speed (> 3800x g at room temperature).

8. Transfer 1500 µl of the upper phase (water phase DNA) to two new tubes respectively (2ml tubes, one A and one B sample)

9. Add 500µl Chloroform. Vortex samples. Centrifuge samples for 5 minutes 12000 x g at room temperature. Transfer 1200 µl of the upper phase (water phase DNA) to new tube.

10. Add 800 cold isopropanol (stored at -20 ° C). Turn the tubes upside down several times to mix and precipitate DNA.

11. Incubate samples for 15 minutes at 4 ° C.

12. Centrifuge samples for 15 minutes at maximum speed (> 16,000 xg).

13. Remove supernatant.

14. Add 500µl ice cold 70% ethanol to purify the DNA pellet. Vortex briefly.

15. Centrifuge samples for 5 minutes at maximum speed (> 16,000 xg) and gently pipette the supernatant without losing the pellet.

16. Dry the pellet (open cap) in a vacuum centrifuge (about 10 min) or heat block (65 ° C) in sterile bench (to avoid potential contamination from the air). It is important that the pellet is dry.

17. Dissolve DNA pellet in 100 µl TE buffer (or sterile milliQ water), vortex and spin down. Let the DNA dissolve for at least 1 hour before further analysis (or store in fridge/freeze)

Paper III

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Simultaneous detection of native and invasive crayfish and *Aphanomyces astaci* from environmental DNA samples in a wide range of habitats in Central Europe

Johannes C. Rusch[1,2], Michaela Mojžišová[3], David A. Strand[1], Jitka Svobodová[4], Trude Vrålstad[1], Adam Petrusek[3]

1 *Norwegian Veterinary Institute, P.O. Box 750, Sentrum, NO-0106 Oslo, Norway* **2** *Department of Biosciences, University of Oslo, P.O. Box 1066, Blindern, NO-0316 Oslo, Norway* **3** *Department of Ecology, Faculty of Science, Charles University, Viničná 7, Prague 2, CZ-12844, Czech Republic* **4** *T. G. Masaryk Water Research Institute, Podbabská 30, Prague 6, CZ-16000, Czech Republic*

Corresponding author: Adam Petrusek (petrusek@natur.cuni.cz)

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Abstract

Crayfish of North American origin are amongst the most prominent high-impact invasive invertebrates in European freshwaters. They contribute to the decline of European native crayfish species by spreading the pathogen causing crayfish plague, the oomycete *Aphanomyces astaci*. In this study we validated the specificity of four quantitative PCR (qPCR) assays, either published or newly developed, usable for environmental DNA (eDNA) screening for widely distributed native and non-native crayfish present in Central Europe: *Astacus astacus*, *Pacifastacus leniusculus*, *Faxonius limosus* and *Procambarus virginalis*. We then conducted an eDNA monitoring survey of these crayfish as well as the crayfish plague pathogen in a wide variety of habitat types representative for Central and Western Europe. The specificity of qPCR assays was validated against an extensive collection of crayfish DNA isolates, containing most crayfish species documented from European waters. The three assays developed in this study were sufficiently species-specific, but the published assay for *F. limosus* displayed a weak cross-reaction with multiple other crayfish species of the family Cambaridae. In the field study, we infrequently detected eDNA of *A. astaci* together with the three non-native crayfish species under examination. We never detected eDNA from *A. astaci* together with native crayfish, but in a few locations eDNA from both native and non-native crayfish was captured, due either to passive transport of eDNA from upstream populations or co-existence

in the absence of infected crayfish carriers of *A. astaci*. In the study, we evaluated a robust, easy-to-use and low-cost version of the eDNA sampling equipment, based mostly on items readily available in garden stores and hobby markets, for filtering relatively large (~5 l) water samples. It performed just as well as the far more expensive equipment industrially designed for eDNA water sampling, thus opening the possibility of collecting suitable eDNA samples to a wide range of stakeholders. Overall, our study confirms that eDNA-based screening for crayfish and their associated pathogen is a feasible alternative to traditional monitoring.

Keywords
crayfish plague, eDNA monitoring, eDNA sampling methods, quantitative PCR, TaqMan assay validation

Introduction

Environmental DNA (hereafter eDNA) is commonly defined as genetic material obtained directly from environmental samples (soil, sediment, water) without any obvious signs of the biological source material (Thomsen and Willerslev 2015). In water samples, eDNA typically originates from single-celled uncultured microorganisms or, in the case of multicellular taxa, from shed cells, faeces, mucus, body fluids, gametes, spores or other propagules (Strand et al. 2014; Deiner et al. 2016; Mächler et al. 2016) or even from recently dead and decomposing organisms (Strand et al. 2019).

During the past decade, different concepts of eDNA analyses have become established for various purposes such as monitoring endangered and elusive targets, invasive species, as well as parasites and pathogens (Kirshtein et al. 2007; Thomsen et al. 2012a; Takahara et al. 2013; Rusch et al. 2018; Strand et al. 2019). There are two essentially different approaches to eDNA monitoring: either broad spectrum metabarcoding for bio-assessments of whole communities (Thomsen et al. 2012a; Valentini et al. 2016; Ruppert et al. 2019) or more targeted approaches for the detection and quantification of one or several species of interest (Jerde et al. 2011; Thomsen et al. 2012b), usually using species-specific quantitative real-time PCR (qPCR) or droplet digital PCR (ddPCR). Since eDNA has a relatively short half-life in the water column of aquatic systems (Dejean et al. 2011), positive detection suggests that the targeted organism is either present or has been present within the system very recently.

One of the pathogens for which monitoring methods based on eDNA have been developed is the oomycete *Aphanomyces astaci* Schikora, the causative agent of crayfish plague (Strand et al. 2011, 2012, 2014; Robinson et al. 2018; Wittwer et al. 2018). Since its initial introduction into Europe in the late 1850s (Alderman 1996), and reinforced by subsequent introductions of several Non-Indigenous Crayfish Species (NICS) of North American origin (Holdich et al. 2009), crayfish plague has ravaged the continent and led to mass mortalities of native crayfish (Alderman 1996; Holdich et al. 2009). *Aphanomyces astaci* is usually carried as a benign infection by its natural crayfish hosts from North America, where both originate. However, crayfish indig-

enous to Europe usually lack efficient defence mechanisms to resist this pathogen and thus whole populations tend to be eliminated as a result of crayfish plague outbreaks (Söderhäll and Cerenius 1999; Holdich et al. 2009; Vrålstad et al. 2014). This explains why *A. astaci* is a disease listed by the World Organisation for Animal Health (OIE 2019) and featured on the list of the "world's 100 worst invasive species" (Lowe et al. 2004).

American crayfish species, such as the spiny cheek crayfish *Faxonius limosus* (Rafinesque, 1817), the signal crayfish *Pacifastacus leniusculus* (Dana, 1852) and the red swamp crayfish *Procambarus clarkii* (Girard, 1852), were originally introduced into Europe for stocking or aquaculture purposes (Holdich et al. 2009). Others, such as the marbled crayfish *Procambarus virginalis* Lyko, 2017, reached European waters through the pet trade (Chucholl 2013; Kouba et al. 2014). All species listed above pose a threat to native European crayfish species and are therefore subject to the EU Regulation on the prevention and management of the introduction and spread of invasive alien species (Regulation (EU) No 1143/2014).

The marbled crayfish, *P. virginalis*, is causing great concern outside of Europe, too. This triploid species seems to have emerged as a thelytokous parthenogenetic form of *Procambarus fallax* (Hagen, 1870), possibly from the pet trade (Gutekunst et al. 2018; Martin et al. 2010). Thus, it produces female-only offspring and a single individual is required to establish a new population. It has been shown to thrive in a very broad range of habitats, recently demonstrated in Madagascar (Andriantsoa et al. 2019).

When non-indigenous crayfish are present, the only conceivable option to eradicate crayfish plague is by treating the entire waterbody with pesticides such as Betamax-VET (Sandodden and Johnsen 2010). This procedure kills the crayfish hosts and subsequently also the crayfish plague pathogen which depends on its host for long-term survival (Söderhäll and Cerenius 1999). However, this is only applicable to smaller aquatic habitats (Peay et al. 2019) and, even there, it is an extremely costly and devastating undertaking, often not compliant with local legislation. Therefore, mitigation strategies must be employed to preserve and protect Indigenous Crayfish Species (ICS) and their natural environment. These mitigation strategies can include the prohibition of fishing in certain areas or the enforcement of decontamination protocols for fishing gear. They could also encompass the creation and management of the so-called "ark sites", where introduction of neither the alien crayfish nor the disease is likely (Peay 2009a). When creating such ark sites or planning restocking and rescue transfers, precise knowledge about the distribution of crayfish plague vectors and presence or absence of the crayfish plague agent in the vicinity is required. For this purpose, the eDNA methodology is a particularly suitable tool (Cowart et al. 2018; Strand et al. 2019).

Recent research has focused on developing eDNA monitoring for early alert of NICS and *A. astaci*, as well as for efficient biomonitoring of ICS. The main goals are safeguarding indigenous crayfish while limiting the spread of both NICS and crayfish plague pathogen (Strand et al. 2014, 2019; Agersnap et al. 2017; Cai et al. 2017; Vrålstad et al. 2017; Harper et al. 2018; Wittwer et al. 2019).

In this study we demonstrate the applicability of eDNA-based screening for crayfish and the crayfish plague pathogen in a wide range of aquatic habitats in Czechia, a Central European country with a long tradition of crayfish conservation and research. Three European crayfish species, the noble crayfish *Astacus astacus* (Linnaeus, 1758), the stone crayfish *Austropotamobius torrentium* (Schrank, 1803) and the narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823) are found in local waters. The two former species are native to the country, the latter being introduced from Eastern Europe to multiple localities in the late 19th century (Štambergová et al. 2009). Crayfish plague has caused large-scale mortalities of native crayfish in the area since the 1890s (Kozubíková et al. 2006). Although not considered a conservation problem throughout most of the 20th century, crayfish plague outbreaks, caused by *A. astaci* genotypes associated with different North American host taxa (Grandjean et al. 2014), are at present rampant in the country (Kozubíková et al. 2008; Kozubíková-Balcarová et al. 2014; Mojžišová et al. 2020).

Czech waters host three documented North American crayfish species. *Faxonius limosus* that invaded the Elbe river as far back as the 1960s (Petrusek et al. 2006) and *P. leniusculus*, introduced for fishery purposes in 1980 (Filipová et al. 2006), are both widespread in at least some regions of the country (Kouba et al. 2014; Mojžišová et al. 2020). *Procambarus virginalis* has recently been documented from two sites, most likely resulting from aquarium releases (Patoka et al. 2016), but there is a high probability that other established populations of *P. virginalis* are yet waiting to be discovered. All these species are confirmed carriers of *A. astaci* (Svoboda et al. 2017). Infections of Czech populations have been documented for *P. leniusculus* and *F. limosus* (Kozubíková et al. 2009), but not for *P. virginalis* (Patoka et al. 2016).

Native and non-native crayfish populations can be found in a wide range of diverse habitats in Czechia: large and smaller rivers and streams as well as artificial still waters including fishponds, flooded quarries and reservoir lakes. There is a wealth of documented data on existing crayfish populations in lentic and lotic waterbodies in the country (Štambergová et al. 2009; Svobodová et al. 2012), together with data on the infection status by *A. astaci* in NICS populations (Kozubíková et al. 2009, 2011). Thus, Czechia is a suitable region to conduct a study focusing on eDNA-based detection of multiple NICS and their pathogen across a broad range of habitats.

The goal of the study presented here is two-fold: firstly, to validate the specificity of presumably species-specific qPCR assays for selected native and non-native crayfish present in Central Europe (Fig. 1). Three assays newly developed for this study and one previously published assay were tested against a broad panel of DNA isolates from various crayfish species present in Europe or available via the ornamental pet trade. Secondly, the presence of the crayfish plague agent *A. astaci* as well as its various crayfish hosts by means of eDNA analysis of water samples was evaluated. These were collected from various Czech localities and some from urban waters from Berlin (Germany) and Budapest (Hungary), which are representative for crayfish habitats in Central and Eastern Europe.

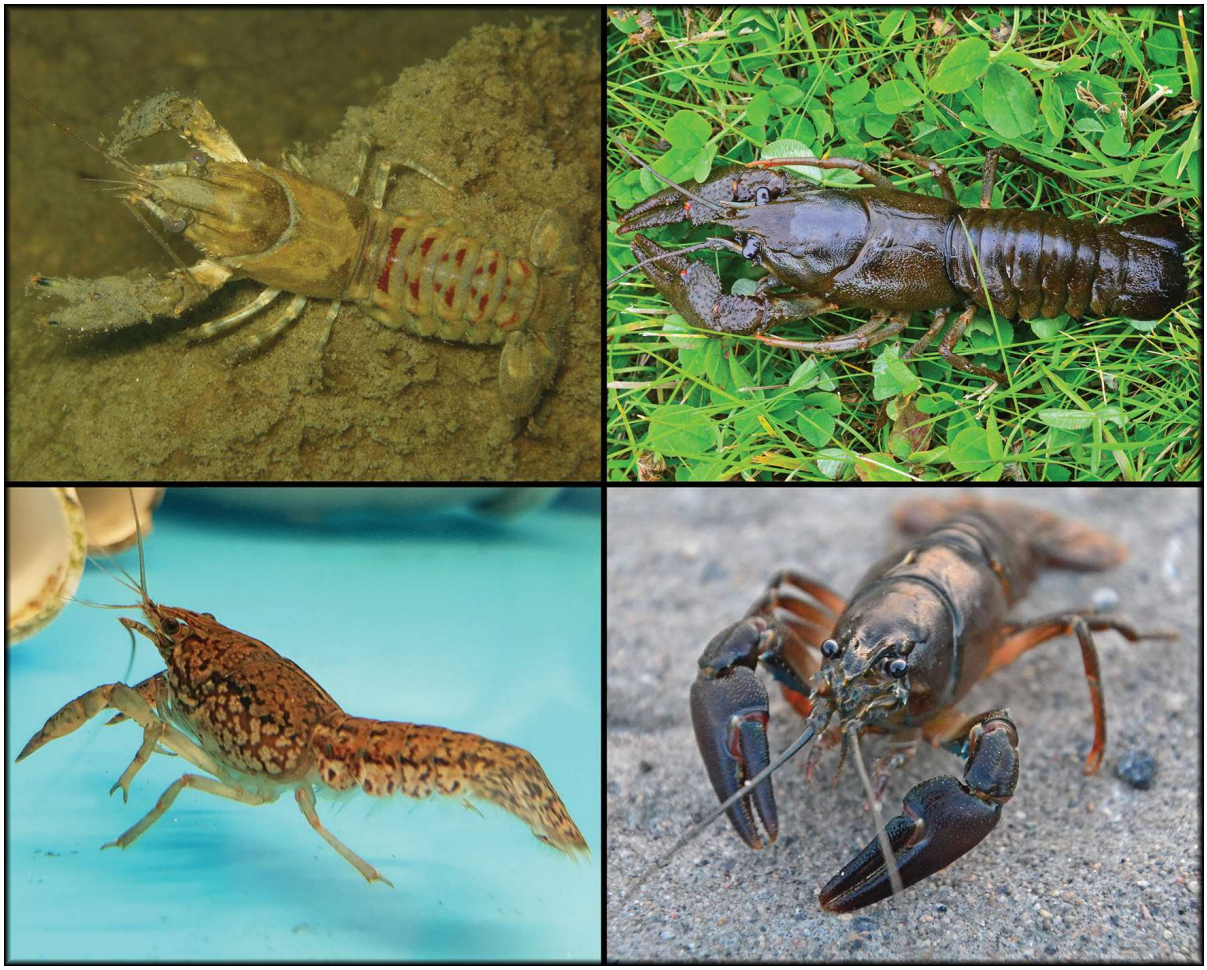

Figure 1. Crayfish species searched for by means of eDNA. Species clockwise from top left: *Faxonius limosus*, *Astacus astacus*, *Pacifastacus leniusculus*, *Procambarus virginalis*. Photos taken by A. Petrusek (Fl) and J. Rusch (Aa, Pl, Pv).

Methods

Study sites and populations

A full range of all relevant habitats for Central and Western Europe was covered, including large rivers and small streams, a thermal stream, natural lakes and man-made reservoirs, flooded quarries and fishponds (in total 32 localities; Suppl. material 1: Table S1). The majority of the samples (28) were taken in August 2017 at various waterbodies within Czechia, for which previous presence of crayfish was reliably known or presumed. The sampling sites were chosen to ensure that each one could be considered negative for at least some of the four target crayfish species, i.e. *F. limosus*, *P. virginalis*, *P. leniusculus* and *A. astacus*. None of the sites was within the known distribution area for stone crayfish in the country (Vlach et al. 2009; Petrusek et al. 2017a). Two samples were collected in December 2018 at two lakes in Berlin with a recently reported or assumed presence of both *P. virginalis* and *F. limosus* (Linzmaier et al. 2018; A. Mrugała, pers. comm.). Two additional water samples were obtained in January 2019 from a

stream in Budapest and its thermal tributary with a confirmed co-existence of the same two (and also additional) NICS (Szendőfi et al. 2018; A. Kouba, pers. comm.). Control eDNA samples were collected from an aquarium housing numerous marbled crayfish individuals, held at the Czech University of Life Sciences, Prague.

For comparison with eDNA results, crayfish were actively searched for at most sampling locations by manual examination of suitable shelters to confirm their *in-situ* presence. At the Czech sites containing NICS, we also attempted to obtain individuals to test for infection with *A. astaci*. After collection of samples for eDNA analysis, these crayfish were either captured directly at the sampling site on the same date or obtained from a nearby site within the same watercourse. Occasionally, we benefited from availability of such samples from previous recent fieldwork, assuming that the infection status of the NICS population does not change dramatically in a short time (Matasová et al. 2011). Crayfish plague diagnostics were carried out according to the method described in Vrålstad et al. (2009) with minor modifications (Mrugała et al. 2015). In brief: the soft abdominal cuticle and part of the tail fan of each crayfish were dissected and ground in liquid nitrogen. Total genomic DNA was then extracted using the DNeasy Blood & Tissue Kit (Qiagen, Düsseldorf, Germany) and the DNA extracts were then screened using the qPCR protocol for detection of *A. astaci* described below.

eDNA sample collection and extraction

Water samples at Czech locations 1 to 28 were obtained according to Strand et al. (2019) by filtering up to 5 l of water through glass fibre filters (47 mm AP25 Millipore, 2 µm pore size; Millipore, Billerica, USA), utilising a portable peristaltic pump (Masterflex E/S portable sampler; Masterflex, Cole-Parmer, Vermon Hills, USA), tygon tubing (Masterflex) and an in-line filter holder (Millipore). The front end of the tube was fastened to the inside of a plastic box which was weighted with lead on the bottom. This box was lowered into the water between 2 m and 5 m from the water's edge or to the centre of smaller streams. Before the filter was placed into the holder, water was pumped through the tubes for several minutes to remove any sediments that could have been disturbed from the waterbed and thus prevent clogging of the filter (Strand et al. 2019). For sampling sites where less than 5 l of water was filtered due to filter clogging, the final volume is noted in Table 2. At each location, two filter samples were taken.

For the samples obtained at locations 29 to 32 (Berlin and Budapest) the same filters (47 mm AP25 Millipore, 2 µm pore size) were used. However, the filters were placed into filter cups (Nalgene Analytical Test Filter Funnel, 145-0045; Thermo Fisher Scientific, Waltham, USA) after removal of the original filter provided by the manufacturer. Pumping was carried out by attaching the provided filter-cup adapter to a ¾ inch garden water hose and a drill-operated pump (product code 1490-20; Gardena, Ulm, Germany) (Fig. 2). As opposed to the protocol described above, the filters and filter cups were submerged into the water since they were situated at the front end of the pumping system (Fig. 2). The samples from the aquarium with *P. virginalis* and from the Barát stream in Budapest (sites 31, 32) were obtained after transporting water

Figure 2. Drill-powered sampling equipment. The low-cost sampling equipment used in this study consisting of a drill-powered pump, single use forceps, filter cups and glass fibre filters. The pump depicted in the bottom right corner is one of many alternative models to the one used in this study.

from the location in disinfected 5 l containers. This water was stored in the dark at low temperatures but not frozen and was filtered upon arrival in the laboratory using the drill-operated pumping system described above.

Filters from locations 1 to 28 were submerged in 4 ml of cetyl trimethyl ammonium bromide (CTAB) buffer in individual 15 ml Falcon tubes immediately after filtration and subsequently stored on ice until their arrival at the laboratory where they were stored at –20 °C prior to further analysis. Filters from locations 29 to 32 were placed into separate zip-lock bags containing ca. 70 g of silica gel following Carim et al. (2016), which ensured efficient desiccation, and stored in an opaque container until further analysis in the laboratory.

To prevent contamination of filters and accidental spreading of crayfish plague, a strict disinfection protocol was followed at each location. After filtering, all the equipment was submerged in, and filled with, a 10% chlorine bleach solution for a minimum of 15 minutes to break down any vital pathogen spores and residual eDNA. Then the tubes and filter holders were rinsed with a 5% sodium thiosulphate ($Na_2S_2O_3$) solution to neutralise the chlorine solution. Prior to water sample filtration, the equipment was thoroughly rinsed with ambient water from the sampling site. While using the drill-operated pumping system, separate tubing and filter holders were used at each respective sampling site, thus eliminating the concern for carryover contamination.

DNA isolation from the filters was performed according to the CTAB method described in Strand et al. (2019). In brief: the samples were lysed on CTAB buffer and proteinase K at 65 °C for one hour, cleaned and separated with chloroform and then precipitated in isopropanol. The pellets were then re-suspended in TE-buffer.

Due to the large volume of eluate from each filter, the samples were split up into two subsamples (technical replicates) to bypass the volume restrictions caused by centrifuge size. These subsamples were subsequently processed separately. Each extraction process incorporated an environmental blank control and an extraction blank control as a precautionary measure to detect any potential contamination during the extraction (Strand et al. 2019).

Molecular detection of target species with qPCR

Molecular eDNA detection of all five target-species (the crayfish plague pathogen *A. astaci* and the crayfish *A. astacus*, *P. leniusculus*, *F. limosus* and *P. virginalis*) was based on TaqMan MGB qPCR assays, either published in the case of *A. astaci* (Vrålstad et al. 2009) and *F. limosus* (Mauvisseau et al. 2018) or developed in this study (*A. astacus*, *P. leniusculus* and *P. virginalis*).

Due to the absence of any published assay for *P. virginalis* while this study was being carried out, we designed a qPCR assay with species-specific primers and a minor grove binder (MGB) probe targeting the mitochondrial gene for the cytochrome c oxidase subunit I (COI) of this asexually reproducing, genetically uniform species (cf. GenBank reference sequence: JF438007). We have since learnt of the existence of a newly-published assay (Mauvisseau et al. 2019) which targets a very similar fragment of the COI gene and thus differs only marginally from the one developed by us.

High specificity of the primers–probe combination was first ensured by checking the variation of the potential primer and probe sites against COI sequences of all crayfish known to occur in European waters, both native and invasive, and various related crayfish species of the family Cambaridae, particularly those available from the pet trade (taxa listed in Suppl. material 2: Table S2). This was accomplished using Geneious version 11.0.1 (Biomatters Limited, Auckland, New Zealand) and MEGA 7.0.26 (Kumar et al. 2016) through visual comparison. The efficacy of the primers and probe was evaluated using the Primer Express software (Version 3.0.1, Applied Biosystems, Foster City, USA).

New assays, differing from those published in Agersnap et al. (2017), Dunn et al. (2017), Larson et al. (2017), Harper et al. (2018), Mauvisseau et al. (2018) and Robinson et al. (2018), were designed for *A. astacus* and *P. leniusculus*. These two assays were developed with particular regard to functionality on both the qPCR and the droplet digital PCR (ddPCR) platform (D.A. Strand, unpublished). However, in this study we have only tested the efficiency and efficacy of the assays on the qPCR platform. Sequences from individual crayfish from several European regions (including North American individuals for *P. leniusculus*; Petrusek et al. 2017b) obtained from GenBank were used to design the assays for *A. astacus* and *P. leniusculus*.

For *in-vitro* validation, to determine the specificity of the assays, we re-used a total of 29 DNA isolates from tissues of crayfish species from previous studies on diversity of both indigenous and non-indigenous crayfish species in Europe that involved COI

Table 1. Primers and probes used in the present study. The probes used are TaqMan MGB probes with either FAM or VIC reporter dyes.

Target species	Target marker	Primer/probe	Sequence (5'-3')	Reference
Aphanomyces astaci	ITS	forward	AAGGCTTGTGCTGGGATGTT	Vrålstad et al. (2009)
		reverse	CTTCTTGCGAAACCTTCTGCTA	Vrålstad et al. (2009)
		probe	FAM-TTCGGGACGACCC-MGBNFQ	Vrålstad et al. (2009)
Astacus astacus	COI	forward	CCCCTTTRGCATCAGCTATTG	current study
		reverse	CGAAGATACACCTGCCAAGTGT	current study
		probe	FAM-CTCATGCAGGCGCAT-MGBFNQ	current study
Pacifastacus leniusculus	COI	forward	GAGTGGGTACTGGATGAACTG	current study
		reverse	GAAGAAACACCCGCTAAATGAAG	current study
		probe	VIC-CAGCGGCTATTGCT-MGBFNQ	current study
Faxonius limosus	COI	forward	CCTCCTCTCGCTTCTGCAAT	Mauvisseau et al. (2018)
		reverse	AACCCCTGCTAAATGCAACG	Mauvisseau et al. (2018)
		probe	FAM-CTCATGCAGGGGCATCAGTGG-MGBFNQ	Mauvisseau et al. (2018)
Procambarus virginalis	COI	forward	ACGGGCAGCTGGTATAACTATG	current study
		reverse	TCTCCTCCACCAGCAGGATC	current study
		probe	FAM-CCGCTATTTGTTTGGTCAGTA-MGBNFQ	current study

sequencing (Filipová et al. 2011; Chucholl et al. 2015; Petrusek et al. 2017a). We also used isolates from surveys of *A. astaci* infections in various carrier species (Tilmans et al. 2014; Mrugała et al. 2015) and crayfish plague outbreaks (Kozubíková-Balcarová et al. 2014) (see additional material, Suppl. material 2: Table S2). The identity of non-indigenous species was confirmed and variation at the target marker (COI) in most of these particular isolates was assessed by DNA barcoding in previous studies (Filipová et al. 2011; Mrugała et al. 2015). The isolate collection, used to test the assay specificity, contained most of the native crayfish known from Western, Central and Northern European countries and the Balkans (see distribution maps in Kouba et al. 2014), with the exception of narrowly-endemic lineages related to *A. torrentium* (Klobučar et al. 2013; Pârvulescu 2019) and the thick-clawed crayfish *Pontastacus pachypus* (Rathke, 1837).

Both newly-developed assays for *A. astacus* and *P. leniusculus*, as well as the published assay for *F. limosus* (Mauvisseau et al. 2018), were subjected to the same *in-vitro* validation procedure as the assay for *P. virginalis*, described above. To ensure optimal performance of all qPCR assays targeting crayfish, we determined the most suitable annealing temperatures through a temperature gradient from 56 °C to 63 °C and multiple primer-probe concentrations were evaluated. Our two objectives were to define the conditions when the assays show efficient amplification of the target DNA but minimal cross-reaction with DNA of related taxa and, if possible, to establish a common protocol for routine application of all assays.

The final protocol used for eDNA screening was identical for the detection of all four crayfish species. The undiluted and diluted samples were run in the following 25 µl reaction: 12.5 µl of TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Foster City, USA), 1.25 µl of each 10 µM primer (forward and reverse), 1.25 µl of 5 µM TaqMan MGB probe, 3.75 µl of PCR-grade water and 5 µl of DNA sample. The following qPCR cycling conditions were used: an initial denaturation at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 1 min.

For all species-specific crayfish assays, we followed recommendations for defining the limit of detection (LOD) and the limit of quantification (LOQ) in qPCR assays used for diagnostic analyses of genetically-modified organisms and microbiological pathogens in foodstuff, tissues and environmental samples (Berdal et al. 2008). These have also been used for previously-published assays for crayfish plague (Vrålstad et al. 2009) and freshwater crayfish ("the Norwegian approach" in Agersnap et al. 2017). Genomic DNA from all target species was extracted according to the protocol in Agersnap et al. (2017) and stock solutions of 50 ng.µl^{-1} genomic DNA (measured using Qubit fluorometer; Invitrogen, Carlsbad, USA) from each species were used to prepare a four-fold dilution series of 13 standard dilutions. In an initial qPCR test, ≥ 3 replicates of the standard dilution 1–8 were run on a Stratagene Mx3005P with qPCR-conditions as described above, while the standard dilutions 9–13 were run in 20 replicates. A template concentration of approximately 1 DNA copy per PCR volume will yield a positive:negative ratio of 7:3 (70% detection success; Berdal et al. 2008). Thus, the copy number in the standard dilutions closest to 70% detection rate were then calculated with most probable number (MPN) calculations (Berdal et al. 2008) and the obtained copy number was then used to calculate copy numbers in the more concentrated standards. The LOD was established for each assay following the criteria that LOD is the lowest concentration that yields a probability of false negatives < 5% (Berdal et al. 2008; Vrålstad et al. 2009). The LOQ was established using the same acceptance level as set for qPCR quantification of the crayfish plague pathogen *A. astaci* (Vrålstad et al. 2009), with observed standard deviation < 0.5 for the Ct-values.

In order to detect *A. astaci* in both eDNA samples and crayfish tissues, we used the assay developed by Vrålstad et al. (2009) with modifications according to Strand (2013). Each undiluted and diluted sample was run in the following 25 µl reaction: 12.5 µl of TaqMan Environmental Master Mix 2.0, 2.5 µl of each 5 µM primer (forward and reverse), 1 µl of 5 µM TaqMan MGB probe, 1.5 µl of PCR-grade water and 5 µl of DNA sample. The following qPCR cycling conditions were used: an initial denaturation at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 15 s and annealing at 62 °C for 30 s.

All qPCR analyses of the eDNA samples were carried out on an Mx3005P qPCR thermocycler (Stratagene, San Diego, USA) at the Norwegian Veterinary Institute, Oslo. The validation of crayfish assays concerning specificity tests against other crayfish species was performed on a BioRad iQ5 (Bio-Rad, Hercules, USA) thermocycler at the Faculty of Science, Charles University, Prague. An analysis of a subset of eDNA

isolates on the BioRad iQ5 thermocycler suggested comparable performance to that on Mx3005P.

As described above, each filter was divided into two technical replicates/subsamples. Both subsamples were analysed as 2x undiluted and 2x 10-fold diluted replicates, in total 4 qPCR replicates per filter. Results for each respective filter were considered positive, only if more than one of the four reactions yielded positive results. A cut-off value was set at Ct 41 following previous recommendations (Agersnap et al. 2017; Kozubíková et al. 2011; Strand et al. 2019) which means that any amplification occurring at or above this value was not considered a positive detection.

The presence or absence of qPCR inhibition was controlled by calculating the difference in Ct values (ΔCt) between the undiluted and corresponding 10-fold diluted DNA replicates as described in Kozubíková et al. (2011) and Agersnap et al. (2017). In case of apparent inhibition (if $\Delta Ct < 2.82$) the estimated eDNA copy number was based on the 10-fold diluted DNA replicates alone, while if $\Delta Ct > 3.82$ (i.e. 10-fold dilution out of range), the estimation of eDNA copy number was based solely on the undiluted DNA replicates (see Suppl. material 4: Table S4 for observed inhibition). If none or only one of the relevant replicates were detected above LOQ, further quantification was not performed and thus qPCR inhibition was not possible to evaluate either.

Results

Optimising and validating the crayfish qPCR assays

We successfully developed new assays for *A. astacus*, *P. leniusculus* and *P. virginalis*. All three assays were apparently species-specific *in-silico* and, for the first two, we also confirmed this *in-vitro*. The assay for *P. virginalis* displayed weak cross-amplification of three other cambarid species (see below). While *in-silico* testing the assays and comparing sequences of the respective crayfish to their closest relatives, we observed the assay for *F. limosus* to differ from a closely-related species *Faxonius* cf. *virilis* (a lineage of the *F. virilis* complex known from Europe; Filipová et al. 2010) by only one mismatch in the forward primer and two mismatches in the probe and the reverse primer, respectively. For subsequent qPCR testing with a temperature gradient, we included DNA isolated from European *F.* cf. *virilis* (labelled *F. virilis* below). While using the PCR conditions (annealing temperature 56 °C) suggested by the authors (Mauvisseau et al. 2018), *F. limosus* and *F. virilis* DNA were amplified at Ct 17.92 and 24.62 respectively. An increase in annealing temperature to 60.5 °C resulted in amplification of *F. limosus* and *F. virilis* DNA at Ct 18.58 and 34.12 respectively, thus increasing the specificity of the assay, although still cross-reacting with *F. virilis*.

Ensuing specificity testing against the collection of all DNA isolates (Suppl. material 2: Table S2) was carried out at 60 °C. The assay for *F. limosus*, which amplified the DNA of the target taxon at Ct 17.7 to 18.5, also amplified DNA of isolates of the following species (lowest Ct stated): *F. virilis* (Ct 30.14), *F. margorectus* (Ct 36.32),

F. rusticus (36.74), *F. harrisonii* (Ct 40.72), *F. punctimanus* (Ct 40.86), *P. virginalis* (Ct 36.13), *P. zonangulus* (Ct 37.91) and *P. acutus* (Ct 35.79). The assay for *P. virginalis*, which amplified the DNA of the target taxon at Ct 18.3 to 23.33 (depending on the starting DNA concentration of isolate), also weakly cross-amplified DNA of isolates from *P. acutus* (Ct 37.29), *P. alleni* (Ct 38.22) and *P. clarkii* (Ct 39.41).

For all crayfish assays, LOD was experimentally established as 5 copies/PCR reaction with good margin; the observed detection success for 20 replicates of a standard dilution corresponding to ~2–4 copies per PCR reaction was between 90–100% (for details see Suppl. material 3: Table S3). Further, LOQ was established as 10 copies per PCR reaction, where the assays demonstrated acceptable repeatability with observed standard deviation for the Ct-values (Suppl. material 3: Table S3).

Environmental DNA monitoring

We detected eDNA of all surveyed crayfish species during our sampling effort (Fig. 3). We also detected eDNA of the crayfish plague pathogen *A. astaci* together with the three investigated non-native crayfish species, but only infrequently. More commonly, eDNA from non-native crayfish was detected alone (Fig. 3, Table 2). A full overview of the qPCR results and eDNA copy estimations is supplied in Suppl. material 4: Table S4.

From the total of 32 surveyed locations, eDNA from native *A. astacus* was unambiguously detected in seven (~22 %) locations. In two of these, however, a positive amplification only occurred in one out of two filter samples. At four locations, the eDNA results were corroborated by observation of *A. astacus* at the sampling sites (Table 2). Simultaneous detection of *A. astacus* and *F. limosus* eDNA was observed in two locations (7 – Všechlapy reservoir and 10 – Pšovka), eDNA from *A. astacus* and *P. leniusculus* was simultaneously detected in location 16 (Oslava). Environmental DNA from the crayfish plague pathogen *A. astaci* was never detected in samples that contained *A. astacus* eDNA. However, in location 10 (Pšovka), we caught specimens of *F. limosus*, whose tissue analyses showed low *A. astaci* prevalence (20%) and very low infection load (agent level 2, A2; Vrålstad et al. 2009).

Non-native *P. leniusculus* was detected by eDNA in eight locations (25%), all where the species was expected according to our prior knowledge (Suppl. material 1: Table S1). All detections occurred in both samples taken at the respective sampling sites. The eDNA results were corroborated by observation of signal crayfish at seven locations on the date of sampling. Environmental DNA from the crayfish plague pathogen *A. astaci* was detected in only two of the locations where *P. leniusculus* was detected (13 – Malše and 15 – Dračice). In these two locations, data from tissue analyses confirmed high prevalence (80% and 100%) and low to high infection load (up to A3 and A5, respectively). For three other *P. leniusculus* positive locations (16 – Oslava, 20 – Žďárka, and 22 – Staviště), the apparent absence of *A. astaci* eDNA was corroborated by no detection of the pathogen in screened crayfish individuals (Table 2). Generally, *P. leniusculus* was the only crayfish species detected through eDNA at the respective

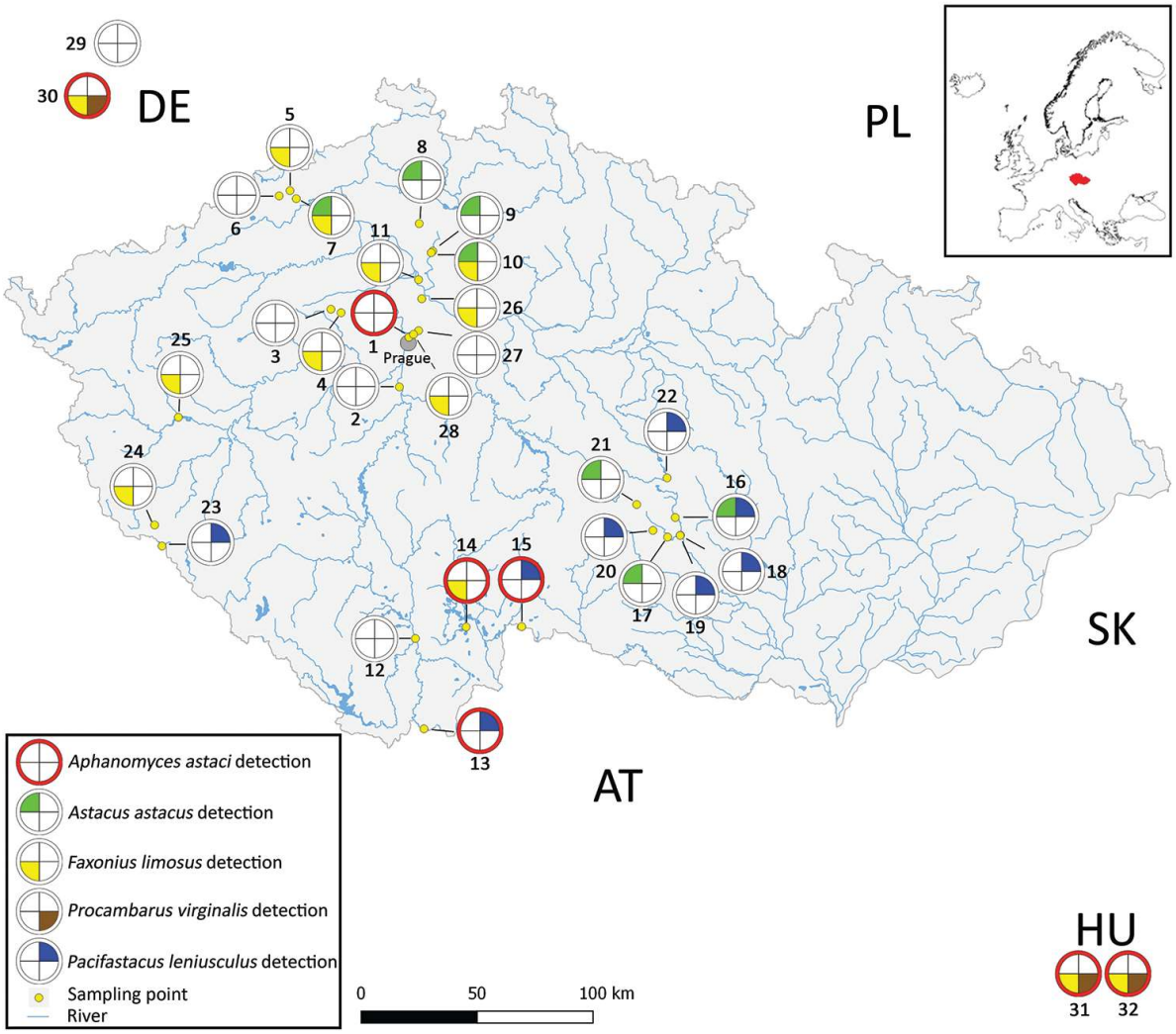

Figure 3. Map of Czechia with results of the eDNA screening at the sampling locations. Blue lines and areas represent the main water bodies, yellow dots represent each respective sampling point with numbers referring to the sampling sites in Table 2 and Suppl. material 1: Table S1. Pie charts: the red ring around the pie charts indicates unambiguous eDNA detection of *A. astaci* whereas a white ring represents non-detection. The green colour indicates detection of *A. astacus*, blue indicates detection of *P. leniusculus*, yellow represents detection of *F. limosus* and brown indicates presence of *P. virginalis*. The neighbouring countries are indicated by their two-letter ISO codes: AT, DE, HU, PL and SK stand for Austria, Germany, Hungary, Poland and Slovakia.

sampling points, except at location 16 (Oslava) where eDNA of *A. astacus* was also detected. Environmental DNA from *P. leniusculus* never co-occurred with other non-native crayfish species.

Environmental DNA of non-native *F. limosus* was unambiguously detected in 13 locations. At one location the detection occurred on only one filter. *In-situ* observation on the day of sampling confirmed the eDNA results at eight locations. Environmental DNA from the crayfish plague pathogen *A. astaci* was detected in four of the *F. limosus*-positive locations, three of which were urban waters of Berlin (site 30 – Hundekehlesee) and Budapest (31 and 32 – Barát); presence of infected crayfish was confirmed at site no.

Table 2. Results of the eDNA analyses from individual sampling sites. Volumes of water filtered (in l) indicated. The target species are abbreviated as follows: AA for *Astacus astacus* (noble crayfish), PL for *Pacifastacus leniusculus* (signal crayfish), PV for *Procambarus virginalis* (marbled crayfish), FL for *Faxonius limosus* (spiny-cheek crayfish) and Aph for *Aphanomyces astaci* (crayfish plague agent). The column labelled "obs" indicates any crayfish observed at the respective site during the sampling, using the same species abbreviations. Sites where manual search for crayfish was impossible to conduct are indicated by "ns". Detection in eDNA samples is stated as unambiguous confirmation on 0 (marked as "–"), 1 or 2 filters per site (for more details, see Suppl. material 4: Table S4). The prevalence of *A. astaci* in NICS populations and maximum agent level in infected crayfish following Vrålstad et al. (2009) is specified. For more details about the sampling sites and specific comments, including past evidence of crayfish presence, see Suppl. material 1: Table S1.

No.	Locations	Habitat	Volume (in l)	qPCR positives in eDNA samples						*A. astaci* screening in NICS	
				AA	PL	FL	PV	Aph	obs	Prevalence	Max. agent level
1	Vltava in Prague	River	4	–	–	–	–	2		88% (15/17)	A4
2	Vltava (Vrané)	Reservoir	2.2	–	–	–	–	–		n/a	
3	Kněžák Pond	Fishpond	1.35	–	–	–	–	–		n/a	
4	Smečno	Urban pond	1.9	–	–	1	–	–		n/a	
5	Barbora	Flooded mine	10	–	–	2	–	–	FL	0% (0/22)	(3 x A1)
6	Osecký Pond	Fishpond	0.7	–	–	–	–	–		n/a	
7	Bouřlivec (Všechlapy)	Reservoir	2.8	1	–	2	–	–	ns	n/a	
8	Liběchovka	Stream	1.5	2	–	–	–	–		n/a	
9	Pšovka (above Harasov)	Stream	4.4	2	–	–	–	–	AA	n/a	
10	Pšovka (Harasov)	Pond out	10	1	–	2	–	–	FL	20% (3/15)	A2
11	Elbe	River	3.8	–	–	2	–	–	FL	35% (6/17)	A4
12	Malše in České Budějovice	River	1.85	–	–	–	–	–		n/a	
13	Malše (border with Austria)	Stream	10	–	2	–	–	2	PL	80% (16/20)	A3
14	Zlatá stoka	Channel	1.6	–	–	2	–	1		12.5% (1/8)	A3
15	Dračice	Stream	1.2	–	2	–	–	2	PL	100% (20/20)	A5
16	Oslava (upstream)	Stream	2.3	2	2	–	–	–	PL	0% (0/23)	A0
17	Balinka (upstream)	Stream	4	2	–	–	–	–	PL	n/a	
18	Oslava (confluence)	Small river	10	–	2	–	–	–	PL	n/a	
19	Balinka (confluence)	Stream	4.1	–	2	–	–	–		n/a	
20	Žďárka	Stream	5.1	–	2	–	–	–	PL	0% (0/28)	A0
21	Ochozský Brook	Stream	0.85	2	–	–	–	–	AA	n/a	
22	Staviště	Stream	4.4	–	2	–	–	–	PL	0% (0/18)	A0
23	Kouba	Stream	3	–	2	–	–	–	PL	n/a	
24	Starý Klíčov – Lomeček	Quarry	10	–	–	2	–	–	ns	n/a	
25	Mže (Hracholusky)	Reservoir	3.2	–	–	2	–	–	FL	29% (2/10)	A3
26	Kojetice	Quarry	10	–	–	2	–	–	FL	70% (14/20)	A2

No.	Locations	Habitat	Volume (in l)	qPCR positives in eDNA samples						*A. astaci* screening in NICS	
				AA	PL	FL	PV	Aph	obs	Prevalence	Max. agent level
27	Prague–Prosek (park)	Urban pond	10	–	–	–	–	–		n/a	
28	Rokytka	Stream	2	–	–	2	–	–		n/a	
29	Krumme Lanke	Lake	10	–	–	–	–	–	ns	n/a	
30	Hundekehlesee	Lake	10	–	–	2	1	1	ns	n/a	
31	Tributary of Barát	Thermal stream	10	–	–	2	2	2	FL, PV	85% (17/20)	A3
32	Barát Brook	Stream	10	–	–	2	2	2	FL, PV	n/a	

31. In four locations (10 – Pšovka, 11 – Elbe, 25 – Mže and 26 – Kojetice), data from *F. limosus* tissue analyses confirmed *A. astaci* prevalence ranging from low to high (20%, 35%, 29% and 70% respectively) and very low to moderate infection load (A2, A4, A3 and A2), but no *A. astaci* spores were detected by eDNA there. Environmental DNA of *F. limosus* and native *A. astacus* was detected together in two locations (mentioned above; Table 2, Fig. 3). *Faxonius limosus* eDNA did not co-occur with that of other non-native crayfish species in Czechia, but did so at both locations in Budapest (31 and 32) and one location in Berlin (30 – Hundekehlesee) (Table 2, Fig. 3). These three urban waters were the only sites where we confirmed eDNA of *P. virginalis* (in all cases together with *A. astaci*). Specimens of this crayfish species were observed at the Hungarian sampling sites.

In 24 subsamples (i.e. technical replicates), eDNA of *A. astaci* was detected (with Ct values in the qPCR reaction not exceeding 41; Suppl. material 4: Table S4), but it was quantifiable only in 12 subsamples. Four of these detections (33%) showed inhibition, mostly weak. *Astacus astacus* eDNA was detected in 27 subsamples of which 17 were above the LOQ. Two of these (12%) displayed weak inhibition. All of the 32 subsamples that were positive for *P. leniusculus* were quantifiable and none of them showed any inhibition. Of 49 subsamples positive for *F. limosus*, ten were quantifiable (above LOQ) and four (40%) showed some inhibition. Ten subsamples were positive for *P. virginalis* of which eight were quantifiable; ΔCt values for these subsamples indicated some qPCR inhibition as well.

Discussion

Crayfish eDNA and assays – our study compared to the state of art

This study explores the use of the eDNA methodology for the detection of the crayfish plague pathogen *A. astaci* and freshwater crayfish in Central and Western Europe, simultaneously covering several species and numerous habitat types. A steadily increasing number of studies use eDNA monitoring to assess the presence of native crayfish or the introduction and spread of non-native crayfish across the globe (Tréguier et al. 2014; Dougherty et al. 2016; Ikeda et al. 2016, 2019; Agersnap et al. 2017; Larson

et al. 2017; Mauvisseau et al. 2018). In Europe, these tend to be complemented by screening for the accompanying conservationally relevant pathogen *A. astaci* (Robinson et al. 2018; Mauvisseau et al. 2019; Strand et al. 2019; Wittwer et al. 2019).

One of the potential pitfalls of eDNA monitoring methods, relying on species-specific qPCR, lies within the development and testing of the assays themselves. Specificity testing, both *in silico* and *in vitro* against isolates of any closely-related species that may cause false-positive results, is therefore imperative. While several previous studies have performed specificity testing on a limited range of locally relevant freshwater crayfish species (Dougherty et al. 2016; Agersnap et al. 2017) and one on a more comprehensive range of non-target species than just those found in the examined area (Larson et al. 2017), we tested the assays used for *A. astacus*, *P. leniusculus*, *P. virginalis* (this study) and *F. limosus* (Mauvisseau et al. 2018) towards most native and non-native freshwater crayfish species known from European waters (Suppl. material 2: Table S2). The three former assays proved sufficiently specific, although a weak cross-amplification with other cambarids was observed when testing the *P. virginalis* assay against DNA isolates from other crayfish. However, the *F. limosus* assay yielded a relatively strong non-target amplification for *F. virilis* with the originally recommended annealing temperature (56 °C). An increase of the annealing temperature to 60 °C reduced its extent, but DNA of several other *Faxonius* and *Procambarus* species also yielded cross-amplification with this assay. We may presume that at 56 °C this effect would be substantially stronger.

The cross-amplification of non-target species at high Ct levels, close to cut-off of both assays for *F. limosus* and *P. virginalis*, should pose no practical problems in eDNA studies, as these were observed while analysing tissue isolates. Environmental samples contain, by their very nature, less DNA of the target species than tissue isolates and thus usually amplify more than 10 cycles later compared to DNA isolates from tissue. A false-positive detection is therefore highly unlikely to occur for most of these taxa, possibly with the exception of *F. virilis* detection by the *F. limosus* assay. Yet, it seems that achieving universal specificity for assays may pose a challenge, especially in regions with higher crayfish species biodiversity than Europe where closely-related species can co-occur that differ only marginally in the target DNA marker. In such cases it may be beneficial to apply the metabarcoding approach with general primers to better capture the overall crayfish biodiversity (Thomsen et al. 2012a).

However, for management purposes in Europe, even the non-specific amplification of *F. virilis* is not likely to pose a substantial problem as non-native *F. virilis* has so far only been found in London (Ahern et al. 2008) and the Netherlands (Soes and van Eekelen 2006). Moreover, even in the case of such a false detection, this still indicates the presence of an invasive crayfish of concern to the EU (Regulation (EU) No 1143/2014) that may act as a crayfish plague carrier (Tilmans et al. 2014).

Environmental DNA monitoring of crayfish – pros and cons

An increasing number of studies, including the present one, demonstrate that the eDNA approach is effective in providing presence/absence data for freshwater crayfish

(Dougherty et al. 2016; Ikeda et al. 2016, 2019; Agersnap et al. 2017; Mauvisseau et al. 2018, 2019; Strand et al. 2019). In contrast to the crayfish plague agent *A. astaci*, where it is possible to determine the rough quantity of spores in the water (Strand et al. 2011, 2012, 2014; Makkonen et al. 2013; Svoboda et al. 2013, 2014), it is not possible to quantify crayfish biomass, population density or population structure on the basis of eDNA detection (Dougherty et al. 2016; Agersnap et al. 2017; Laurendz 2017; Rice et al. 2018).

For conservation purposes, for example when determining the suitability of an unpopulated habitat as an ark site, the critical information is nevertheless the presence or absence of the crayfish plague pathogen and any potential vectors thereof. For this purpose, eDNA monitoring provides an efficient alternative for confirming the presence of target organisms (Strand et al. 2019). However, caution must be exercised regarding the interpretation of samples that do not yield any positive detection. Many samples and large volumes should be analysed to substantiate the high likelihood of absence of a rare target organism convincingly (Strand et al. 2014, 2019).

In this study, we failed to detect *A. astaci* eDNA in four of eight locations where crayfish tissue analyses confirmed the presence of this pathogen, albeit in either a low prevalence or low infection load. Here, we have no knowledge about the density of the carrier-population, but the combination of low pathogen prevalence and low crayfish population density is obviously a challenge to reveal *A. astaci* presence in a random water sample. At location 29 (Krumme Lanke), we were unable to detect eDNA of any of the five target organisms despite reports of the presence of both *F. limosus* and *P. virginalis* somewhere in the lake in the recent past (Linzmaier et al. 2018). This might be explained by spatial mismatch (Harper et al. 2018) and low ambient temperatures which may have led to decreased activity of crayfish (Bubb et al. 2004; Rusch and Füreder 2015) and thus decreased emission of eDNA.

Dilution of the eDNA amount in large waterbodies is a factor that may lead to the failure to detect the target taxa, even if present. This is also exemplified in location 1 (the river Vltava in Prague) where we detected the crayfish plague agent but none of the host species. At this sampling site, the Vltava is more than 115 m wide and the flow rate on the date of sampling was ~50 m^3/s, so any eDNA signal would be subject to significant dilution, a common problem reported in previous studies (Strand et al. 2014, 2019). The presence of *F. limosus* in the Vltava in Prague has previously been confirmed, with crayfish displaying high levels of infection with *A. astaci* (Table 2) only a short distance downstream from the sampling site. Furthermore, *A. astac*i spores are alive and active and will more likely withstand chemical and biological processes in the water that lead to degradation of eDNA (Laurendz 2017), compared to cells shed from crayfish, a group reported to release only a very low amount of eDNA (Rice et al. 2018).

Strand et al. (2019) monthly monitored a watercourse for more than a year during an ongoing crayfish plague outbreak in Norway. There, the very scarce population of *P. leniusculus* that had caused the plague outbreak was detected by eDNA only in July and October, concurring with the presumed periods of moulting and reproduction, when more eDNA from the crayfish is likely to be released to the water. Dunn et al.

(2017) examined the relationship between eDNA concentration and crayfish biomass and were able to detect a relationship only when female *P. leniusculus* crayfish were ovigerous. Laurendz (2017) found no clear correlation between number of crayfish and eDNA emission in aquaria experiments with *P. leniusculus*, but observed peaks during moulting and huge quantitative variation depending on various environmental and biological factors. Similarly, Buxton et al. (2017) observed peaks of eDNA of the great crested newt (*Triturus cristatus*) towards the end of the adult breeding period and when newt larval abundance was at its highest. While studying seasonal variation of eDNA emission by freshwater pearl mussel (*Margaritifera margaritifera*), Wacker et al. (2019) measured the highest concentrations of eDNA in August, corresponding to the period these mussels release large amounts of larvae into the water. These studies and our results demonstrate that sample number, coverage, season, inhibition and other environmental factors can substantially influence the results and that eDNA methods may fail to detect elusive or rare targets. A robust knowledge of the biology of the target species is thus required for improving sampling success. In our study, although using large volumes that to some degree compensate for few samples, we would most likely increase the detection success with more samples.

A useful tool to help determine the number of samples required for maximising detection probability could be occupancy modelling. Schmidt et al. (2013) analysed data obtained while examining the presence of the chytrid fungus *Batrachochytrium dendrobatidis*. Based on an index similar to "catch-per-unit-effort", which is also obtainable for crayfish, they were able to calculate the amount of samples required for a detection probability to exceed 95%. Dougherty et al. (2016) used relative abundance and site characteristics as covariates to model the detection probability for *F. rusticus* using eDNA sampling. A similar tool for occupancy modelling, an R package for multiscale occupancy modelling of eDNA data, was recently presented by Dorazio and Erickson (2017).

Detection of the host-pathogen complex

In the screening of crayfish habitats, we successfully managed to detect eDNA of European noble crayfish and all three North American crayfish species investigated in this study. Here, we infrequently detected eDNA of the crayfish plague pathogen *A. astaci* together with the three investigated non-native crayfish species. More commonly, only eDNA from non-native crayfish was detected alone, suggesting low prevalence and infection load or possibly even absence of the pathogen (as also corroborated by analyses of the host crayfish tissues).

The eDNA monitoring methodology has been promoted as a reliable, non-invasive, ethical and animal welfare-friendly alternative to cage monitoring for early detection of crayfish plague (Wittwer et al. 2017; Strand et al. 2019). Indeed, when eDNA fails to detect *A. astaci*, although present at the location, it is likely that the pathogen spore concentration is too low to infect caged susceptible crayfish anyway. Strand et al. (2019) demonstrated that eDNA monitoring reveals the presence of *A. astaci* in the water earlier

than cages with live crayfish put out for disease surveillance. According to Strand et al. (2019), the simultaneous monitoring of native and non-native crayfish also provides additional information on habitat status, which otherwise requires trapping surveys.

We never detected eDNA from *A. astaci* together with native *A. astacus*, which is a good sign for the habitat status for these locations. However, in a few locations, eDNA from both native and non-native crayfish co-occurred. This could, in some cases, result from passive downstream transport of eDNA (Deiner and Altermatt 2014; Rice et al. 2018) from one of the target species that was geographically separated – even with migration barriers. However, in other cases it could reflect co-existence of native and non-native crayfish in the absence of infected crayfish carriers, or with very low *A. astaci* prevalence in the non-native crayfish population. In the latter case, it might only be a matter of time before the low-prevalent crayfish plague agent eradicates the native population. In a Norwegian lake, populations of *A. astacus* and *A. astaci*-carrying *P. leniusculus* presumably occurred at the same time for more than a decade before crayfish plague struck the native population (Vrålstad et al. 2011, 2014). This might be explained by low infection pressure and geographic separation within the lake.

The observed co-occurrence of eDNA from *A. astacus* and *F. limosus* in two locations, as well as *A. astacus* and *P. leniusculus* in one location, could suggest a possible syntopic presence of native and non-native species, although in at least one of the cases (location 10), downstream transport of *A. astacus* eDNA from a population upstream of the *F. limosus* population (location 9) is more likely. However, co-existence can occur in the absence of *A. astaci* infection in the non-native population. This has been thoroughly documented in Central Europe for *F. limosus* populations co-occurring with *A. astacus* (Schrimpf et al. 2013) and also for *P. leniusculus* populations co-occurring with *A. astacus* in Denmark (Skov et al. 2011). In our study, 70% and 80% of the *P. leniusculus* and *F. limosus* locations did not yield positive eDNA results for *A. astaci*, respectively. However, the number of individuals directly tested by us for infection was too low to conclude about the absence of the pathogen even at places where none was detected (see Schrimpf et al. 2013).

The co-occurrence of NICS in urban waters, represented by an inner-city lake (30 – Hundekehlensee) and a thermal stream (31 and 32 – Barát stream and its thermal inflow), demonstrates the importance these habitats play for the spread of NICS. The ornamental pet trade has been shown to be a major introduction pathway for non-native crayfish species into Europe (Peay 2009b; Chucholl 2013) and the species found at these locations are available through the pet trade (Mrugała et al. 2015). Additionally, eDNA of the crayfish plague pathogen *A. astaci* was detected at all three locations. Our findings highlight both the risks emanating from these habitats as well as the possibilities of monitoring similar habitats using eDNA.

Methods and sample strategies

The use of eDNA plays an important role in the present efforts to introduce advanced molecular tools into monitoring and bio-assessment of aquatic ecosystems (Leese et

al. 2016). This is particularly important with regard to the protection, preservation and restoration of aquatic ecosystems, which for European Union countries is legally binding through the Water Framework Directive (EU directive 2000/60/EC). Current approaches are still largely based on traditional sampling of organisms followed by identification by morphology, which is time-consuming and error-prone due to the varying and diminishing taxonomic expertise (Leese et al. 2016). While metabarcoding of environmental samples is the most promising approach for bioassessment and biodiversity inventory studies (de Vargas et al. 2015; Visco et al. 2015; Fujii et al. 2019), the more targeted qPCR approaches are specifically relevant for the monitoring of rare and red-listed native species and/or harmful invasive species of particular focus.

For both approaches, sampling strategies are of great importance for the quality and outcome regarding results. The choice of sample method, filter and volume might be of vital importance for maximising the detection probability of rare targets (Strand et al. 2014; Kumar et al. 2019). Crustaceans are more challenging to detect (Forsström and Vasemägi 2016; Rice et al. 2018) than fish, for example, that shed multiple sources of eDNA into the water (Jo et al. 2019). It appears, therefore, that efficient eDNA sampling for crayfish and their pathogen requires a substantially larger volume of water than for fish and amphibians. However, we are not aware of any study directly comparing these organisms.

The cost of the sampling equipment, as used for example in Strand et al. (2014, 2019) or Thomas et al. (2018), may be a limiting factor that prevents collection of suitable samples by a wider body of stakeholders. While conducting the fieldwork, we thus also evaluated the applicability of a robust, easy-to-use and low-cost version of the eDNA sampling equipment, based mostly on items readily available in garden stores and hobby markets. Most importantly, we exchanged the costly Masterflex E/S portable peristaltic pump-based sampler (retail price exceeding 2000 USD) with the drill-powered pumping system (ca. 26 USD without drill). This low-cost alternative provided very satisfactory results since it was possible to filter the same amount of water as sampled with the Masterflex E/S sampler and the target organisms were usually detected where expected. The difference between the two systems, which use exactly the same filter, is that water is pumped through the filter with suction, rather than pressure, since the filter is situated at the front of the drill-pump system. All parts of the entire setup can be detached and disinfected and the easy-to-replace filter cups eliminate issues with potential carry-over contamination. The low price of the equipment is a particularly important benefit for various stakeholders with limited budgets (e.g. nature conservancy agencies, NGOs, fishery managers).

Compared to the traditional methods used to determine presence or absence of crayfish which consist of either manual searching or trapping, this method requires less time in the field at each sampling site and it allows for sampling at locations unsuitable for traditional monitoring. For example, some of the sampling points visited by us were inaccessible for manual searching crayfish and would have required trapping or scuba diving, neither of which was possible during the fieldwork for this study. The eDNA methodology also enables the user to detect crayfish species when only small-sized in-

dividuals which might neither be caught in traps nor easily detected by manual search are dominant. Additionally, the extracted eDNA filter samples contain a broad variety of species from each location, both microorganisms and macroorganisms, and can be, at a later date, screened for entirely different targets (Dysthe et al. 2018). There is thus a potential for savings of both effort and costs if relevant stakeholders synchronise and/or collaborate on the eDNA sampling for multiple research and monitoring purposes.

Conclusions

The eDNA method based on targeted species-specific qPCR is suitable for detecting several invasive and native crayfish species as well as the crayfish plague pathogen in relevant habitat types in Central and Western Europe. The assays presented here performed well and yielded results that mostly corroborated our knowledge on the presence of native and non-native crayfish in the visited habitats.

It is particularly the positive data on the presence of crayfish and crayfish plague that yield valuable information, while negative results have to be interpreted with great caution. The latter should preferably be followed up with analyses of more samples collected in suitable periods, taking into account the time of year, temperature, water flow and the biology of the target species. This is of paramount importance if the absence of a specific species needs to be unambiguously established.

Including further assays of other crayfish species native to Central Europe, such as the stone crayfish, into this already broad panel will enable relevant stakeholders and authorities to use this method as a routine monitoring tool for all relevant crayfish species or in preparation of restocking operations.

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Supplementary material 1

Table S1

Authors: Johannes C. Rusch, Michaela Mojžišová, David A. Strand, Jitka Svobodová, Trude Vrålstad, Adam Petrusek

Data type: details on localities

Explanation note: Detailed information about the eDNA sampling sites visited during the study.

Link: https://doi.org/10.3897/neobiota.58.49358.suppl1

Supplementary material 2

Table S2

Authors: Johannes C. Rusch, Michaela Mojžišová, David A. Strand, Jitka Svobodová, Trude Vrålstad, Adam Petrusek

Data type: species list

Explanation note: List of crayfish species used for in-vitro testing of the assay specificity.

Link: https://doi.org/10.3897/neobiota.58.49358.suppl2

Supplementary material 3

Table S3

Authors: Johannes C. Rusch, Michaela Mojžišová, David A. Strand, Jitka Svobodová, Trude Vrålstad, Adam Petrusek

Data type: data for methods

Explanation note: Standard dilutions from crayfish genomic DNA.

Link: https://doi.org/10.3897/neobiota.58.49358.suppl3

Supplementary material 4

Table S4

Authors: Johannes C. Rusch, Michaela Mojžišová, David A. Strand, Jitka Svobodová, Trude Vrålstad, Adam Petrusek

Data type: detailed results

Explanation note: Overview of the qPCR results, eDNA copy number estimation and PCR inhibition.

Link: https://doi.org/10.3897/neobiota.58.49358.suppl4

Supplementary Table S1. Detailed information about the eDNA sampling sites visited during the study.

The crayfish species abbreviations: AA for noble crayfish (*Astacus astacus*), PL for signal crayfish (*Pacifastacus leniusculus*), PV for marbled crayfish (*Procambarus virginalis*) and FL for spiny cheek crayfish (*Faxonius limosus*). Characters after the species abbreviation indicate the confirmed presence of crayfish in the respective waterbody at the sampling date, or reliable report within one year from the sampling date (+), historic presence documented several years before the sampling (*) or reported but highly doubtful and unconfirmed presence (?). Sites with presumed alien species presence where manual search for crayfish was impossible to conduct are indicated by (!).

Date	Site no.	Waterbody	Settlement	Waterbody type	Crayfish species	Geographic coordinates
7.8.2017	1	Vltava	Prague	river	FL (+)	50.0970N, 14.4404E
7.8.2017	2	Vltava – Vrané Reservoir	Měchenice	reservoir	FL (*)	49.9112N, 14.3852E
7.8.2017	3	pond Kněžák on Drnecký Brook	Drnek	fishpond	FL (?)	50.2010N, 13.9879E
7.8.2017	4	pond in Smečno	Smečno	urban pond	FL (*)	50.1881N, 14.0469E
8.8.2017	5	Barbora	Oldřichov	flooded mine	FL (+)	50.6402N, 13,7503E
8.8.2017	6	Osecký rybník	Osek	fishpond	PL (?)	50.6209N, 13,6871E
8.8.2017	7	Bouřlivec – Všechlapy Reservoir	Všechlapy	reservoir	FL (*)(!)	50.6106N, 13,7865E
8.8.2017	8	Liběchovka	Zakšín	stream	AA (+)	50.5186N, 14.5001E
8.8.2017	9	Pšovka (upstream of the Harasov Pond)	Harasov	stream	AA (+)	50.4168N, 14,5790E
8.8.2017	10	Pšovka (outflow of the Harasov Pond)	Harasov	pond outflow	FL (+)	50.4109N, 14.5698E
8.8.2017	11	Elbe	Kly	river	FL (+)	50.3109N, 14.4961E
9.8.2017	12	Malše (above confluence with the Vltava)	České Budějovice	river	FL (*)	48.9605N, 14.4779E
9.8.2017	13	Malše (border with Austria)	Leopoldschlag	stream	PL (+)	48.6146N, 14.5279E
9.8.2017	14	Zlatá stoka (ancient artificial channel)	Třeboň	channel	FL (*)	49.0043N, 14.7727E
9.8.2017	15	Dračice	Nová Bystřice	stream	PL (+)	49.0056N, 15.0951E

10.8.2017	16	Oslava (above the Mostiště Reservoir)	Netín – Hora	stream	AA & PL (+)	49.4201N, 15.9864E
10.8.2017	17	Balinka	Uhřínov	stream	AA (+)	49.3461N, 15.9419E
10.8.2017	18	Oslava (confluence with the Balinka)	Velké Meziříčí	small river	PL (+)	49.3527N, 16.0161E
10.8.2017	19	Balinka (confluence with the Oslava)	Velké Meziříčí	stream	PL (+)	49.3521N, 16.0151E
10.8.2017	20	Žďárka	Chlumek – Benešov	stream	PL (+)	49.3713N, 15.8569E
10.8.2017	21	Ochozský Brook	Dobroutov	stream	AA (+)	49.4690N, 15.7626E
10.8.2017	22	Staviště	Žďár nad Sázavou	stream	AA (*), PL (+)	49.5696N, 15.9391E
11.8.2017	23	Kouba	Sruby	stream	PL (+)	49.3121N, 13.0075E
11.8.2017	24	Starý Klíčov – dive site Lomeček	Starý Klíčov	quarry	AA (*), FL (+)(!)	49.3916N, 12.9646E
11.8.2017	25	Mže – Hracholusky Reservoir	Čerňovice	reservoir	FL (+)	49.7976N, 13.1024E
12.8.2017	26	quarry in Kojetice	Kojetice	quarry	FL (+)	50.2401N, 14.5149E
12.8.2017	27	Prague – Prosek (Přátelství park)	Prague	urban pond	PV (?)	50.1215N, 14.4966E
12.8.2017	28	Rokytka (confluence with the Vltava)	Prague	stream		50.1077N, 14,4670E
04.12.2018	29	Krumme Lanke	Berlin	lake	PV, FL (*)(!)	52.4478N, 13.2302E
04.12.2018	30	Hundekehlesee	Berlin	lake	PV (+)(!)	52.4803N, 13.2586E
10.01.2019	31	thermal water tributary of Barát patak	Budapest	thermal stream	PV, FL (+)	47.6070N, 19.0606E
10.01.2019	32	Barát patak	Budapest	stream	PV, FL (+)	47.6093N, 19.0624E

Further comments:

#3: Stocking of *F. limosus* into the Drnecký Brook was reported in 2005 (Petrusek et al. 2006) but according to the locals, the released individuals were re-captured and removed. No presence of this or any other crayfish species was documented from the stream afterwards.

#4: The established *F. limosus* populations in the urban pond in Smečno served as the source of individuals highly infected by *A. astaci* for the research purposes (Matasová et al. 2011; Svoboda et al. 2013) for years. In several recent years, attempts to capture additional individuals failed (A. Petrusek, pers. obs.) but there are ample inaccessible shelters in the pond bank.

#6: Presence of *P. leniusculus* in the Osecký fishpond was reported by local anglers but no evidence was provided. The pond is located far from other known localities of the species and the report should be considered doubtful.

#7: Presence of *F. limosus* was reported before 2014; the steep banks of the reservoir prevented on-site manual search for crayfish at the sampling date.

#9 + #10: The stream Pšovka has been apparently struck by a crayfish plague outbreak that eliminated the local noble crayfish in 1998-1999 almost entirely (Kozubíková et al. 2006). However, some specimens survived in an upstream refugium and the recolonisation of the stream was assisted by relocation of some individuals downstream (Kozubíková-Balcarová et al. 2014). The noble crayfish population extends almost to the pond Harasov, built on the Pšovka, which hosts an established *F. limosus* population. The site #9 was located between the stretch inhabited by *A. astacu*s and Harasov, site #10 was just below the outflow of the Pšovka from the pond.

#12: Infected *F. limosus* had been sampled above the mouth of the Malše River in České Budějovice in the past (Kozubíková et al. 2011) but local anglers did not confirm any recent presence of crayfish in the section of the river where eDNA samples were taken, ca 1.6 km upstream and separated by a migration barrier (weir).

#16: A syntopic presence of *A. astacus* and *P. leniusculus* was documented from the stream Oslava in 2017 (Hladovec 2018), although not on the sampling date.

#22: A syntopic presence of *P. leniusculus* with a few remaining individuals of *A. astacus* was documented from the stream Staviště around 2014 (A. Petrusek, pers. obs.); *A. astacus* still persisted in the reservoir upstream of Žďár nad Sázavou in 2018 (J. Svobodová, pers. obs.) but not in the stream below it.

#24: The flooded quarry Lomeček in Starý Klíčov is a popular dive site with a well-established population of *F. limosus*. Its coexistence with rare individuals of *A. astacus* was documented in the 2000s (Kozubíková et al. 2009). The present status of other crayfish than *F. limosus* is unclear but considering the negligible infection load by *A. astaci* (Matasová et al. 2011) and frequent attempts to release various aquatic species by divers (A. Petrusek, pers. obs.), their presence is not unlikely. On-site sampling is impossible without scuba gear, and only feasible during night dives.

#27: Overwintering *P. virginalis* were documented from the park pond in Prague–Prosek between 2015-2016 (Patoka et al. 2016). An eradication was then attempted by conservation authorities after draining the pond system for winter and liming the exposed concrete bottom of the pond. However, the draining itself might have resulted in exporting crayfish to the stream Rokytka (sampled downstream at site #28).

#28: No crayfish were reported from the Rokytka but colonization of its lowermost section by *Faxonius limosus* from the Vltava, or potential colonization from the *P. virginalis* population in the park pond (site #27) could not be ruled out.

#29 + #30: Both *P. virginalis* and *F. limosus* have been documented from Krumme Lanke in 2015 or 2016 (Linzmaier et al. 2018), present status of the population is unclear. *Procambarus virginalis* has been captured in the Hundekehlesee in October 2018. On-site sampling in December 2018 was impossible; both lakes sampled in Berlin are however part of a larger system interconnected by a stream, allowing crayfish dispersal.

#31 + #32: The Barát Brook and its tributary warmed by the thermal water inflow in Budapest hosts additional exotic species; in addition to our target species *P. virginalis* and *F. limosus*, alien crayfish *Procambarus clarkii* and *Cherax quadricarinatus* and numerous aquarium fish taxa were documented from its thermal water tributary (Szendőfi et al. 2018).

Supplementary Table S2. List of crayfish species used for *in vitro* testing of the assay specificity

Species	**origin of specimen**	**Reference (if applicable)**
Astacus astacus	Czechia	Mojžišová et al. (2020)
Austropotamobius italicus	Germany	Chucholl et al. (2015)
Austropotamobius pallipes	Germany	Chucholl et al. (2015)
Austropotamobius torrentium	Czechia	Mojžišová et al. (2020)
Cambarellus diminutus	Czechia – pet trade	Mrugała et al. (2015)
Cambarellus patzcuarensis	Germany – pet trade	Mrugała et al. (2015)
Cambarellus shufeldtii	Germany – pet trade	Mrugała et al. (2015)
Cambarellus texanus	Czechia – pet trade	Mrugała et al. (2015)
Cherax destructor	Czechia – lab culture	Mrugała et al. (2016)
Cherax quadricarinatus	Germany – pet trade	Mrugała et al. (2015)
Faxonius limosus	Czechia	
Faxonius harrisonii	Germany – pet trade	Mrugała et al. (2015)
Faxonius immunis	Germany	
Faxonius leptogonopodus	Germany – pet trade	Mrugała et al. (2015)
Faxonius margorectus	Germany – pet trade	Mrugała et al. (2015)
Faxonius palmeri	Germany – pet trade	Mrugała et al. (2015)
Faxonius punctimanus	Germany – pet trade	Mrugała et al. (2015)
Faxonius rusticus	Germany – pet trade	Mrugała et al. (2015)
Faxonius virilis	Netherlands	Tilmans et al. (2014)
Pacifastacus leniusculus	Czechia	
Pontastacus leptodactylus	Turkey	Svoboda et al. (2012)
Procambarus alleni	Czechia – pet trade	Mrugała et al. (2015)
Procambarus cf. zonangulus	Netherlands	Tilmans et al. (2014)
Procambarus cf. acutus	Netherlands	Tilmans et al. (2014)
Procambarus clarkii	Netherlands	Tilmans et al. (2014)
Procambarus enoplosternum	Germany – pet trade	Mrugała et al. (2015)
Procambarus llamasi	Germany – pet trade	Mrugała et al. (2015)
Procambarus vasquezae	Germany – pet trade	Mrugała et al. (2015)
Procambarus virginalis	Czechia – pet trade	Mrugała et al. (2015)

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Supplementary Table S3. Standard dilutions from crayfish genomic DNA.

Standard curves were established from several calibrant points using qPCR replicates to define the dynamic/quantitative range and to calculate DNA copy number on the basis of positive/negative ratios (single molecule quantification; SIMQUANT).

Standard dilutions[a]	Mean Ct-values[b]	N (# PCR replicates)[c]	% detection[d]	DNA (ng/µl) in calibrants[e]	ng DNA (5 µl) in PCR[f]	Estimated DNA copies in PCR[g]
PV - Marbled crayfish						
4^1	21.65 (±0.09)	3	100	12.5	62.5	726663.2
4^2	23.79 (±0.05)	3	100	3.125	15.63	181665.8
4^3	25.93 (±0.07)	3	100	7.81×10^{-1}	3.91	45416.4
4^4	27.91 (±0.06)	3	100	1.95×10^{-1}	9.76×10^{-1}	11354.1
4^5	29.99 (±0.03)	3	100	4.88×10^{-2}	2.44×10^{-1}	2838.5
4^6	32.07 (±0.06)	3	100	1.22×10^{-2}	6.10×10^{-2}	709.6
4^7	34.44 (±0.18)	3	100	3.05×10^{-3}	1.52×10^{-2}	177.4
4^8	36.43 (±0.40)	3	100	7.63×10^{-4}	3.81×10^{-3}	44.4
4^9	38.58 (±0.56)	20	100	1.91×10^{-4}	9.54×10^{-4}	11.1
4^10	40.67 (±1.22)	20	100	4.77×10^{-5}	2.38×10^{-4}	2.8
4^11	41.68 (±0.69)	20	50	0.19×10^{-5}	5.96×10^{-5}	0.7
4^12	41.89 (±0.40)	20	20	0.47×10^{-6}	1.49×10^{-5}	0.2
AA - Noble crayfish						
4^1	18.00 (±0.07)	6	100	12.5	62.5	1807745.0
4^2	09.95 (±0.06)	8	100	3.125	15.63	451936.3
4^3	22.01 (±0.04)	8	100	7.81×10^{-1}	3.91	112984.1
4^4	24.08 (±0.07)	8	100	1.95×10^{-1}	9.76×10^{-1}	28246.0
4^5	26.11 (±0.05)	8	100	4.88×10^{-2}	2.44×10^{-1}	7061.5
4^6	28.09 (±0.07)	8	100	1.22×10^{-2}	6.10×10^{-2}	1765.4
4^7	30.13 (±0.08)	8	100	3.05×10^{-3}	1.52×10^{-2}	441.3
4^8	32.14 (±0.17)	8	100	7.63×10^{-4}	3.81×10^{-3}	110.3
4^9	34.37 (±0.30)	20	100	1.91×10^{-4}	9.54×10^{-4}	27.6
4^10	36.68 (±0.60)	20	100	4.77×10^{-5}	2.38×10^{-4}	6.9
4^11	38.83 (±1.01)	20	100	0.19×10^{-5}	5.96×10^{-5}	1.7
4^12	39.71 (±0.56)	20	35	0.47×10^{-6}	1.49×10^{-5}	0.4
4^13	39.96 (±0.03)	20	20	0.47×10^{-6}	3.73×10^{-6}	0.1
PL - Signal crayfish						
4^1	19.54 (±0.14)	3	100	12.5	62.5	2143289.3
4^2	21.69 (±0.11)	3	100	3.125	15.63	535822.3
4^3	23.65 (±0.03)	3	100	7.81×10^{-1}	3.91	133955.6
4^4	25.65 (±0.11)	3	100	1.95×10^{-1}	9.76×10^{-1}	33488.9
4^5	27.67 (±0.02)	3	100	4.88×10^{-2}	2.44×10^{-1}	8372.2
4^6	29.69 (±0.11)	3	100	1.22×10^{-2}	6.10×10^{-2}	2093.1
4^7	31.81 (±0.05)	3	100	3.05×10^{-3}	1.52×10^{-2}	523.3
4^8	33.91 (±0.15)	3	100	7.63×10^{-4}	3.81×10^{-3}	130.8
4^9	36.44 (±0.37)	20	100	1.91×10^{-4}	9.54×10^{-4}	32.7
4^10	38.10 (±0.37)	20	100	4.77×10^{-5}	2.38×10^{-4}	8.2
4^11	39.87 (±1.13)	20	90	0.19×10^{-5}	5.96×10^{-5}	2.0
4^12	41.13 (±0.75)	20	40	0.47×10^{-6}	1.49×10^{-5}	0.5

FI - Spiny cheek crayfish						
4^1	19.81 (±0.05)	3	100	12.5	62.5	275251.2
4^2	21.83 (±0.06)	11	100	3.125	15.63	68812.8
4^3	23.80 (±0.06)	11	100	7.81×10^{-1}	3.91	17203.2
4^4	25.91 (±0.27)	11	100	1.95×10^{-1}	9.76×10^{-1}	4300.8
4^5	27.85 (±0.11)	11	100	4.88×10^{-2}	2.44×10^{-1}	1075.2
4^6	29.94 (±0.13)	3	100	1.22×10^{-2}	6.10×10^{-2}	268.8
4^7	31.89 (±0.08)	3	100	3.05×10^{-3}	1.52×10^{-2}	67.2
4^8	34.15 (±0.41)	20	100	7.63×10^{-4}	3.81×10^{-3}	16.8
4^9	36.27 (±0.66)	20	100	1.91×10^{-4}	9.54×10^{-4}	4.2
4^10	38.00 (±0.87)	20	65	4.77×10^{-5}	2.38×10^{-4}	1.1
4^11	38.71 (±0.95)	20	40	1.19×10^{-5}	5.96×10^{-5}	0.3

[a] In total of 11-13 standard dilutions were made from a four-fold dilution series where the stock solutions contained 50 ng/ µL genomic DNA from marbled crayfish. noble. signal crayfish and spiny cheek crayfish. A standard curve was established from several calibrant points (standard dilutions from 4^3 - 4^9). cf. Fig 3a-b.

[b] Mean Ct-values are based on the qPCR replicates of each standard.

[c] The standards from the 1rd to the 8th dilution were run in 3 replicates. while the 9th - 11th dilution in 20. to get a larger sample for positive/negative ratio.

[d] The percentage of RT-PCR replicates yielding positive results (detection) for each standard.

[e] Theoretical content of DNA in ng/ µL for each standard calculated from the concentration assigned to the DNA stock (50 ng/ml).

[f] Quantity of template DNA in each qPCR replicate in (2 µL template multiplied by assigned concentration per µL).

[g] Number of detected DNA copies in each PCR replicate (2 µL template DNA) estimated on the basis of application of single molecule quantification (SIMQUANT; Berdal et al. 2008).

Supplementary Table S4. Overview of the qPCR results and eDNA copy number estimations.

Respective Ct values and eDNA copies (undiluted and 10x dilution) are stated for each target on each filter. Volume of filtered water is stated in litres. The potential effect of qPCR inhibition was controlled by calculating the difference in Ct values (ΔCt) between the undiluted and corresponding 10-fold diluted DNA replicates as described in Kozubíková et al. (2011) and Agersnap et al. (2017). In case of inhibition (if ΔCt <2.82), the estimated eDNA copy number was based on the 10-fold diluted DNA replicates alone, while if ΔCt > 3.82 (i.e. 10-fold dilution out of range), the estimation of eDNA copy number was based solely on the undiluted DNA replicates. If none or only one of the relevant replicates were detected above LOQ, further quantification was not performed. If detection was below LOQ, inhibition was not calculated.

			Aphanomyces astaci						
Location	**Subsample**	**Vol (L)**	**Ct 1x**	**Ct 10x**	**eDNA copies 1x**	**eDNA copies 10x**	**ΔCt**	**eDNA copies (A+B PCR)**	**eDNA copies/L**
1	A	2	37,21	40,61	35,5	3,72		<LOQ	
1	B	2	37,95	No Ct	21,69	No Ct		<LOQ	
1	C	2	40,49	40,51	2,96	2,92		<LOQ	
1	D	2	40,65	40,64	2,66	2,67		<LOQ	
2	A	1	No Ct	No Ct	No Ct	No Ct			
2	B	1	No Ct	No Ct	No Ct	No Ct			
2	C	1,2	No Ct	No Ct	No Ct	No Ct			
2	D	1,2	No Ct	No Ct	No Ct	No Ct			
3	A	0,7	No Ct	No Ct	No Ct	No Ct			
3	B	0,65	No Ct	No Ct	No Ct	No Ct			
3	C	0,7	No Ct	No Ct	No Ct	No Ct			
3	D	0,65	No Ct	No Ct	No Ct	No Ct			
4	A	0,9	No Ct	No Ct	No Ct	No Ct			
4	B	0,9	No Ct	No Ct	No Ct	No Ct			
4	C	1	No Ct	No Ct	No Ct	No Ct			
4	D	1	No Ct	No Ct	No Ct	No Ct			
5	A	5	No Ct	No Ct	No Ct	No Ct			
5	B	5	No Ct	No Ct	No Ct	No Ct			
5	C	5	No Ct	No Ct	No Ct	No Ct			
5	D	5	No Ct	No Ct	No Ct	No Ct			
6	A	0,3	No Ct	No Ct	No Ct	No Ct			
6	B	0,3	No Ct	No Ct	No Ct	No Ct			
6	C	0,4	No Ct	No Ct	No Ct	No Ct			
6	D	0,4	No Ct	No Ct	No Ct	No Ct			
7	A	1,3	No Ct	No Ct	No Ct	No Ct			
7	B	1,3	No Ct	No Ct	No Ct	No Ct			
7	C	1,4	No Ct	No Ct	No Ct	No Ct			
7	D	1,4	No Ct	No Ct	No Ct	No Ct			
8	A	0,75	No Ct	No Ct	No Ct	No Ct			
8	B	0,75	No Ct	No Ct	No Ct	No Ct			
8	C	0,75	No Ct	No Ct	No Ct	No Ct			
8	D	0,75	No Ct	No Ct	No Ct	No Ct			
9	A	2,3	No Ct	No Ct	No Ct	No Ct			
9	B	2,3	No Ct	No Ct	No Ct	No Ct			
9	C	2,1	No Ct	No Ct	No Ct	No Ct			
9	D	2,1	No Ct	No Ct	No Ct	No Ct			
10	A	5	No Ct	No Ct	No Ct	No Ct			
10	B	5	No Ct	No Ct	No Ct	No Ct			
10	C	5	No Ct	No Ct	No Ct	No Ct			
10	D	5	No Ct	No Ct	No Ct	No Ct			
11	A	1,8	No Ct	No Ct	No Ct	No Ct			
11	B	1,8	No Ct	No Ct	No Ct	No Ct			
11	C	2	No Ct	No Ct	No Ct	No Ct			

11	D	2	No Ct	No Ct	No Ct	No Ct			
12	A	0,85	No Ct	No Ct	No Ct	No Ct			
12	B	0,85	No Ct	No Ct	No Ct	No Ct			
12	C	1	No Ct	No Ct	No Ct	No Ct			
12	D	1	No Ct	No Ct	No Ct	No Ct			
13	A	5	33,93	37,17	310	36,39	3,24	6200,00	2870,40
13	B	5	33,52	37,61	407,6	27,16	4,09	8152,00	
13	C	5	33,41	36,65	382,7	41,18	3,24	7654,00	3057,20
13	D	5	33,41	37,46	381,6	23,63	4,05	7632,00	
14	A	0,8	38,34	No Ct	16,75	No Ct		<LOQ	
14	B	0,8	37,8	No Ct	23,97	No Ct		<LOQ	
14	C	0,8	No Ct	No Ct	No Ct	No Ct			
14	D	0,8	No Ct	No Ct	No Ct	No Ct			
15	A	0,6	37,54	41,44	28,43	2,16		<LOQ	
15	B	0,6	37,46	39,94	30,05	5,82		<LOQ	
15	C	0,6	36,7	39,1	39,81	7,70		<LOQ	
15	D	0,6	36,85	39,49	36,04	5,89		<LOQ	
16	A	1,1	No Ct	No Ct	No Ct	No Ct			
16	B	1,1	No Ct	No Ct	No Ct	No Ct			
16	C	1,2	No Ct	No Ct	No Ct	No Ct			
16	D	1,2	No Ct	No Ct	No Ct	No Ct			
17	A	2	No Ct	No Ct	No Ct	No Ct			
17	B	2	No Ct	No Ct	No Ct	No Ct			
17	C	2	No Ct	No Ct	No Ct	No Ct			
17	D	2	No Ct	No Ct	No Ct	No Ct			
18	A	5	No Ct	No Ct	No Ct	No Ct			
18	B	5	No Ct	No Ct	No Ct	No Ct			
18	C	5	No Ct	No Ct	No Ct	No Ct			
18	D	5	No Ct	No Ct	No Ct	No Ct			
19	A	2	No Ct	No Ct	No Ct	No Ct			
19	B	2	No Ct	No Ct	No Ct	No Ct			
19	C	2,1	No Ct	No Ct	No Ct	No Ct			
19	D	2,1	No Ct	No Ct	No Ct	No Ct			
20	A	1,8	No Ct	No Ct	No Ct	No Ct			
20	B	1,8	No Ct	No Ct	No Ct	No Ct			
20	C	3,3	No Ct	No Ct	No Ct	No Ct			
20	D	3,3	No Ct	No Ct	No Ct	No Ct			
21	A	0,45	No Ct	No Ct	No Ct	No Ct			
21	B	0,45	No Ct	No Ct	No Ct	No Ct			
21	C	0,4	No Ct	No Ct	No Ct	No Ct			
21	D	0,4	No Ct	No Ct	No Ct	No Ct			
22	A	2,2	No Ct	No Ct	No Ct	No Ct			
22	B	2,2	No Ct	No Ct	No Ct	No Ct			
22	C	2,2	No Ct	No Ct	No Ct	No Ct			
22	D	2,2	No Ct	No Ct	No Ct	No Ct			
23	A	1,5	No Ct	No Ct	No Ct	No Ct			
23	B	1,5	No Ct	No Ct	No Ct	No Ct			
23	C	1,5	No Ct	No Ct	No Ct	No Ct			
23	D	1,5	No Ct	No Ct	No Ct	No Ct			
24	A	5	No Ct	No Ct	No Ct	No Ct			
24	B	5	No Ct	No Ct	No Ct	No Ct			
24	C	5	No Ct	No Ct	No Ct	No Ct			
24	D	5	No Ct	No Ct	No Ct	No Ct			
25	A	1,6	No Ct	No Ct	No Ct	No Ct			
25	B	1,6	No Ct	No Ct	No Ct	No Ct			
25	C	1,6	No Ct	No Ct	No Ct	No Ct			
25	D	1,6	No Ct	No Ct	No Ct	No Ct			

26	A	5	No Ct	No Ct	No Ct	No Ct			
26	B	5	No Ct	No Ct	No Ct	No Ct			
26	C	5	No Ct	No Ct	No Ct	No Ct			
26	D	5	No Ct	No Ct	No Ct	No Ct			
27	A	5	No Ct	No Ct	No Ct	No Ct			
27	B	5	No Ct	No Ct	No Ct	No Ct			
27	C	5	No Ct	No Ct	No Ct	No Ct			
27	D	5	No Ct	No Ct	No Ct	No Ct			
28	A	1	No Ct	No Ct	No Ct	No Ct			
28	B	1	No Ct	No Ct	No Ct	No Ct			
28	C	1	No Ct	No Ct	No Ct	No Ct			
28	D	1	No Ct	No Ct	No Ct	No Ct			
29	A	5	No Ct	No Ct	No Ct	No Ct			
29	B	5	No Ct	No Ct	No Ct	No Ct			
29	C	4	No Ct	No Ct	No Ct	No Ct			
29	D	4	No Ct	No Ct	No Ct	No Ct			
30	A	5	44,62	45	0,08	0,06		cut off	
30	B	5	41,62	43,04	0,56	0,23		cut off	
30	C	5	40,13	43,95	1,46	0,13		<LOQ	
30	D	5	40,22	No Ct	1,38	No Ct		<LOQ	
31	A	5	32,86	36,09	112,30	12,62	3,23	2246,00	907,20
31	B	5	32,83	35,91	114,50	14,19	3,08	2290,00	
31	C	5	30,55	33,87	535,60	56,63	3,32	10712,00	10790,40
31	D	5	31,24	31,89	336,30	216,20	0,65	43240,00	
32	A	5	29,62	32,42	1004,00	151,20	2,80	30240,00	12052,00
32	B	5	30,23	32,43	665,00	150,10	2,20	30020,00	
32	C	5	29,89	32,93	835,50	107,00	3,04	16710,00	11806,00
32	D	5	30,23	31,92	664,50	211,60	1,69	42320,00	

			Astacus astacus						
Location	**Subsample**	**Vol (L)**	**Ct 1x**	**Ct 10x**	**eDNA copies 1x**	**eDNA copies 10x**	**ΔCt**	**eDNA copies (A+B PCR)**	**eDNA copies/L**
1	A	2	No Ct	No Ct	No Ct	No Ct			
1	B	2	No Ct	No Ct	No Ct	No Ct			
1	C	2	No Ct	No Ct	No Ct	No Ct			
1	D	2	No Ct	No Ct	No Ct	No Ct			
2	A	1	No Ct	No Ct	No Ct	No Ct			
2	B	1	No Ct	No Ct	No Ct	No Ct			
2	C	1,2	No Ct	No Ct	No Ct	No Ct			
2	D	1,2	No Ct	No Ct	No Ct	No Ct			
3	A	0,7	No Ct	No Ct	No Ct	No Ct			
3	B	0,65	No Ct	No Ct	No Ct	No Ct			
3	C	0,7	No Ct	No Ct	No Ct	No Ct			
3	D	0,65	No Ct	No Ct	No Ct	No Ct			
4	A	0,9	No Ct	No Ct	No Ct	No Ct			
4	B	0,9	No Ct	No Ct	No Ct	No Ct			
4	C	1	No Ct	No Ct	No Ct	No Ct			
4	D	1	No Ct	No Ct	No Ct	No Ct			
5	A	5	No Ct	No Ct	No Ct	No Ct			
5	B	5	No Ct	No Ct	No Ct	No Ct			
5	C	5	No Ct	No Ct	No Ct	No Ct			
5	D	5	No Ct	No Ct	No Ct	No Ct			
6	A	0,3	No Ct	No Ct	No Ct	No Ct			
6	B	0,3	No Ct	No Ct	No Ct	No Ct			
6	C	0,4	No Ct	No Ct	No Ct	No Ct			
6	D	0,4	No Ct	No Ct	No Ct	No Ct			
7	A	1,3	37,71	No Ct	5,09	No Ct		<LOQ	

7	B	1,3	38,64	No Ct	2,7	No Ct		<LOQ	
7	C	1,4	No Ct	No Ct	No Ct	No Ct			
7	D	1,4	No Ct	No Ct	No Ct	No Ct			
8	A	0,75	34,5	No Ct	45,57	No Ct		911,40	2788,53
8	B	0,75	34,79	37,49	37,26	5,90	2,70	1180,00	
8	C	0,75	34,95	39,65	32,62	1,29	4,70	652,40	1635,73
8	D	0,75	35,14	38,2	28,72	3,49	3,06	574,40	
9	A	2,3	29,86	33,28	1077	104,70	3,42	21540,00	18495,65
9	B	2,3	29,9	32,94	1050	131,60	3,04	21000,00	
9	C	2,1	29,42	32,65	1472	159,30	3,23	29440,00	27019,05
9	D	2,1	29,53	33,01	1365	124,30	3,48	27300,00	
10	A	5	39,74	No Ct	1,28	No Ct		<LOQ	
10	B	5	39,84	No Ct	1,19	No Ct		<LOQ	
10	C	5	No Ct	No Ct	No Ct	No Ct			
10	D	5	No Ct	No Ct	No Ct	No Ct			
11	A	1,8	No Ct	No Ct	No Ct	No Ct			
11	B	1,8	No Ct	No Ct	No Ct	No Ct			
11	C	2	No Ct	No Ct	No Ct	No Ct			
11	D	2	No Ct	No Ct	No Ct	No Ct			
12	A	0,85	No Ct	No Ct	No Ct	No Ct			
12	B	0,85	No Ct	No Ct	No Ct	No Ct			
12	C	1	No Ct	No Ct	No Ct	No Ct			
12	D	1	No Ct	No Ct	No Ct	No Ct			
13	A	5	No Ct	No Ct	No Ct	No Ct			
13	B	5	No Ct	No Ct	No Ct	No Ct			
13	C	5	No Ct	No Ct	No Ct	No Ct			
13	D	5	No Ct	No Ct	No Ct	No Ct			
14	A	0,8	No Ct	No Ct	No Ct	No Ct			
14	B	0,8	No Ct	No Ct	No Ct	No Ct			
14	C	0,8	No Ct	No Ct	No Ct	No Ct			
14	D	0,8	No Ct	No Ct	No Ct	No Ct			
15	A	0,6	No Ct	No Ct	No Ct	No Ct			
15	B	0,6	No Ct	No Ct	No Ct	No Ct			
15	C	0,6	No Ct	No Ct	No Ct	No Ct			
15	D	0,6	No Ct	No Ct	No Ct	No Ct			
16	A	1,1	34,62	38,18	41,84	3,70	3,56	836,80	1664,00
16	B	1,1	34,37	37,79	49,68	4,83	3,42	993,60	
16	C	1,2	33,77	37,29	73,57	6,55	3,52	1471,40	2699,67
16	D	1,2	33,5	36,59	88,41	10,59	3,09	1768,20	
17	A	2	32,61	36,72	164,5	9,97	4,11	3290,00	3205,00
17	B	2	32,69	36,63	156	10,61	3,94	3120,00	
17	C	2	32,34	35,32	197,1	25,41	2,98	3942,00	3599,00
17	D	2	32,62	35,93	162,8	16,60	3,31	3256,00	
18	A	5	No Ct	No Ct	No Ct	No Ct			
18	B	5	No Ct	No Ct	No Ct	No Ct			
18	C	5	No Ct	No Ct	No Ct	No Ct			
18	D	5	No Ct	No Ct	No Ct	No Ct			
19	A	2	No Ct	No Ct	No Ct	No Ct			
19	B	2	No Ct	No Ct	No Ct	No Ct			
19	C	2,1	No Ct	No Ct	No Ct	No Ct			
19	D	2,1	No Ct	No Ct	No Ct	No Ct			
20	A	1,8	No Ct	No Ct	No Ct	No Ct			
20	B	1,8	No Ct	No Ct	No Ct	No Ct			
20	C	3,3	No Ct	No Ct	No Ct	No Ct			
20	D	3,3	39,93	No Ct	0,9757	No Ct		<LOQ	
21	A	0,45	39,79	No Ct	1,08	No Ct		<LOQ	
21	B	0,45	37,66	No Ct	4,68	No Ct		<LOQ	

21	C	0,4	36,68	38,71	9,23	2,26		<LOQ	
21	D	0,4	36,5	38,7	10,29	2,28	2,18	102,90	
22	A	2,2	No Ct	No Ct	No Ct	No Ct			
22	B	2,2	No Ct	No Ct	No Ct	No Ct			
22	C	2,2	39,07	No Ct	1,76	No Ct		<LOQ	
22	D	2,2	No Ct	No Ct	No Ct	No Ct			
23	A	1,5	No Ct	No Ct	No Ct	No Ct			
23	B	1,5	No Ct	No Ct	No Ct	No Ct			
23	C	1,5	No Ct	No Ct	No Ct	No Ct			
23	D	1,5	No Ct	No Ct	No Ct	No Ct			
24	A	5	No Ct	No Ct	No Ct	No Ct			
24	B	5	No Ct	No Ct	No Ct	No Ct			
24	C	5	No Ct	No Ct	No Ct	No Ct			
24	D	5	38,72	No Ct	2,25	No Ct		<LOQ	
25	A	1,6	No Ct	No Ct	No Ct	No Ct			
25	B	1,6	No Ct	No Ct	No Ct	No Ct			
25	C	1,6	No Ct	No Ct	No Ct	No Ct			
25	D	1,6	No Ct	No Ct	No Ct	No Ct			
26	A	5	No Ct	No Ct	No Ct	No Ct			
26	B	5	No Ct	No Ct	No Ct	No Ct			
26	C	5	No Ct	No Ct	No Ct	No Ct			
26	D	5	No Ct	No Ct	No Ct	No Ct			
27	A	5	No Ct	No Ct	No Ct	No Ct			
27	B	5	No Ct	No Ct	No Ct	No Ct			
27	C	5	No Ct	No Ct	No Ct	No Ct			
27	D	5	No Ct	No Ct	No Ct	No Ct			
28	A	1	No Ct	No Ct	No Ct	No Ct			
28	B	1	No Ct	No Ct	No Ct	No Ct			
28	C	1	No Ct	No Ct	No Ct	No Ct			
28	D	1	No Ct	No Ct	No Ct	No Ct			
29	A	5	No Ct	No Ct	No Ct	No Ct			
29	B	5	No Ct	No Ct	No Ct	No Ct			
29	C	4	No Ct	No Ct	No Ct	No Ct			
29	D	4	No Ct	No Ct	No Ct	No Ct			
30	A	5	No Ct	No Ct	No Ct	No Ct			
30	B	5	No Ct	No Ct	No Ct	No Ct			
30	C	5	No Ct	No Ct	No Ct	No Ct			
30	D	5	No Ct	No Ct	No Ct	No Ct			
31	A	5	No Ct	No Ct	No Ct	No Ct			
31	B	5	No Ct	No Ct	No Ct	No Ct			
31	C	5	No Ct	No Ct	No Ct	No Ct			
31	D	5	No Ct	No Ct	No Ct	No Ct			
32	A	5	No Ct	No Ct	No Ct	No Ct			
32	B	5	No Ct	No Ct	No Ct	No Ct			
32	C	5	No Ct	No Ct	No Ct	No Ct			
32	D	5	No Ct	No Ct	No Ct	No Ct			

			Pacifastacus leniusculus						
Location	**Subsample**	**Vol (L)**	**Ct 1x**	**Ct 10x**	**eDNA copies 1x**	**eDNA copies 10x**	**ΔCt**	**eDNA copies (A+B PCR)**	**eDNA copies/L**
1	A	2	No Ct	No Ct	No Ct	No Ct			
1	B	2	No Ct	No Ct	No Ct	No Ct			
1	C	2	No Ct	No Ct	No Ct	No Ct			
1	D	2	No Ct	No Ct	No Ct	No Ct			
2	A	1	No Ct	No Ct	No Ct	No Ct			
2	B	1	No Ct	No Ct	No Ct	No Ct			
2	C	1,2	No Ct	No Ct	No Ct	No Ct			

2	D	1,2	No Ct	No Ct	No Ct	No Ct			
3	A	0,7	No Ct	No Ct	No Ct	No Ct			
3	B	0,65	No Ct	No Ct	No Ct	No Ct			
3	C	0,7	No Ct	No Ct	No Ct	No Ct			
3	D	0,65	No Ct	No Ct	No Ct	No Ct			
4	A	0,9	No Ct	No Ct	No Ct	No Ct			
4	B	0,9	No Ct	No Ct	No Ct	No Ct			
4	C	1	No Ct	No Ct	No Ct	No Ct			
4	D	1	No Ct	No Ct	No Ct	No Ct			
5	A	5	No Ct	No Ct	No Ct	No Ct			
5	B	5	No Ct	No Ct	No Ct	No Ct			
5	C	5	No Ct	No Ct	No Ct	No Ct			
5	D	5	No Ct	No Ct	No Ct	No Ct			
6	A	0,3	No Ct	No Ct	No Ct	No Ct			
6	B	0,3	No Ct	No Ct	No Ct	No Ct			
6	C	0,4	No Ct	No Ct	No Ct	No Ct			
6	D	0,4	No Ct	No Ct	No Ct	No Ct			
7	A	1,3	No Ct	No Ct	No Ct	No Ct			
7	B	1,3	No Ct	No Ct	No Ct	No Ct			
7	C	1,4	No Ct	No Ct	No Ct	No Ct			
7	D	1,4	No Ct	No Ct	No Ct	No Ct			
8	A	0,75	No Ct	No Ct	No Ct	No Ct			
8	B	0,75	No Ct	No Ct	No Ct	No Ct			
8	C	0,75	No Ct	No Ct	No Ct	No Ct			
8	D	0,75	No Ct	No Ct	No Ct	No Ct			
9	A	2,3	No Ct	No Ct	No Ct	No Ct			
9	B	2,3	No Ct	No Ct	No Ct	No Ct			
9	C	2,1	No Ct	No Ct	No Ct	No Ct			
9	D	2,1	No Ct	No Ct	No Ct	No Ct			
10	A	5	No Ct	No Ct	No Ct	No Ct			
10	B	5	No Ct	No Ct	No Ct	No Ct			
10	C	5	No Ct	No Ct	No Ct	No Ct			
10	D	5	No Ct	No Ct	No Ct	No Ct			
11	A	1,8	No Ct	No Ct	No Ct	No Ct			
11	B	1,8	No Ct	No Ct	No Ct	No Ct			
11	C	2	No Ct	No Ct	No Ct	No Ct			
11	D	2	No Ct	No Ct	No Ct	No Ct			
12	A	0,85	No Ct	No Ct	No Ct	No Ct			
12	B	0,85	No Ct	No Ct	No Ct	No Ct			
12	C	1	No Ct	No Ct	No Ct	No Ct			
12	D	1	No Ct	No Ct	No Ct	No Ct			
13	A	5	31,27	34,94	221,7	16,09	3,67	4434,00	1948,80
13	B	5	31,02	34,21	265,5	27,21	3,19	5310,00	
13	C	5	31,17	34,25	65,96	4,29	3,08	1319,20	595,32
13	D	5	30,91	34,06	82,87	5,09	3,15	1657,40	
14	A	0,8	No Ct	No Ct	No Ct	No Ct			
14	B	0,8	No Ct	No Ct	No Ct	No Ct			
14	C	0,8	No Ct	No Ct	No Ct	No Ct			
14	D	0,8	No Ct	No Ct	No Ct	No Ct			
15	A	0,6	28,47	31,36	1640	207,50	2,89	32800,00	101233,33
15	B	0,6	28,7	31,77	1397	155,10	3,07	27940,00	
15	C	0,6	28,94	32,25	476,2	25,20	3,31	9524,00	33020,00
15	D	0,6	28,85	31,88	514,4	34,97	3,03	10288,00	
16	A	1,1	30,28	33,78	448,5	36,87	3,50	8970,00	15880,00
16	B	1,1	30,36	33,49	424,9	45,53	3,13	8498,00	
16	C	1,2	29,56	32,77	273,6	15,93	3,21	5472,00	7991,67
16	D	1,2	29,88	33,16	205,9	11,30	3,28	4118,00	

17	A	2	No Ct	No Ct	No Ct	No Ct			
17	B	2	No Ct	No Ct	No Ct	No Ct			
17	C	2	No Ct	No Ct	No Ct	No Ct			
17	D	2	No Ct	No Ct	No Ct	No Ct			
18	A	5	29,68	32,52	693,5	90,99	2,84	13870,00	5076,00
18	B	5	29,94	33,15	575,5	57,77	3,21	11510,00	
18	C	5	28,86	31,89	511,7	34,70	3,03	10234,00	4110,00
18	D	5	28,85	32,09	515,8	29,11	3,24	10316,00	
19	A	2	32,07	34,9	125,1	16,58	2,83	2502,00	2668,00
19	B	2	31,9	36,21	141,7	6,48	4,31	2834,00	
19	C	2,1	31,37	34,49	55,28	3,48	3,12	1105,60	1058,57
19	D	2,1	31,36	34,33	55,87	4,02	2,97	1117,40	
20	A	1,8	28,99	32,39	1193	99,51	3,40	23860,00	26611,11
20	B	1,8	28,97	31,92	1202	140,00	2,95	24040,00	
20	C	3,3	28,36	31,59	1885	178,90	3,23	37700,00	24151,52
20	D	3,3	28,21	31,54	2100	185,60	3,33	42000,00	
21	A	0,45	No Ct	No Ct	No Ct	No Ct			
21	B	0,45	No Ct	No Ct	No Ct	No Ct			
21	C	0,4	No Ct	No Ct	No Ct	No Ct			
21	D	0,4	No Ct	No Ct	No Ct	No Ct			
22	A	2,2	29,24	32,19	992,1	115,20	2,95	19842,00	18655,45
22	B	2,2	29,15	32,57	1060	87,43	3,42	21200,00	
22	C	2,2	29,4	32,58	878,8	87,04	3,18	17576,00	14898,18
22	D	2,2	29,6	32,75	760	76,49	3,15	15200,00	
23	A	1,5	28,08	31,26	2305	226,60	3,18	46100,00	60080,00
23	B	1,5	28,14	31,2	2201	237,30	3,06	44020,00	
23	C	1,5	28,51	31,68	1685	167,20	3,17	33700,00	48573,33
23	D	1,5	28,31	31,55	1958	183,50	3,24	39160,00	
24	A	5	No Ct	No Ct	No Ct	No Ct			
24	B	5	No Ct	No Ct	No Ct	No Ct			
24	C	5	No Ct	No Ct	No Ct	No Ct			
24	D	5	No Ct	No Ct	No Ct	No Ct			
25	A	1,6	No Ct	No Ct	No Ct	No Ct			
25	B	1,6	No Ct	No Ct	No Ct	No Ct			
25	C	1,6	No Ct	No Ct	No Ct	No Ct			
25	D	1,6	No Ct	No Ct	No Ct	No Ct			
26	A	5	No Ct	No Ct	No Ct	No Ct			
26	B	5	No Ct	No Ct	No Ct	No Ct			
26	C	5	No Ct	No Ct	No Ct	No Ct			
26	D	5	No Ct	No Ct	No Ct	No Ct			
27	A	5	No Ct	No Ct	No Ct	No Ct			
27	B	5	No Ct	No Ct	No Ct	No Ct			
27	C	5	No Ct	No Ct	No Ct	No Ct			
27	D	5	No Ct	No Ct	No Ct	No Ct			
28	A	1	No Ct	No Ct	No Ct	No Ct			
28	B	1	No Ct	No Ct	No Ct	No Ct			
28	C	1	No Ct	No Ct	No Ct	No Ct			
28	D	1	No Ct	No Ct	No Ct	No Ct			
29	A	5	No Ct	No Ct	No Ct	No Ct			
29	B	5	No Ct	No Ct	No Ct	No Ct			
29	C	4	No Ct	No Ct	No Ct	No Ct			
29	D	4	No Ct	No Ct	No Ct	No Ct			
30	A	5	No Ct	No Ct	No Ct	No Ct			
30	B	5	No Ct	No Ct	No Ct	No Ct			
30	C	5	No Ct	No Ct	No Ct	No Ct			
30	D	5	No Ct	No Ct	No Ct	No Ct			
31	A	5	No Ct	No Ct	No Ct	No Ct			

31	B	5	No Ct	No Ct	No Ct	No Ct			
31	C	5	No Ct	No Ct	No Ct	No Ct			
31	D	5	No Ct	No Ct	No Ct	No Ct			
32	A	5	No Ct	No Ct	No Ct	No Ct			
32	B	5	No Ct	No Ct	No Ct	No Ct			
32	C	5	No Ct	No Ct	No Ct	No Ct			
32	D	5	No Ct	No Ct	No Ct	No Ct			

			Faxonius limosus						
Location	**Subsample**	**Vol (L)**	**Ct 1x**	**Ct 10x**	**eDNA copies 1x**	**eDNA copies 10x**	**ΔCt**	**eDNA copies (A+B PCR)**	**eDNA copies/L**
1	A	2	No Ct	No Ct	No Ct	No Ct			
1	B	2	38,47	No Ct	0,68	No Ct		<LOQ	
1	C	2	No Ct	No Ct	No Ct	No Ct			
1	D	2	No Ct	No Ct	No Ct	No Ct			
2	A	1	No Ct	No Ct	No Ct	No Ct			
2	B	1	No Ct	No Ct	No Ct	No Ct			
2	C	1,2	No Ct	No Ct	No Ct	No Ct			
2	D	1,2	No Ct	No Ct	No Ct	No Ct			
3	A	0,7	No Ct	No Ct	No Ct	No Ct			
3	B	0,65	No Ct	No Ct	No Ct	No Ct			
3	C	0,7	No Ct	No Ct	No Ct	No Ct			
3	D	0,65	No Ct	No Ct	No Ct	No Ct			
4	A	0,9	39,85	No Ct	0,26	No Ct		<LOQ	
4	B	0,9	No Ct	No Ct	No Ct	No Ct			
4	C	1	38,85	39,35	0,52	0,37		<LOQ	
4	D	1	39,56	No Ct	0,32	No Ct		<LOQ	
5	A	5	38,4	No Ct	0,71	No Ct		<LOQ	
5	B	5	39,35	No Ct	0,37	No Ct		<LOQ	
5	C	5	38,17	No Ct	0,83	No Ct		<LOQ	
5	D	5	39,31	No Ct	0,38	No Ct		<LOQ	
6	A	0,3	No Ct	No Ct	No Ct	No Ct			
6	B	0,3	No Ct	No Ct	No Ct	No Ct			
6	C	0,4	No Ct	No Ct	No Ct	No Ct			
6	D	0,4	No Ct	No Ct	No Ct	No Ct			
7	A	1,3	34,65	36,42	9,55	2,79		<LOQ	
7	B	1,3	33,79	37,36	17,32	1,46	3,57	346,40	
7	C	1,4	32,68	35,95	37,26	3,86	3,27	745,20	1044,86
7	D	1,4	32,74	35,65	35,88	4,75	2,91	717,60	
8	A	0,75	No Ct	No Ct	No Ct	No Ct			
8	B	0,75	No Ct	No Ct	No Ct	No Ct			
8	C	0,75	No Ct	No Ct	No Ct	No Ct			
8	D	0,75	No Ct	No Ct	No Ct	No Ct			
9	A	2,3	No Ct	No Ct	No Ct	No Ct			
9	B	2,3	No Ct	No Ct	No Ct	No Ct			
9	C	2,1	No Ct	No Ct	No Ct	No Ct			
9	D	2,1	No Ct	No Ct	No Ct	No Ct			
10	A	5	35,66	37,03	4,72	1,83		<LOQ	
10	B	5	35,41	37,62	5,63	1,22		<LOQ	
10	C	5	35,48	No Ct	5,34	No Ct		<LOQ	
10	D	5	35,57	No Ct	5,03	No Ct		<LOQ	
11	A	1,8	35,04	37,42	7,29	1,39		<LOQ	
11	B	1,8	34,77	39,3	8,77	0,38		<LOQ	
11	C	2	35,63	38,83	4,83	0,53		<LOQ	
11	D	2	35,09	38,26	7,01	0,78		<LOQ	
12	A	0,85	No Ct	No Ct	No Ct	No Ct			
12	B	0,85	No Ct	No Ct	No Ct	No Ct			

12	C	1	No Ct	No Ct	No Ct	No Ct			
12	D	1	No Ct	No Ct	No Ct	No Ct			
13	A	5	No Ct	No Ct	No Ct	No Ct			
13	B	5	No Ct	No Ct	No Ct	No Ct			
13	C	5	No Ct	No Ct	No Ct	No Ct			
13	D	5	No Ct	No Ct	No Ct	No Ct			
14	A	0,8	34,46	39,25	12,05	0,45	4,79	241,00	788,75
14	B	0,8	34,68	37,11	10,36	1,95	2,43	390,00	
14	C	0,8	34,61	39,17	10,87	0,48	4,56	217,40	
14	D	0,8	34,8	37,15	9,5	1,90		<LOQ	
15	A	0,6	No Ct	No Ct	No Ct	No Ct			
15	B	0,6	No Ct	No Ct	No Ct	No Ct			
15	C	0,6	No Ct	No Ct	No Ct	No Ct			
15	D	0,6	No Ct	No Ct	No Ct	No Ct			
16	A	1,1	No Ct	No Ct	No Ct	No Ct			
16	B	1,1	No Ct	No Ct	No Ct	No Ct			
16	C	1,2	No Ct	No Ct	No Ct	No Ct			
16	D	1,2	No Ct	No Ct	No Ct	No Ct			
17	A	2	No Ct	No Ct	No Ct	No Ct			
17	B	2	No Ct	No Ct	No Ct	No Ct			
17	C	2	No Ct	No Ct	No Ct	No Ct			
17	D	2	No Ct	No Ct	No Ct	No Ct			
18	A	5	No Ct	No Ct	No Ct	No Ct			
18	B	5	No Ct	No Ct	No Ct	No Ct			
18	C	5	No Ct	No Ct	No Ct	No Ct			
18	D	5	No Ct	No Ct	No Ct	No Ct			
19	A	2	No Ct	No Ct	No Ct	No Ct			
19	B	2	No Ct	No Ct	No Ct	No Ct			
19	C	2,1	No Ct	No Ct	No Ct	No Ct			
19	D	2,1	No Ct	No Ct	No Ct	No Ct			
20	A	1,8	No Ct	No Ct	No Ct	No Ct			
20	B	1,8	No Ct	No Ct	No Ct	No Ct			
20	C	3,3	No Ct	No Ct	No Ct	No Ct			
20	D	3,3	No Ct	No Ct	No Ct	No Ct			
21	A	0,45	No Ct	No Ct	No Ct	No Ct			
21	B	0,45	No Ct	No Ct	No Ct	No Ct			
21	C	0,4	No Ct	No Ct	No Ct	No Ct			
21	D	0,4	No Ct	No Ct	No Ct	No Ct			
22	A	2,2	No Ct	No Ct	No Ct	No Ct			
22	B	2,2	No Ct	No Ct	No Ct	No Ct			
22	C	2,2	No Ct	No Ct	No Ct	No Ct			
22	D	2,2	No Ct	No Ct	No Ct	No Ct			
23	A	1,5	No Ct	No Ct	No Ct	No Ct			
23	B	1,5	No Ct	No Ct	No Ct	No Ct			
23	C	1,5	No Ct	No Ct	No Ct	No Ct			
23	D	1,5	No Ct	No Ct	No Ct	No Ct			
24	A	5	36,61	No Ct	2,76	No Ct		<LOQ	
24	B	5	36,27	No Ct	3,49	No Ct		<LOQ	
24	C	5	36	No Ct	4,18	No Ct		<LOQ	
24	D	5	36,54	No Ct	2,89	No Ct		<LOQ	
25	A	1,6	35,96	38,29	4,31	0,87		<LOQ	
25	B	1,6	39,17	No Ct	0,4763	No Ct		<LOQ	
25	C	1,6	35,92	39,4	4,41	0,41		<LOQ	
25	D	1,6	35,43	39,5	6,19	0,38		<LOQ	
26	A	5	37,72	No Ct	1,29	No Ct		<LOQ	
26	B	5	36,98	No Ct	2,14	No Ct		<LOQ	
26	C	5	36,68	No Ct	2,62	No Ct		<LOQ	

26	D	5	36,75	No Ct	2,5	No Ct		<LOQ	
27	A	5	No Ct	No Ct	No Ct	No Ct			
27	B	5	No Ct	No Ct	No Ct	No Ct			
27	C	5	No Ct	No Ct	No Ct	No Ct			
27	D	5	No Ct	No Ct	No Ct	No Ct			
28	A	1	No Ct	39,42	No Ct	0,37		<LOQ	
28	B	1	38,02	No Ct	0,967	No Ct		<LOQ	
28	C	1	No Ct	No Ct	No Ct	No Ct			
28	D	1	39,93	No Ct	0,2605	No Ct		<LOQ	
29	A	5	41,54	No Ct	0,09	No Ct		Cut off	
29	B	5	43,66	No Ct	0,02	No Ct		Cut off	
29	C	4	No Ct	No Ct	No Ct	No Ct			
29	D	4	No Ct	No Ct	No Ct	No Ct			
30	A	5	38,81	No Ct	0,56	No Ct		<LOQ	
30	B	5	38,32	No Ct	0,79	No Ct		<LOQ	
30	C	5	40,07	No Ct	0,24	No Ct		<LOQ	
30	D	5	No Ct	No Ct	No Ct	No Ct			
31	A	5	36,89	No Ct	1,84	No Ct		<LOQ	
31	B	5	37,72	39,45	1,03	0,31		<LOQ	
31	C	5	38,02	No Ct	0,835	No Ct		<LOQ	
31	D	5	45,86	42,44	0,00	0,04		cut off	
32	A	5	32,81	35,21	31,74	5,94	2,40	1188,00	570,00
32	B	5	32,83	34,73	31,35	8,31	1,90	1662,00	
32	C	5	32,57	35,24	37,58	5,82	2,67	1164,00	435,20
32	D	5	32,14	35,36	50,6	5,35	3,22	1012,00	

			Procambarus virginalis						
Location	**Subsample**	**Vol (L)**	**Ct 1x**	**Ct 10x**	**eDNA copies 1x**	**eDNA copies 10x**	**Inhibition**	**eDNA copies (A+B PCR)**	**eDNA copies/L**
1	A	2	No Ct	No Ct	No Ct	No Ct			
1	B	2	No Ct	No Ct	No Ct	No Ct			
1	C	2	No Ct	No Ct	No Ct	No Ct			
1	D	2	No Ct	No Ct	No Ct	No Ct			
2	A	1	No Ct	No Ct	No Ct	No Ct			
2	B	1	No Ct	No Ct	No Ct	No Ct			
2	C	1,2	No Ct	No Ct	No Ct	No Ct			
2	D	1,2	No Ct	No Ct	No Ct	No Ct			
3	A	0,7	No Ct	No Ct	No Ct	No Ct			
3	B	0,65	No Ct	No Ct	No Ct	No Ct			
3	C	0,7	No Ct	No Ct	No Ct	No Ct			
3	D	0,65	No Ct	No Ct	No Ct	No Ct			
4	A	0,9	No Ct	No Ct	No Ct	No Ct			
4	B	0,9	No Ct	No Ct	No Ct	No Ct			
4	C	1	No Ct	No Ct	No Ct	No Ct			
4	D	1	No Ct	No Ct	No Ct	No Ct			
5	A	5	No Ct	No Ct	No Ct	No Ct			
5	B	5	No Ct	No Ct	No Ct	No Ct			
5	C	5	No Ct	No Ct	No Ct	No Ct			
5	D	5	No Ct	No Ct	No Ct	No Ct			
6	A	0,3	No Ct	No Ct	No Ct	No Ct			
6	B	0,3	No Ct	No Ct	No Ct	No Ct			
6	C	0,4	No Ct	No Ct	No Ct	No Ct			
6	D	0,4	No Ct	No Ct	No Ct	No Ct			
7	A	1,3	No Ct	No Ct	No Ct	No Ct			
7	B	1,3	No Ct	No Ct	No Ct	No Ct			
7	C	1,4	No Ct	No Ct	No Ct	No Ct			
7	D	1,4	No Ct	No Ct	No Ct	No Ct			

8	A	0,75	No Ct	No Ct	No Ct	No Ct
8	B	0,75	No Ct	No Ct	No Ct	No Ct
8	C	0,75	No Ct	No Ct	No Ct	No Ct
8	D	0,75	No Ct	No Ct	No Ct	No Ct
9	A	2,3	No Ct	No Ct	No Ct	No Ct
9	B	2,3	No Ct	No Ct	No Ct	No Ct
9	C	2,1	No Ct	No Ct	No Ct	No Ct
9	D	2,1	No Ct	No Ct	No Ct	No Ct
10	A	5	No Ct	No Ct	No Ct	No Ct
10	B	5	No Ct	No Ct	No Ct	No Ct
10	C	5	No Ct	No Ct	No Ct	No Ct
10	D	5	No Ct	No Ct	No Ct	No Ct
11	A	1,8	No Ct	No Ct	No Ct	No Ct
11	B	1,8	No Ct	No Ct	No Ct	No Ct
11	C	2	No Ct	No Ct	No Ct	No Ct
11	D	2	No Ct	No Ct	No Ct	No Ct
12	A	0,85	No Ct	No Ct	No Ct	No Ct
12	B	0,85	No Ct	No Ct	No Ct	No Ct
12	C	1	No Ct	No Ct	No Ct	No Ct
12	D	1	No Ct	No Ct	No Ct	No Ct
13	A	5	No Ct	No Ct	No Ct	No Ct
13	B	5	No Ct	No Ct	No Ct	No Ct
13	C	5	No Ct	No Ct	No Ct	No Ct
13	D	5	No Ct	No Ct	No Ct	No Ct
14	A	0,8	No Ct	No Ct	No Ct	No Ct
14	B	0,8	No Ct	No Ct	No Ct	No Ct
14	C	0,8	No Ct	No Ct	No Ct	No Ct
14	D	0,8	No Ct	No Ct	No Ct	No Ct
15	A	0,6	No Ct	No Ct	No Ct	No Ct
15	B	0,6	No Ct	No Ct	No Ct	No Ct
15	C	0,6	No Ct	No Ct	No Ct	No Ct
15	D	0,6	No Ct	No Ct	No Ct	No Ct
16	A	1,1	No Ct	No Ct	No Ct	No Ct
16	B	1,1	No Ct	No Ct	No Ct	No Ct
16	C	1,2	No Ct	No Ct	No Ct	No Ct
16	D	1,2	No Ct	No Ct	No Ct	No Ct
17	A	2	No Ct	No Ct	No Ct	No Ct
17	B	2	No Ct	No Ct	No Ct	No Ct
17	C	2	No Ct	No Ct	No Ct	No Ct
17	D	2	No Ct	No Ct	No Ct	No Ct
18	A	5	No Ct	No Ct	No Ct	No Ct
18	B	5	No Ct	No Ct	No Ct	No Ct
18	C	5	No Ct	No Ct	No Ct	No Ct
18	D	5	No Ct	No Ct	No Ct	No Ct
19	A	2	No Ct	No Ct	No Ct	No Ct
19	B	2	No Ct	No Ct	No Ct	No Ct
19	C	2,1	No Ct	No Ct	No Ct	No Ct
19	D	2,1	No Ct	No Ct	No Ct	No Ct
20	A	1,8	No Ct	No Ct	No Ct	No Ct
20	B	1,8	No Ct	No Ct	No Ct	No Ct
20	C	3,3	No Ct	No Ct	No Ct	No Ct
20	D	3,3	No Ct	No Ct	No Ct	No Ct
21	A	0,45	No Ct	No Ct	No Ct	No Ct
21	B	0,45	No Ct	No Ct	No Ct	No Ct
21	C	0,4	No Ct	No Ct	No Ct	No Ct
21	D	0,4	No Ct	No Ct	No Ct	No Ct
22	A	2,2	No Ct	No Ct	No Ct	No Ct

22	B	2,2	No Ct	No Ct	No Ct	No Ct			
22	C	2,2	No Ct	No Ct	No Ct	No Ct			
22	D	2,2	No Ct	No Ct	No Ct	No Ct			
23	A	1,5	No Ct	No Ct	No Ct	No Ct			
23	B	1,5	No Ct	No Ct	No Ct	No Ct			
23	C	1,5	No Ct	No Ct	No Ct	No Ct			
23	D	1,5	No Ct	No Ct	No Ct	No Ct			
24	A	5	No Ct	No Ct	No Ct	No Ct			
24	B	5	No Ct	No Ct	No Ct	No Ct			
24	C	5	No Ct	No Ct	No Ct	No Ct			
24	D	5	No Ct	No Ct	No Ct	No Ct			
25	A	1,6	No Ct	No Ct	No Ct	No Ct			
25	B	1,6	No Ct	No Ct	No Ct	No Ct			
25	C	1,6	No Ct	No Ct	No Ct	No Ct			
25	D	1,6	No Ct	No Ct	No Ct	No Ct			
26	A	5	No Ct	No Ct	No Ct	No Ct			
26	B	5	No Ct	No Ct	No Ct	No Ct			
26	C	5	No Ct	No Ct	No Ct	No Ct			
26	D	5	No Ct	No Ct	No Ct	No Ct			
27	A	5	No Ct	No Ct	No Ct	No Ct			
27	B	5	No Ct	No Ct	No Ct	No Ct			
27	C	5	No Ct	No Ct	No Ct	No Ct			
27	D	5	No Ct	No Ct	No Ct	No Ct			
28	A	1	No Ct	No Ct	No Ct	No Ct			
28	B	1	No Ct	No Ct	No Ct	No Ct			
28	C	1	No Ct	No Ct	No Ct	No Ct			
28	D	1	No Ct	No Ct	No Ct	No Ct			
29	A	5	No Ct	No Ct	No Ct	No Ct			
29	B	5	No Ct	No Ct	No Ct	No Ct			
29	C	4	No Ct	No Ct	No Ct	No Ct			
29	D	4	No Ct	No Ct	No Ct	No Ct			
30	A	5	42,88	43,73	0,3987	0,22		Cut off	
30	B	5	42,05	No Ct	0,7065	No Ct		Cut off	
30	C	5	40,81	43,39	1,66	0,28		<LOQ	
30	D	5	39,76	No Ct	3,43	No Ct		<LOQ	
31	A	5	37,56	37,23	15,76	19,72	-0,33	3944	1777,6
31	B	5	39,35	36,9	4,56	24,72	-2,45	4944	
31	C	5	36,84	39,59	25,86	3,85	2,75	770	1180,8
31	D	5	37,41	36,85	17,41	25,67	-0,56	5134	
32	A	5	35,27	37,36	76,23	17,99	2,09	3598	1495,6
32	B	5	35,46	37,26	67,05	19,40	1,80	3880	
32	C	5	34,49	36,87	131,4	25,34	2,38	5068	2373,6
32	D	5	34,47	36,44	133	34,00	1,97	6800	

Paper IV

Paper V