Norwegian Environment Agency

The use of eDNA and DNA-based methods to assess and monitor alien and doorknocker species

Report

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Colophon

Title – Norwegian and English: Bruk av miljø-DNA og DNA-baserte metoder for å vurdere og overvåke fremmede arter og dørstokkarter. The use of eDNA and DNA-based methods to assess and monitor alien and doorknocker species

Summary – sammendrag:
All organisms that interact with an environment leave traces of their genetic material in some form. This DNA/RNA can be used to assess the presence of species using a variety of molecular tools and can potentially be part of an effective early warning system for alien species. This report, commissioned by the Norwegian Environment Agency, aims to present an overview of methods using eDNA to detect and monitor alien species. This report is based on an initial commissioned project from the Norwegian Environment Agency to a consortium of Norwegian institutions (2021) where background literature on this topic was assembled and made available for future assessments.

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Preface

All organisms that interact with an environment leave traces of their genetic material in some form. This DNA/RNA can be used to assess the presence of species using a variety of molecular tools and can potentially be part of an effective early warning system for alien species. This report, commissioned by the Norwegian Environment Agency, aims to present an overview of methods using eDNA to detect and monitor alien species. We also present a list of species-specific assays that are developed for taxa considered alien species (which are already here) or doorknocker species (which have a high potential to establish) in Norway. Minimum criteria for the use of eDNA to assess the presence of alien species are suggested and a comprehensive decision diagram for detection and monitoring of alien species is presented. This report is based on an initial commissioned project from the Norwegian Environment Agency to a consortium of Norwegian institutions (2021) where background literature on this topic was assembled and made available for future assessments.

Utvidet norsk sammendrag

Alle organismer som er i kontakt med sine omgivelser etterlater seg DNA-spor. Disse sporene (miljø-DNA) kan brukes til å vurdere om en art er til stede i et gitt miljø og kan potensielt tas i bruk som et tidlig varslingssystem for introduksjon av fremmede arter. På oppdrag fra Miljødirektoratet, gir vi i denne rapporten en oversikt over ulike molekylære tilnærminger egnet til bruk for påvisning og overvåkning av fremmede arter ved bruk av miljø-DNA. Rapporten inneholder en oversikt over artsspesifikke analyser som er utviklet for taxa regnet som fremmede og allerede er til stede i norsk natur, eller som har stort potensiale til å etablere seg her (dørstokkarter). Vi legger vekt på behovet for testing av protokoller på flere nivå før artsspesifikke analyser operasjonaliseres og gir seks informative eksempler fra eksisterende overvåkingsprogram i ulike typer miljø for: virvelløse dyr og karplanter i jord fra importerte planter, ferskvannskreps, krepsepest, *Gyrodactylus salaris*, pukkellaks, vasspest og amerikahummer. Vi foreslår minimumskrav til arbeidet ved vurdering av en fremmed arts tilstedeværelse, presenterer et utfyllende beslutningsdiagram, og gir til slutt anbefalinger med hensyn på hvilke forutsetninger, krav og arbeidsmetodikk som bør følges når miljø-DNA tas i bruk for detektering og overvåking av fremmede arter. Minimumskravene inkluderer krav til prøvetakingsmetodikk og program, laboratoriepraksis, bioinformatikk-, statistikk- og referansebibliotek-håndtering samt rapportering. Beslutningsdiagrammet omfatter vurderinger som må gjøres tidlig i prosessen, blant annet kost/nytte, mål for overvåkningen, risikotoleranse, alternativ metodikk, tilgjengelige protokoller, laboratorier og kvalitetskontroll, definisjon av positiv påvisning, samt aspekter knyttet til beslutningsstøtte slik som gjentakende prøvetaking, igangsetting av ikke-molekylær prøvetaking, og prøvetakingsintensitet i forhold til risiko- og toleransegrenser (Norsk sammenfatning av kapittel 3 i supplement 1).

Ved igangsetting av overvåkingsprogrammer som tar i bruk av miljø-DNA anbefaler vi at:
1) Referansesekvenser for aktuell og nært beslektede taxa må være, eller gjøres, tilgjengelig for utforming av artsspesifikke analyser; 2) Nøyaktige og kvalitetssikrede standarder tas i bruk både ved innsamling og laboratoriearbeidet, som negative kontroller i alle trinn i prosessen (feltprøver, DNA ekstrahering, PCR, indeksering) og positiv kontroll for PCR effektivitet; 3) Rapporteringen bør blant annet inneholde filtertype/størrelse, vannvolum og vanntemperatur, oppbevaringsforhold for prøver, detaljerte opplysninger om laboratorieprotokoll, samt valg av fragmentstørrelse og sekvenseringsplattform; 4) Tilfeldig påvisning gjennom metabarcoding sjekkes grundig og undersøkes videre når metabarcodingresultatene indikerer robusthet; 5) Positiv påvisning defineres og man vurderer betydning av positiv påvisning; 6) Beslutningsdiagram (operasjonelle prosedyrer, forvaltnings krav og ønsker, kommunikasjonsplaner) tas i bruk og vurderes i hvert enkelt tilfelle, før igangsetting av overvåkingsprogram med bruk av miljø-DNA.

Rapporten bygger på en oversikt over bakgrunnslitteratur innsamlet gjennom et tidligere oppdragsprosjekt fra Miljødirektoratet fra 2021 (Supplement 3).

Summary

All organisms leave traces of their DNA in the environment they live in. This environmental DNA (eDNA) can be used to detect and monitor single species as well as communities, and potentially be utilized in early warning systems to detect alien species. In this report, we give an overview of eDNA-based methods used to detect and monitor alien species, and give examples of species-specific assays designed to identify species that are alien to Norwegian nature or have the potential to establish viable populations in Norway (doorknockers). We emphasize the need for several stages of testing before species-specific assays can be operational, and discuss the importance of including models to assess detection probabilities. Going through standards for sampling and analyses, we suggest a number of minimum requirements for eDNA sampling, laboratory practice, bioinformatics and the use of reference libraries, as well as for reporting results from eDNA studies.

The origin and fate of eDNA in different environments can influence its usefulness in detecting and monitoring alien species. We outline factors for terrestrial, freshwater and marine ecosystems and provide examples from six case studies where eDNA has been used to detect and/or monitor alien invasive species: invertebrates and vascular plants in soil of imported ornamental plants, freshwater crayfish and crayfish plague, *Gyrodactylus salaris,* pink salmon, Canadian pondweed, and American lobster.

We provide a decision diagram for detection and monitoring of invasive species starting with early considerations for implementation of a monitoring program, and ending with management decision points depending on detection outcomes at different stages. Finally, we provide some key recommendations for the use of eDNA in assessments of alien and doorknocker species.

1. Background knowledge

1.1 Definition of environmental DNA

There are several definitions for eDNA, including "DNA extracted from environmental samples (such as soil, water or air), without first isolating any target organisms" (Taberlet et al. 2012) or "genetic material obtained directly from environmental samples (soil, sediment, water, etc.) without any obvious signs of biological source material" (Thomsen and Willerslev 2015). Both definitions include organisms that sheds cells with genetic information (DNA) into its environment, and those that are invisibly present such as meiobiota and 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, while for unicellular organisms or other multicellular microorganisms, the entire organisms or their propagules can be extracted and identified by means of eDNA analyses directly from an environmental sample. As it is difficult, and often impossible, to separate organismal and environmental DNA when analysing DNA from an environmental sample, Pawlowski et al. (2020) argued that all DNA from the sample must be considered eDNA. Although this definition might mask the origin of eDNA and thus the ecological meaning of its presence, since detection of a species through its extra-organismal DNA can be interpreted differently than detection by organismal DNA (Rodriguez-Ezpeleta et al. 2021), it is the most appropriate and practical definition of eDNA also for detecting and monitoring alien species.

1.2 Definitions of alien, invasive, and doorknocker species, pathways and source populations

Identification of relevant alien species at different stages of their invasion process is a key to any targeted management. Species never previously identified as aliens anywhere is a major challenge for biosecurity interventions, especially since our ability to predict the identity of future invasive alien species have largely been based upon knowledge of prior invasion history (Seebens et al. 2018). In order to make a knowledge-based choice for which species to include in a horizon scan and further for ecological risk assessment and monitoring, we need to be able to identify the relevant species.

We use the term **alien species** as defined by IUCN (2000):
"Alien species (non-native, non-indigenous, foreign, exotic) means a species, subspecies, or lower taxon occurring outside of its natural range (past or present) and dispersal potential (i.e., outside the range it occupies naturally or could not occupy without direct or indirect introduction or care by humans) and includes any part, gametes or propagule of such species that might survive and subsequently reproduce."

However, this definition is very broad, and four additional delimitations have been necessary to operationalize the term for ecological risk assessments (Sandvik et al. 2020): i) a *historical* delimitation excludes any alien species stably reproducing in Norway before the year 1800; ii) a *geographical* delimitation requires the alien species to have crossed national borders during its

introduction (excluding regional alien species); iii) an *ecological* delimitation requires the alien species to reproduce without human management in the wild in Norway; and iv) a *taxonomical* delimitation excludes unicellular, genetically modified organisms, and most alien taxa below the species level from being assessed.

Most alien species have little or no known ecological effects, while **invasive alien species (IAS)** are negatively impacting native biodiversity and ecosystems. In Norway, all alien species (defined and delimited as described above) are regularly impact assessed using the Generic Ecological Impact Assessment of Alien Species (GEIAA) protocol (Sandvik et al. 2019). Using a set of criteria to assess invasion potential and the ecological effect of the species, species are assigned to five ecological impact categories from 'no impact' (NK), via 'low impact' (LO), 'potentially high impact' (PH), 'high impact' (HI), to 'severe impact' (SE) (Artsdatabanken 2018).

Doorknocker species are alien species not currently reproducing in the wild in Norway, but that can be expected to do so within 50 years (Sandvik et al. 2020). They may not yet be present in Norway, or they may be present but not able to reproduce in Norway, or they may currently only reproduce indoors or in cultivation.

Pathways of introduction are "the processes that result in the introduction of alien species from one location to another" (Hulme et al. 2008). These processes are classified into six main categories (with 44 subcategories): intentional release, escape from confinement, contaminants of commodities, stowaways on transport vectors, spread through human-made corridors and unaided dispersal via natural means from alien populations elsewhere (Hulme et al. 2008, CBD 2014). As part of the impact assessment of alien species in Norway, the known and assumed pathways have been listed for every alien and doorknocker species (Artsdatabanken 2018).

Source populations are the pools of species from which the introduced species originate.

1.3 Methods for the use of eDNA in the detection and monitoring of species

eDNA detection methods can be broadly divided into two main types: species-specific detection using qPCR or ddPCR technologies, and eDNA metabarcoding using high-throughput sequencing technology (Taberlet et al. 2012). Sample collection, filtration and DNA-extraction largely follow the same protocols for the two main types of detection methods, but laboratory analysis methodologies differ. Currently, single-species detection is considered the most suitable method for detection of alien- and doorknocker species because it has higher detection sensitivity (Tsuji et al 2019, but see McCarthy et al. 2022). The drawback of this method is that one has to know what to look for, and introduction of unknown or unpredicted species will go unnoticed. In recent years the use of eDNA metabarcoding has become more commonplace. eDNA metabarcoding uses high-throughput sequencing to generate DNA sequences that can represent a much broader taxonomic scope or that can also be targeted to closely related taxa. Both of these main types of detection methods are described in more detail below.

1.3.1 Species specific detection

The principle behind applying species-specific assays is the amplification of selected DNA markers (typically 80–200 bp) specific to the target organism. Various platforms including different protocols may enable the detection of the target species, including PCR-based amplification techniques such as real-time quantitative PCR (qPCR), droplet digital PCR (ddPCR) and isothermal amplification techniques such as loop-mediated isothermal amplification (LAMP). While endpoint PCR was commonly used in early studies, qPCR and ddPCR clearly form the preferred analysis platforms at present and easily provide the possibility of detecting several species (multiplexing) in one run, and can also provide information on eDNA quantities. Furthermore, the majority of scientific research on the area has been focused on developing robust assays for qPCR and ddPCR both in terms of sensitivity and specificity, and a switch in amplification technique would require all of this work to be re-done and optimized to the requirements of the new amplification technique (such as LAMP). Thus, the following paragraphs mainly focus on species-specific assays developed for qPCR and ddPCR.

Currently, qPCR is the most commonly used method for investigating eDNA samples and determining the occurrence of selected species (Tsuji et al. 2019). In general, the qPCR approach is more sensitive and specific than the metabarcoding approach (Tsuji et al 2019, but see McCarthy et al. 2022) but is usually restricted to one or a few taxa at a time. This is a clear advantage for example for monitoring programs that target specific alien species. The advantage of the qPCR assays lies in the use of a highly specific probe that is very sensitive to mismatches at the annealing site, in addition to the primers which enhances specificity (e.g., Yao et al. 2006; Vrålstad et al. 2009). During qPCR, a fluorometer detects the fluorescent dye binding to the targeted sequence (typically 80–200 bp) which is amplified during the PCR. Readings of the fluorescence-levels are displayed as the thermal cycler operates allowing for evaluation of the target species' DNA levels in real-time. Combined with standard curves and reference values, the resulting reaction data for the samples of interest may give information about relative amounts of the target DNA present. In recent years, droplet digital PCR (ddPCR) has emerged as an even more precise and sensitive tool for species detection than standard qPCR (Hindson et al. 2011, Mauvisseau et al. 2019a). 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). It also provides several reads from the same sample making its results more statistically robust. For ddPCR, a sample is partitioned into tens or hundreds of thousands micro-droplets which are analyzed independently. The fraction of the PCR-positive droplets is then used to calculate the target DNA template concentration in the original sample enabling species detection limits of < 0.1 DNA copies μl^{-1} (Brys et al. 2021).

To analyze samples with qPCR and similar methods, species-specific assays are designed and tested for each target organism. These species-specific assays, however, are time-consuming to develop since both the genetic marker and its primers and fluorescent probe sequences need to be selected and optimized to prevent cross-amplification with closely related species. This is because false positives due to too low specificity or false negatives due to too high specificity

render an assay unsuitable for routine monitoring at larger scales. Hence, accurate eDNA detection including appropriate interpretation of results is crucial. The MIQE guidelines for qPCR (Bustin et al. 2009) define essential information about minimum standards for qPCR runs; however, to ensure optimal functionality and applicability of eDNA assays, several additional actions are recommended including *in silico*, *in vitro* and *in situ* testing (Thalinger et al. 2021). Target sequences of the respective taxon including different genotypes/haplotypes, sister species and other closely related species as well as naturally co-occurring species, either available from public sequence depositories based on reliably identified voucher specimens or generated by individual DNA sequencing, should be compiled into an alignment. This sequence information is compared and analyzed to find the optimal site for primers and probes with a maximum of differing bases between relevant organisms. *In vitro* testing of the assay involves optimization of the PCR chemistry and cycling conditions as well as running DNA tissue samples from all relevant organisms to ensure correct amplification and increase robustness. In this phase, the limit of detection (LOD) and the limit of quantification (LOQ) are important parameters for the detection of low target DNA concentrations (Klymus et al. 2020): The LOD is established by running diluted concentrations of the target DNA until no detection is reported, whereas the LOQ states the lowest amount of target DNA which can be quantified with a given precision enabling inferences between the amount of target species DNA and its abundance. Finally, the assay is tested in the field spanning both positive and negative eDNA samples. Sequencing of eDNA amplicons is advisable to confirm assay specificity. Appropriate numbers of physical and technical replicates should accompany the various steps to ensure repeatability and increase reliability (Mauvisseau et al. 2019a).

To assure the applicability of the assay, Thalinger et al. (2021) developed a 5-stage validation scale (Fig. 1) based on 122 validation variables facilitating the successful development of species-specific assays. According to this scale, interpretation of results from stage 1 and 2 in an assay makes it impossible to tell if a target species is present or absent. If the target species is not detected at stage 3, it is impossible to say if the species is present or absent, but if detected it is likely present if a) the field negative is blank, b) an eDNA-appropriate laboratory procedure was used, and c) positive detections were sequenced. If the target species is not detected at stage 4 or 5, the species is likely not present given that appropriate timing and sufficient replicates in the sampling (stage 5 provides the probability of species presence despite negative results). If the target species is detected at the substantial stage 4 or the operational stage 5, it is likely present. No assays below validation stage 4 are recommended to be utilized for routine monitoring purposes. As technology improves and new analytical platforms and protocols are developed, assays should be reinvestigated or modified to fit platforms different from those which they originally were designed for.

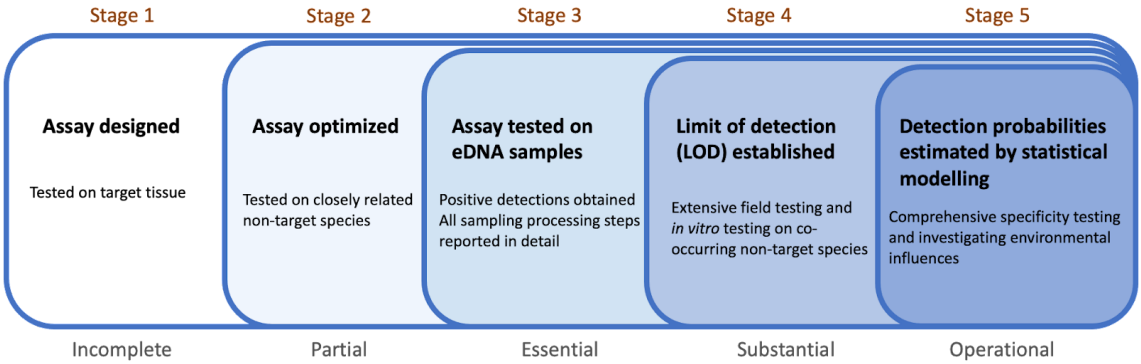


Figure 1. Stages of species-specific assays for detection of target species using eDNA. Modified from Thalinger et al. (2021).

1.3.2 DNA metabarcoding

The principle behind DNA metabarcoding is the amplification of selected DNA markers (of various lengths) that are not specific to just one target organism (Comtet et al. 2015; Taberlet et al. 2018). Depending on the primers chosen for the amplifications during PCR, this broader approach can target a vast array of organisms (e.g., all or most eukaryotes), or it can target a narrower group of organisms (e.g., just ray-fin fish) (Sales et al. 2020a, 2020b, Polanco Fernández et al. 2021, Burian et al. 2022, Lynggaard et al. 2022a, Mas-Carrió et al. 2022). The end result of the DNA metabarcoding is many hundreds of thousands to many thousands of millions of sequences that then need to be bioinformatically processed through numerous steps, including taxonomic identification.

The key aspect to DNA metabarcoding is the choice of which primers to use to amplify the selected DNA markers during the PCR step (Vaulot et al. 2022). During PCR, the forward and reverse primers anneal to targeted DNA, allowing for polymerases to copy the targeted fragment. The exponential copying of DNA can be halted from 20 to 30 or even more cycles, depending on the starting concentration of the total as well as target DNA, and on the potential need to reduce copying-induced sequencing errors.

Users must first decide how taxonomically narrow or how taxonomically broad they want primers to target potential invasive or doorknocker species (Pawlowski et al. 2012). Considerable efforts *in silico* and *in vivo* have been made to identify which taxa will or will not be amplified by different primer sets (Vaulot et al. 2022). Even primers that are designed to target numerous taxa, can still fail to amplify closely related taxa because of variable rates in the evolution of the gene sequences; in this case, invasive species could be missed because the chosen primers are biased against them.

The indexing strategy used to assign sequences back to individual samples can also affect the quality of results, due to "tag/index jumping" (Schnell et al. 2015). Tag/index jumping occurs during the process of PCR or pooling PCR products before sequencing, where tags/indexes from PCR products generated from one sample are erroneously incorporated or switched with tags/indexes from another sample. Unless tag/index jumps can be controlled via laboratory

experimental design or discovered bioinformatically, they can be an insidious form of false positives from metabarcoding data. There are various methods and recommendations for controlling for, reducing or eliminating tag/index jumps (Zinger et al. 2019; Bohmann et al. 2022), ranging from the most expensive and stringent - that each sample or PCR replicate has unique dual indexes - to the least expensive, where some index combinations are not used so that if these combinations are subsequently identified during bioinformatics they indicate the rate of tag/index jumping in a metabarcoding library. It is important that careful consideration is given to this facet of designing metabarcoding laboratory workflows and that the techniques used to eliminate or control for the possibility of tag/index jumping are reported.

Users must also decide the size of the fragment of the DNA marker they want to amplify. Most DNA metabarcoding studies have used primers that amplify short markers that are just a couple of hundred to a few hundred base pairs long (Turon et al. 2022). The sequencing platforms that best handle these short sequences (e.g., Illumina) can result in hundreds of millions of sequences with very low error rates; the analytical per-sequence cost using these platforms therefore tend to be relatively cheap. Some DNA metabarcoding studies are starting to amplify long markers that can be up to thousands of base pairs long (Latz et al. 2022). The sequencing platforms that best handle these long sequences (e.g., PacBio and Nanopore) can result in a few million sequences with low to high error rates.

There are several approaches to bioinformatically analyzing the resulting vast number of sequences produced by the high-throughput platforms (e.g., Mathon et al. 2021). Numerous pipelines have been developed for these analyses to fully or partially handle each step in the process, and the different bioinformatic approaches have been adopted by different researchers depending on which taxonomic groups that are targeted. These pipelines do, though, have some commonalities. First, low-quality and low-abundance sequences are often filtered out (Kunin et al. 2010). Presumably these types of sequences that are filtered out are errors induced during PCR amplification or during sequencing, although low abundant sequences could actually be derived from low abundant species; some researchers keep low abundant sequences if they are sufficiently similar to sequences in a taxonomic reference database (Egge et al. 2021). Second, similar sequences are often clustered together using global similarity (e.g., 100% or 97%) or local similarity thresholds. These clusters can be called by numerous names (e.g., OTUs, ASVs), and are often considered to represent species found in nature (Santoferrara et al. 2020; Taberlet et al. 2018). Third, the clusters of similar sequences are then taxonomically assigned to taxa presented in a reference database (e.g., to the BOLD database). Taxonomic assignments are often performed using pairwise sequence comparisons, although phylogenetic comparisons are increasingly being performed.

1.3.3 Models to assess probability of detection

Surveys relying on presence-absence records are often used to estimate the spatial distribution of a species. However, to account for false negative errors due to the difficulties of detecting rare or elusive species, repeated surveys at defined locations and use of occupancy models are often necessary (Dorazio & Erickson 2018). An occupancy model is a mixed model, which associates

two probability distributions to describe the two sources of uncertainty during a survey (MacKenzie et al. 2018, Burian et al. 2021). They are based on a hierarchical structure, either frequentist, which apply maximum likelihood estimation or Bayesian statistical frameworks, which are mainly based on Markov chain Monte Carlo simulation (Burian et al. 2021). They recognize that the probability to detect a species is linked to the species being present at a given site (Burian et al. 2021) and require multiple observations per investigated site within a defined time frame with an assumed constant occupancy (Rota et al. 2009). Occupancy models can be used to evaluate the probability to detect a species depending on environmental factors and/or other species occurrence (Burian et al. 2021, Goldberg et al. 2018), and models dedicated to the analyzing of eDNA-based data can also evaluate the probability of eDNA detection (Doi et al. 2019). Standard occupancy models assume that false positive detection does not occur, such results can still be obtained in case of contamination or insufficient method validation (Burian et al. 2021). However, recent models accounting for both false positive and negative errors now allow for multiscale occupancy models to be applied (Griffin et al. 2020, Buxton et al. 2022). As eDNA based surveys often rely on the collection of multiple field replicates (or filters at each site) to increase the probability of retrieving DNA and therefore species detection, this allows an easy implementation of occupancy modelling to further increase their reliability (Burian et al. 2021). Indeed, capture probability is based on the chance to collect the target species' eDNA in a field replicate (or filter), while the detection probability is linked to the probability of detecting the captured eDNA in the PCR replicates at the analysis stage. Such a framework facilitates the consideration of complex ecological and environmental interactions when large data sets of presence–absence records are available to support the model structures (MacKenzie and Royle 2005). Various eDNA-based studies have investigated sampling effort, or the effects of covariates on occurrence and detection of eDNA in samples (Erickson et al. 2019; Fossøy et al. 2019, 2020; Mauvisseau et al. 2019a; Johnsen et al. 2020b; Martel et al. 2020; Baudry et al. 2021; Dubreuil et al. 2021; Keller et al. 2022; Dimond et al. 2022), and have highlighted important covariate impacts on eDNA capture or detection. Such knowledge can be used to address monitoring challenges and investigate species population dynamics at larger scale, allowing to evaluate the range or distribution front of invasive species or monitor the spread of pathogens. However, it should be noted that conclusions obtained for a single study, with specific conditions, analytical workflow or environment are often difficult to extrapolate to other workflows or environments (Bruce et al. 2021). In the case of eDNA detection using species-specific based approaches, tools such as the R Shiny application *eDNA* (Griffin et al. 2020, https://seak.shinyapps.io/eDNA/) or the "eDNAoccupancy" R package (Dorazio and Erickson 2018) can be used for the analysis of eDNA based detection using occupancy models, while Fukaya et al. (2021) provide a similar option for eDNA metabarcoding based detection.

1.3.4 Portable and autonomous remote platform for monitoring of alien species

The full benefit of eDNA can be achieved by automation of the different steps, from collection to analysis, which to date, with very few exceptions, poses a number of challenges. Nevertheless, developing unmanned robotic sample handling systems, capable of processing samples with extremely high throughput *in situ* is a promising area (Darling 2020; Sepulveda et al. 2020) to overcome spatial and temporal limitations from manual sampling. *In situ* sampling can overcome

many of the challenges associated with collection of material for metabarcoding, and provide a sampling methodology that is equivalent to, and in some instances preferable to, more traditional sampling methods (Truelove et al. 2022). Devices can be quite advanced such as the Environmental Sample Processor (ESP) (Scholin et al. 2017) suited for oceanic deployment or 'lighter' technology for autonomous filtration in rivers (e.g., Formel et al. 2021). They can be combined with portable eDNA analysers for field use, some of which are currently commercially available. This includes simplified DNA-isolation techniques (Biomeme and Quantabio eDNA isolation cartridge and kits), and qPCR devices (Biomeme; Ubiquitome; Quantabio). These technologies are promising instrumentation for *in situ* analysis of alien species in the field. They allow for adaptive sampling strategies, as one can consider the need for additional samples or sampling locations while in the field (Thomas et al. 2020), with the possibility to upload the result to a cloud server. However, studies (e.g., Seah et al. 2020, Thomas et al. 2020) demonstrate that the rapid DNA extraction method provides lower DNA yield compared to commonly used laboratory DNA kits and that the qPCR chemistry is prone to inhibition from waters with high levels of suspended particulate. These factors increase the likelihood of false negative results, especially when targeting organisms of low abundance. Other challenges posed by autonomous sampling are rapid and large volume sampling, and a larger “intake diameter” of the sampling device (ESP intake 150x smaller than Niskin) to collect larger organisms or particles. This could ease the collection of rare DNA and the detection probability of organisms that do not release much DNA in their surroundings (Sepulveda et al. 2019; Truelove et al. 2022). Good preservation and conservation methods are also critical for autonomous sampling. Conservation may in practice limit the potential of robotic for eDNA and the deployment time if DNA/RNA yield is less performant after conservation and exhibit differences compared to standard freezing or immediate processing methods for alpha and beta diversity (Yamahara et al. 2019). Moreover, there are many new logistical challenges and needs related to the automatic solutions, including advanced technical expertise, that should not be underestimated from a cost-benefit perspective.

Hence, the inclusion of autonomous samplers- and analysis in monitoring programs still need development before use in regular monitoring programs and replacement of standard laboratory routines. Yet, this is seen as a vision to pursue and great progress is already achieved in that direction (Truelove et al. 2022).

1.4 Standards for sampling and analyses

1.4.1 Minimum requirements for sampling of eDNA

eDNA sampling needs to first and foremost consider the biology and ecology of the target species to maximize the potential and effectiveness of detection, as well as the relevant research or management question (Dunshea et al. 2021). This means that sampling should be done at a time of year when the organism is most active (for example during fish spawning periods) and in suitable habitats for the target species. In general, the chance of detecting a species will increase with the number of samples collected and the volume of water filtered, and is related to the spatial representativeness of the targeted organism. It is recommended to collect a minimum of

two biological replicates per sampling event, and each sample should be run in a minimum of three technical replicates. If the goal is to quantify the amount of eDNA from the target organism, more samples may be necessary. In that case, a minimum of three biological replicates and six technical replicates are recommended. A field blank sample should be collected every sampling day, or at every location (if several locations are visited on the same day). A European Standard for Sampling, capture and preservation of environmental DNA from water has been developed and is currently under votation in CEN (prEN 17805:2021). This document will outline general minimum requirements for sampling of eDNA in water but is not specifically targeted towards sampling of eDNA from alien invasive species. Putative publication date if ratified is February/March 2023.

A number of measures should be taken to avoid contamination during sampling of eDNA:

- Field personnel should always wear DNA target-free single use gloves.
- Field personnel should never handle live- or dead specimens of the target species while also conducting sampling.
- If operators in the field are unfamiliar with molecular workflow, single use plastic equipment should be used.
- Encapsulated filters should be used to protect the sample against the surrounding environment.
- Collected samples should never be in contact with one another.
- If the same equipment is utilized in several locations, it must be decontaminated (e.g., by immersing in >0.1% bleach over at least 2 min and thoroughly rinsed by target-DNA free water) between sampling locations.
- Field blank samples must be taken.

Likewise, a number of metadata variables should be recorded during sampling for downstream quality control:

- A field protocol that includes mitigations to avoid contamination
- Filter type and mesh size
- Water volume filtered
- Water temperature
- Storage medium/preservative (e.g., buffer type, kept on ice, frozen)
- Storage temperature and conditions in field and lab

1.4.2 Minimum requirements for laboratory practice

Typically, DNA from environmental samples occurs in considerably lower quantities than DNA extracted from collected specimens. Depending on the environment and exposure time, the DNA often is more fragmented. These factors make eDNA samples more prone to contamination in the laboratory than DNA extracted from well preserved tissues. Thus, a well-structured laboratory practice in a dedicated space isolated from potential contamination sources is important. Moreover, the use of decontaminated and target DNA-free pipettes, DNA-free consumables and protective wear (gloves, coats, shoe covers, etc.) is considered minimum standards for the eDNA laboratory. In addition, the use of negative controls in all potentially

contaminating steps in the protocol is crucial (i.e., during field sampling, during DNA isolation and PCR), and several points are considered minimum requirements for a laboratory report (from Bruce et al. 2021):

- Total DNA concentration for all samples (including negative controls)
- Results of inhibition test and exogenous internal positive controls (e.g., testing extraction efficiency)
- Barcode marker used (only relevant for metabarcoding) including length of target amplicon
- Primers, indexes and library preparation protocol (last two only relevant for metabarcoding)
- Number of PCR replicates performed (technical replicates)
- Documentation of PCR success and method for determining this
- Performance of field blank, DNA extraction and PCR no-template negative controls in PCR
- Description of positive controls and their performance in PCR
- Details of any spike-in added to achieve heterogeneity (only relevant for metabarcoding)
- Final concentration of libraries loaded onto flow cell (only relevant for metabarcoding)

1.4.3 Minimum requirements for bioinformatics and reference libraries for DNA-metabarcoding

For DNA-metabarcoding, eDNA bioinformatics and reference libraries need to be optimized in order to uncover a large enough group of organisms while at the same time as also uncovering potential invasive or doorknocker species. For the bioinformatics pipelines, every step can affect the outcome, although there is no general agreement about what should be done at each step. There are some programs that allow you to perform most of these steps with a few simple commands (e.g., Bolyen et al. 2019; Boyer et al, 2016), although many researchers analyzing eDNA data write their own scripts to configure the output from one bioinformatic step to another. Importantly for all bioinformatic analyses, the different steps taken by one researcher should be repeatable by others. Although bioinformatics is a powerful tool to analyses eDNA metabarcoding data, it cannot solve some problems that were introduced during sampling and sequencing stages; for example, if the chosen barcode does not provide enough differences between species or higher taxa, then cleaning and clustering cannot make these differences.

A number of steps can be taken to ensure repeatability in the bioinformatics:

- All programs and their version numbers that were using in the analyses should be reported
- All computer codes used in the analyses, especially if written for the project, should be made available for other researchers (e.g., by placing them into a supplementary file or on Dryad)
- When possible, use open-sourced programs, so it is clear what is done in each program

Ideally one would want to know what species or higher taxa were uncovered in an eDNA metabarcoding study. Do to this, sequences, or clusters of similar sequences, are compared to taxonomically curated reference libraries. These libraries are usually composed of sequences of a large group of organisms and often contain just one barcode region (e.g., Guillou et al. 2013; Ratnasingham and Hebert, 2007). A key aspect of reference libraries is that many species may not be in the library, including potential invasive or doorknocker species; if they are not in the library, you will not know you uncovered it during eDNA metabarcoding because you will not be able to taxonomically identify it.

A number of steps can taken to improve reference libraries:

- Evaluate the library to make sure potential alien species are included
- If they are not, include available sequences of that species, or make new sequences if non available
- More than one individual per species should be sequenced to consider genetic variation within a species. For widely distributed species, reference libraries should ideally include representatives from the geographic region that is sampled in the field
- Reference libraries should also include closely related taxa to target species to minimize the possibility of taxonomic misassignment

1.4.4 Minimum requirements for reporting eDNA-data

Reporting species occurrence using eDNA and DNA-metabarcoding has been addressed both nationally (Finstad et al. 2020) and internationally (Nilsson et al. 2022) previously. Guidelines for published DNA-based records include templates and field names adapted to GBIF for qPCR, ddPCR and metabarcoding data (Andersson et al. 2021). Recommendations include reporting details concerning sample collection, DNA-extraction, PCR-amplification, sequencing platform, bioinformatic pipeline and reference database behind the detection of any particular species for metabarcoding data (Figure 2).

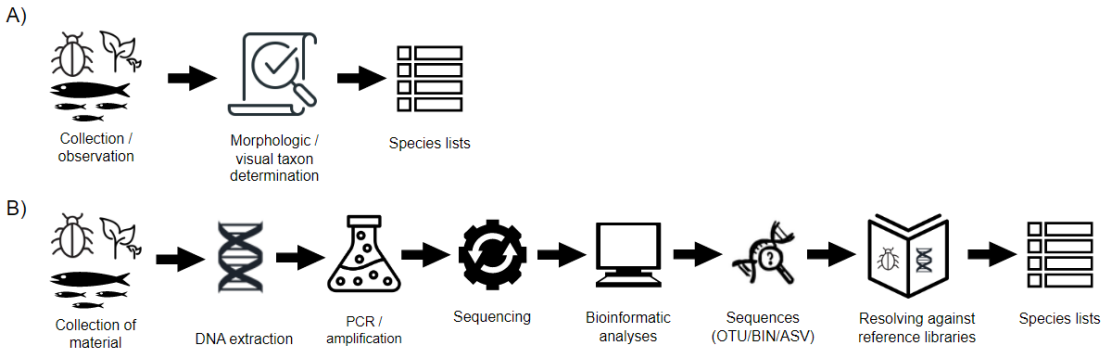


Figure 2. Differences in the sampling and analysis processes when reporting species detections using A) morphology and B) DNA-based methods (from Andersson et al. 2021, CC-BY-SA 4.0).

We suggest that minimum requirements include:

- Field protocol for collecting samples including, filter type and mesh size, water volume filtered, water temperature, storage medium, and storage conditions until further analyses.

- Lab protocol including DNA-extraction, PCR/amplification and programme details, library prep clean-up and fragment size selection for metabarcoding
- Sequencing platform for high-throughput sequencing
- Bioinformatic pipeline with details on reference database for metabarcoding

2. The use of eDNA in detection and monitoring of alien species

2.1 The nature of eDNA

2.1.1 eDNA in terrestrial environments

Aerial eDNA analyses of fungal or oomycete spores from various kinds of traps can be effective in early detection of pathogens (Aguayo et al. 2021; Nicolaisen et al. 2017), including alien species. A variety of passive or active spore traps have been used for this purpose, including analyses of the preservative fluid of insect traps (Bérubé et al. 2022 and references therein). Technical solutions appear to be well established and have been used to document how invasive species replace native biota in North America (Garbelotto et al. 2022). A few recent studies have also been investigating the use of airborne eDNA for either plant (Johnson et al. 2019, 2020, 2021) or vertebrate (Lynggaard et al. 2022a, 2022b) community monitoring. Despite this application looking promising, and already showing a high potential for the detection of invasive plant species compared to traditional methods (Johnson et al. 2021), it still unknown how far airborne eDNA can travel or persist, and additional studies will be needed for a better understanding of airborne eDNA before its eventual addition to the invasive species monitoring toolkit.

2.1.2 eDNA in freshwater environments

eDNA biomonitoring and inventory studies are particularly common and explored for freshwater systems. The potential for monitoring aquatic species without using invasive methods or methods jeopardizing animal welfare (such as trapping, electrofishing, killing), or often time-consuming morphological examination and/or cultivation methods from water samples, are among the drivers for developing the eDNA biomonitoring approach for aquatic environments (e.g. EU COST Action CA15219 “Developing new genetic tools for bioassessment of aquatic ecosystems in Europe” - https://dnaqua.net; Leese et al. 2016). Although both sediment samples and freshwater biofilms are used, filtered water sampling is among the most common methods for eDNA analyses in freshwater environments, and is largely dominated by studies focusing on fish and other aquatic vertebrates (Ruppert et al. 2019). There is a steadily growing number of studies and monitoring programs targeting alien invasive species in freshwater habitats (e.g., Supplement 3). In Norway, there are several examples of assessments and monitoring programs involving eDNA detection of alien species in freshwater habitats (Fossøy et al. 2018, 2019; Hansen et al. 2022a, 2022b; Taugbøl et al. 2021; Ahmed et al. 2022; Engesmo et al. 2022; Strand et al. 2021 – see below).

Detectability of eDNA in natural water systems is influenced by a multitude of factors, such as UV radiation, dilution, inhibition through humic acids, microbial and enzymatic degradation, retention in substrate and transport that expedite its degradation or disappearance from the system (Jerde et al. 2016; Shogren et al. 2017; Stewart 2020; Rusch 2021; Rusch et al. 2022; see Figure 2). Even under reasonably controlled conditions, Rusch et al. (2022) found that a limited set of controlled factors substantially changed the detectable amount of eDNA from signal crayfish and its parasite *A. astaci*, even though the physical presence of the target organisms remains the same. For example increased temperature and murky water with presumed high microbial activity markedly reduced detectability and eDNA quantity compared to cold, clear water.

Special considerations regarding eDNA spatial dynamics should be taken into account when analyzing eDNA from rivers and streams. eDNA is transported by advection (i.e., bulk flow of water) from the source, but is removed from the water column by degradation and adsorption to the benthic substrate (Nukazawa et al. 2018). Detected eDNA from a specific organism at a particular location in a lotic system may thus come from source organisms at the site, or further upstream. eDNA may also be re-suspended in the water column. Detection range of eDNA from an upstreams point source of target species has been measured to range from less than 100 m (Pilliod et al. 2014), to more than 10 km (Deiner & Altermatt 2014). eDNA transport and degradation in streams depend on properties of the stream, such as flow rate, and environmental conditions such as temperature. In addition, increase in water flow during periods of precipitation can dilute eDNA concentration and thus increase the likelihood for false negative detection (Curtis et al. 2020). To be able to better infer the spatial distribution of source organisms, it is thus important to collect this information. However, lotic systems hold the advantage that the movement of water is unidirectional. Thus, the maximum inland migration of an invasive species, e.g., an anadromous salmonid, can be inferred if samples are taken sufficiently far inland.

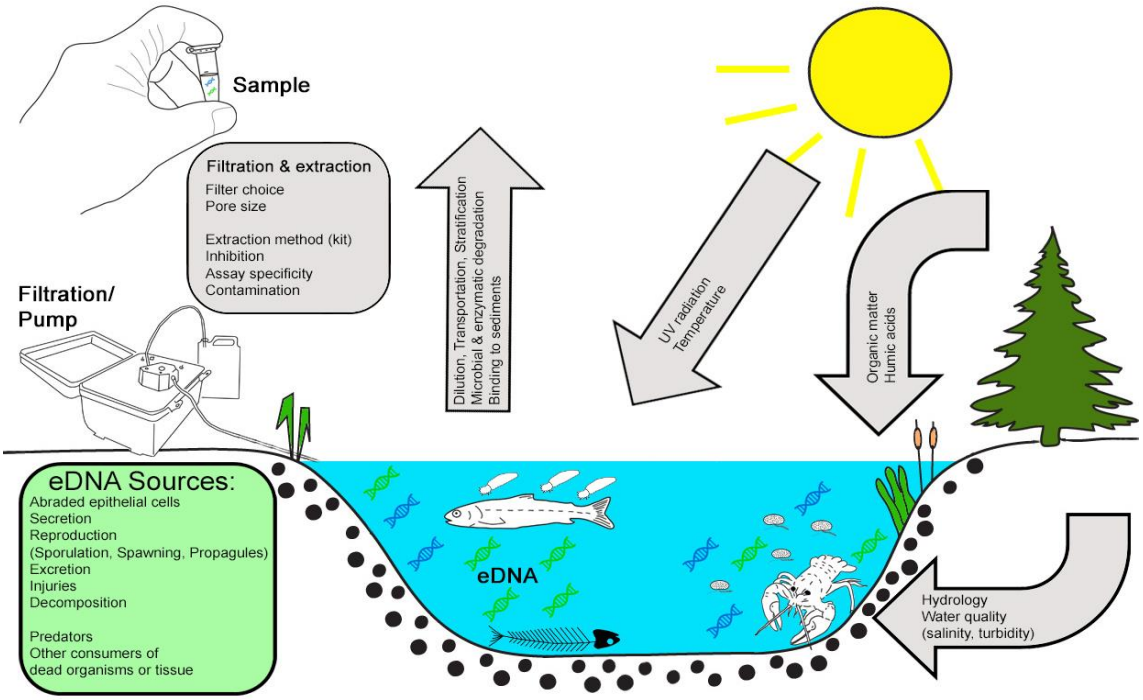


Figure 2 (previous page). A non-exhaustive overview of sources (underlined in light green) and influences on environmental DNA (underlined in grey). Cells with DNA are shed by both living and recently deceased organisms, while microorganisms are represented as single- or minor multicellular units containing their DNA. In this figure, target organisms are 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 ® Johannes C. Rusch. Figure from Rusch (2021) with permission.

2.1.3 eDNA in marine environments

Although the number of studies using eDNA for biodiversity monitoring have increased significantly over the last decade (Sepulveda et al. 2020), eDNA for monitoring invasive species in marine habitats is still in its infancy (Dunshea et al. 2021). In Norway, marine eDNA has been used to map the distribution of sea vomit (*Didemnum vexillum*, Fossøy et al. 2022a), and an ongoing pilot study commissioned by the Norwegian Environment Agency is developing a national monitoring program for marine alien species (Husa et al. 2022a). Depending on the organism type, the possibilities and challenges connected to the methodology can vary and are for some species more similar than for others. Regardless of the species, advantages include the cost- and time efficiency. The only equipment needed for sampling are devices for collecting water and to concentrate the DNA, filtering being recommended (Xing et al. 2022). Further, water sampling is not dependent on the species being present in the exact location of traps nor successful capture, making it less destructive and potentially more efficient than traditional methods used for biomonitoring which are is typically dependent on catch or visual observation (Rees et al. 2014, Kutti et al. 2020). Such approaches also have higher costs and low detection precision, particularly if the population density of the target species is low. Moreover, the continuous release of PCR-detectable DNA (eDNA) by organisms into the ocean (Lawson-Handley

2015) enables detection of rare and low-density populations (Lugg et al. 2018, Fraija-Fernández et al. 2020), typically present just after introduction, and it is thus suitable for early discovery and tracking of invasive species even before established in a new location. Once an assay for quantitative detection is established the subsequent laboratory analyses call for general molecular laboratory skills and can therefore to a much greater extent be more streamlined and "mass-produced" than labor intensive traditional morphological methods, which is also more dependent on expertise and experience - less and less available (Ruppert et al. 2019 and references therein). It should be noted however, that taxonomic expertise is necessary to develop the reference libraries needed for identification of species using eDNA, and that effective use of eDNA in biodiversity assessment and biomonitoring must consider the management questions of importance (Dunshea et al. 2021).

Other special challenges connected to marine habitats include current patterns and possibly density stratification which may both distribute eDNA up to tens of kilometers away from the source population in a few days (Barnes et al. 2016, Thomsen et al. 2015, Andruszkiewicz et al. 2019) and at the same time cause DNA diluted to the extent that it could reach undetectable levels. It has also been shown that DNA degrades at a higher rate in marine than in freshwater systems, presumably due to higher microbial activity (Thomsen et al. 2012, Strickler et al. 2015).

2.2 Introduction pathways

2.2.1 Terrestrial habitats

Introduction of terrestrial alien species to Norway occurs by natural- and anthropogenically mediated dispersal pathways. Although sometimes deliberate (e.g., introduction of alien plants to parks), human mediated introductions that are unintentional or accidental are of greatest relevance for using eDNA for detection and monitoring. The most common pathways for terrestrial alien species in Norway are escape from horticulture or other ornamental purposes, contamination of traded goods and stowaways (Sandvik et al. 2020).

2.2.2 Freshwater habitats

International reviews point to a major contribution from aquaculture, pet/aquarium trade and stocking activities as pathways of introduction for freshwater alien species (Nunes et al. 2015; Bernery et al. 2022). In Norway, the spread of invasive freshwater fish species is particularly linked to recreational fishing and the intentional illegal transport of regional alien species to new lakes and rivers (Bærum et al. 2021). Some alien fish species are used as live bait, and are subsequently unintentionally released into the environment. The introduction and spread of alien invasive freshwater crayfish, so far only involving American signal crayfish in Norway, is also a result of several illegal, human-assisted introductions (Johnsen et al. 2007; Vrålstad et al. 2011; Johnsen et al. 2020a). Such introductions simultaneously lead to the introduction of the crayfish plague pathogen *Aphanomyces astaci*, an oomycetes that is invisibly carried and transmitted by American crayfish (Vrålstad et al. 2014). The Atlantic salmon parasite *Gyrodactylus salaris*, was also introduced on several occasions via import of Atlantic salmon and rainbow trout from

Sweden (Johnsen & Jensen 1999; Hansen et al. 2003). It later spread via brackishwater migration of infected hosts.

2.2.3 Marine habitats

For marine non-indigenous marine species (NIMS), a recent report identified and mapped the most important vectors for introduction in Norway (Husa et al. 2022b). Historically, the transport of alien species in ballast water has been the main pathway into Norway, but with the implementation of the Ballast Water Convention this risk is reduced. Biofouling on vessels coming into the Norwegian coast is thus currently considered to be the most important vector for marine introduction of new alien species. An analysis of the frequency and origin (last port call) for 158 000 vessel arrivals into Norwegian ports in the period 2020-2021 revealed that the Oslofjord area and the west coast have the highest risk for introductions. Other vectors for alien marine species are floating debris which can carry fouling organisms, larvae and eggs to new areas, transport of live cleaner fish for the aquaculture industry, import and sale of living seafood and trade of pet fish for saltwater aquaria (Husa et al. 2022b).

2.3 Early detection of recently established species

Type of organism and environment, fate, and transport of eDNA, type of shed DNA and rates of shedding and decay have an influence on the presence (and quantity) of eDNA and therefore they are tightly related to the chances of early detection. Within this, eDNA shedding and decay rates themselves depend directly on many factors, including the organism itself (e.g., age, maturity, species, size) and external factors (e.g., water temperature, pH, presence of predators, water turbidity and PCR-inhibitors such as humic acids) (Andruszkiewicz Allan et al. 2021). The shed eDNA starts to decay immediately due to numerous processes, including microbial grazing, enzymatic breakdown, and UV light exposure (Strickler et al. 2015; Andruszkiewicz Allan et al. 2021; Mauvisseau et al. 2022). The Global Register of introduced and invasive species in Norway already contains 1591 species within Animalia, Chromista, Fungi and Plantae, therefore a more systematic assessment of eDNA shedding for individual taxonomic groups is needed to estimate possible biases in data sets and detection matrixes. In addition to the natural factors impacting eDNA detection rates, technical aspects should be taken into consideration that in fact follow general good practice strategies when working with eDNA i.e., significant number of replicates and usage of validated assays of high sensitivity i.e., assays with sufficiently low LOD, selection of the best sampling procedure and DNA collection and extraction practices. Last but not least, the probability of early detection will rise with increased sampling effort (sample numbers, water volumes, etc.).

2.3.1 Scanning for detection of potential invasive alien species

Scanning for detection of IAS can be considered a special case of early detection, where the IAS taxa in question are not specifically targeted or *a priori* expected as a nascent threat. As opposed to a specific program aimed at early detection of particular taxa, the methodology employed in a scanning context without *a priori* taxonomic expectation will necessarily be a general approach: usually metabarcoding with general primers that amplify a diverse range of organisms, or potentially a broad panel of species-specific approaches. Employing either of these approaches

requires that the rigorous operational and quality assurance standards outlined in previous sections are adhered to. In this particular context, an understanding of imperfect detection (false positives and false negatives) and how this impacts interpretation of eDNA IAS scanning data is critical. That is, unless detection errors are characterized for the specific scanning method employed, it can be difficult to decipher whether negative detection means no IAS present or whether a positive detection represents IAS presence. There is commentary on this issue in the literature as it relates to metabarcoding (Darling et al. 2020), which in essence argues that both primary researchers and managers alike must consider novel scanning IAS detections cautiously. Such caution would generally translate to a qualitative indication that requires further investigation, starting with the source data itself (Darling et al. 2020) and expanding to further field sampling if warranted.

2.3.2 Terrestrial environments

The project "Early detection of terrestrial alien species" is implemented for detecting newly established species of insects and plants (Jacobsen et al. 2021). For insects, specimens are collected using Malaise traps and classification of species is based on DNA-metabarcoding using a soft-lysis protocol that preserves the specimens for future analyses. The same approach is used in the project "National monitoring of insects" which is not specifically intended for monitoring early detection, but the large geographical scope has so far detected several new species for Norway, and many alien species (Åström et al. 2022). A continuation of this project will likely involve national geographical coverage of the monitoring program and early detection of many new insect species. In general, national monitoring programs using DNA-metabarcoding will likely be an important source for early detection of recently established species. For many groups of organisms, we still lack a comprehensive knowledge of the Norwegian species diversity, and large-scale mapping projects are likely to detect many new species for Norway, including both already established and newly established species.

2.3.3 Freshwater environments

Early detection is a challenge regardless of method as it demands being at the right place at the right time to reveal the unexpected event of an (often illegal) introduction of an alien species. However, if we ignore this problem and look at possibilities for improvements, eDNA monitoring is potentially a promising tool for early detection of invasive species in freshwater compared to classic monitoring methods.

In the autumn 2014, during the national surveillance of noble crayfish, the invasive alien signal crayfish was trapped alongside the native noble crayfish in Lake Rødenesjøen. This opened up the possibility to compare traditional trapping of crayfish and traditional caged-based surveillance (canary in a coalmine approach) of crayfish plague with eDNA monitoring of the signal crayfish and the spread of the causative agent of crayfish plague, the invasive alien parasite *A. astaci*. The study (Strand et al. 2019a) demonstrated the detection of eDNA from *A. astaci* several weeks before caged-based crayfish showed signs of *A. astaci* infection. eDNA from signal crayfish was detected in only 11% of the total of 69 collected water samples (~6,5 L of water per sample) and a total of 110 signal crayfish was caught using 960 trap nights (CPUE 0.12)

from the lake. This demonstrates the possibility of early detection of both the recently established signal crayfish and the spread of *A. astaci* in a large lake using eDNA monitoring. These samples was also screened for noble crayfish eDNA, and no eDNA detections together 0 CPUE of noble crayfish with the intensive trapping effort substantiated the local extinction of noble crayfish from the lake by autumn 2015 (Strand et al. 2019a).

The invasive fungal pathogen *Batrachochytrium dendrobatidis* (Bd), one of the key drivers of amphibian population declines worldwide since the 1970s, is an alien fungal freshwater species most likely spreading through pet trade (Scheele et al. 2019). The species, still listed as a SE door-knocker species (Artsdatabanken 2018), was recently detected in Norway (Taugbøl et al. 2021) by means of eDNA in newt populations without any sign of population decline, and is a good example of early eDNA detection of a new alien species.

2.3.4 Marine environments

In marine environments with large areas and relatively low density of potential alien species, environmental DNA could be an important early step to detect rare invasive species. However, this may vary with the different types of organisms and the methodology used (Rey et al. 2020, Husa et al. 2022a, Sepulveda et al. 2019). Several studies have demonstrated marine vertebrate detection using eDNA from relatively small volumes of ocean water (Kelly et al. 2014; Miya et al. 2015; Port et al. 2015; Thomsen et al. 2016; Andruszkiewicz et al. 2017). Both decay and dispersal of eDNA signals are, however, complex matters that calls for further work to better understand its temporal variation in marine environments (Akatsuka et al. 2018, Andruszkiewicz et al. 2019) and for different groups of organisms, before the methodology confidently can be used for early detection of recently established alien species. The work recently performed by Ellis et al. (2022) using eDNA to assess the geographic range of alien kelp (*Undaria pinnatifida*) and seastar (*Asteria amurensis*), two marine pest species in south-eastern Australia shows however, its potential. The methodology was also recently tested for the detection of the rapidly spreading sea vomit (*Didemnum vexillum*), for the first time observed in Norway in 2020, and the well-established Pacific oyster (*Crassotrea gigas*) (Anglès d'Auriac et al. 2017. The *D. vexiullum* pilot included locations where the species was identified visually as well as new locations. In 2022, a pilot study based on the two reports of Husa et al. (2022a, b) tested DNA-metabarcoding of several environmental materials for early detection of marine alien species as part of a future national monitoring program.

2.4 Case studies and monitoring programs of established populations in Norway

There are some examples of surveillance programs in Norway directed towards alien species that utilize environmental DNA (eDNA) monitoring as a sole or supplementary method. These include the alien crayfish pathogen *Aphanomyces astaci* (crayfish plague; Strand et al. 2022) and the alien invasive *Pacifastacus leniusculus* (signal crayfish; Johnsen et al. 2021). In addition there are case studies demonstrating the use of eDNA for the Atlantic salmon parasite *Gyrodactylus salaris* (Rusch et al. 2018, Fossøy et al. 2019; Hansen et al. 2022a, 2022b), the alien amphibian pathogen

Batrachochytrium dendrobatidis (Taugbøl et al. 2021, Ahmed et al. 2022), several alien fish species (Fossøy et al. 2018, 2022b; Engesmo et al. 2019, 2021, 2022) and the marine invasive carpet sea squirt *Didemnum vexillum*, also known as sea vomit (Fossøy et al. 2022a).

2.4.1 Imported ornamental plants with soil

The ornamental horticulture trade has been identified as a primary pathway for invasive alien plant introductions worldwide, and with traded plants and their potting soils this also includes high numbers of contaminant species. Identification of relevant species for alien species management requires utilizing all available resources of species information in a hierarchical way from source population, via species presence along pathways, to identification of relevant potential doorknocker species and established alien species. A basic monitoring program for the ornamental horticulture pathway based on morphological identification of live contaminant invertebrates and vascular plants is established in Norway (see e.g., Bruteig et al 2017, Westergaard et al 2020a, 2020b). Within this monitoring program, identification of live contaminant species and source populations using eDNA and DNA metabarcoding have recently proven successful, adding an additional tool enabling identification of species otherwise difficult to identify, e.g., Collembola, Diptera, and fungi (Farsund 2022, Westergaard et al. 2020a, 2020b). By analyzing the eDNA in soil samples from the imported pots of ornamental plants one can identify the species composition of the source population. However, the species in the source population may not be directly relevant for alien species or pathway management unless they are detected along the pathway. By extracting the live contaminants within the pathway (e.g., seeds and invertebrates from the soils of potted plants, or invertebrates from the leaves of the plants), one can identify relevant alien species to a receiving area.

2.4.2 Freshwater crayfish and *Aphanomyces astaci* (the crayfish plague pathogen)

Freshwater crayfish of North American origin are amongst the most prominent high-impact invasive invertebrates in European freshwaters. Populations of native European noble crayfish (*Astacus astacus*) are currently being lost at an alarming rate, largely because of North-American invasive crayfish that carry and transmit the crayfish plague pathogen *Aphanomyces astaci* (Holdich et al. 2009; Kouba et al. 2014, Johnsen et al. 2020b). In Norway, the North-American *Pacifastacus leniusculus* (signal crayfish) and *A. astaci* are both listed as an alien invasive species of very high risk (SE) (Artsdatabanken 2018). One of the first established surveillance programmes that adopted eDNA monitoring tools in Norway is the surveillance programme for *A. astaci* that combines qPCR screening of both the crayfish plague pathogen, the threatened native noble crayfish and the alien invasive signal crayfish that serves as a live reservoir of the pathogen. The *A. astaci* surveillance programme in Norway (commissioned by Norwegian Food Safety Authority - NFSA and conducted by the Norwegian Veterinary Institute - NVI) relied until 2015 on cage experiments with noble crayfish serving as “live baits” for disease monitoring. From 2016, classical cage experiments were combined with eDNA monitoring of *A. astaci* (Vrålstad et al. 2017), and from 2017, the cage experiments were excluded from the surveillance programme based on an overall assessment taking crayfish welfare and cost-benefit into account (Vrålstad et al. 2018; Strand et al. 2019a). From 2018, the program has collaborated with the National surveillance programme for freshwater crayfish, commissioned by the Norwegian Environment

Agency (NEA) and coordinated by the Norwegian Institute of Nature Research (NINA). This involves joint fieldwork and joint exploitation of water samples and molecular results in overlapping surveillance areas (Strand et al. 2019b; Johnsen et al. 2019; Fossøy et al. 2020). These synergies enable analyses of a slightly larger sample size than each program would allow separately. The crayfish surveillance involves a combination of classic trapping methods (catch per unit effort, CPUE), and eDNA data. The presence/absence of eDNA from noble crayfish and signal crayfish supplement the CPUE-results but is also used to broaden the range of monitored lakes/rivers (Johnsen et al. 2021). Detection of noble crayfish eDNA, combined with no eDNA detection from *A. astaci* and signal crayfish, substantiate the presence of non-infected noble crayfish which constitutes the desired habitat status. On the contrary, detection of signal crayfish eDNA and/or *A. astaci* eDNA alert on a possible spread of these alien species. In the Halden watercourse, a situation with infected signal crayfish downstream in the watercourse is monitored. Here, there has been a stable situation over the past 7 years with noble crayfish populations higher up in the system.

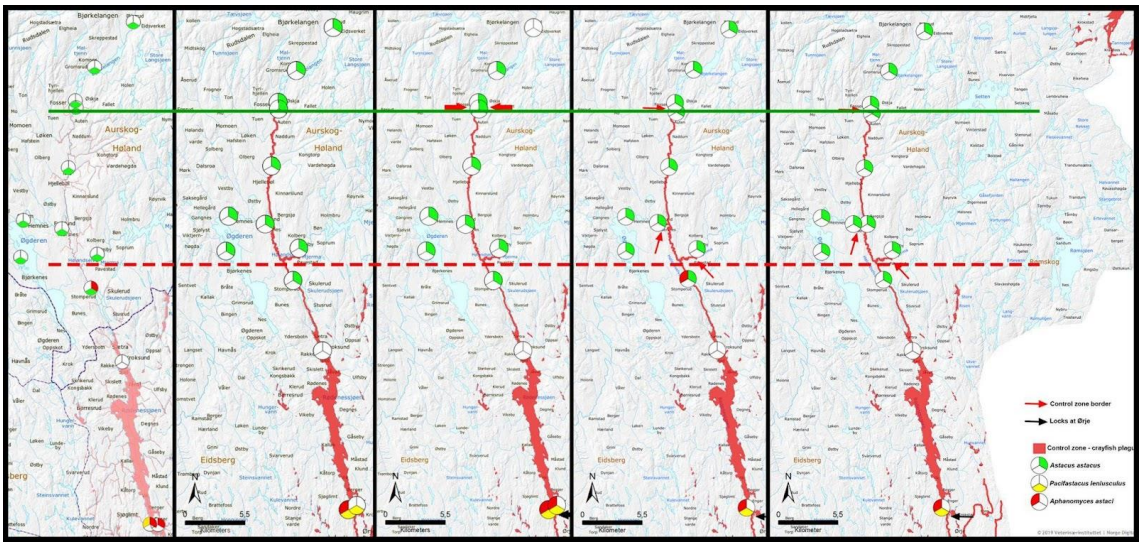


Figure 3. Presence/absence data of eDNA from noble crayfish (green) and its alien threats *A. astaci* (red) and signal crayfish (yellow). Figure from the surveillance programme for *Aphanomyces astaci* in Norway 2021 (Strand et al. 2021).

Figure 3 shows how eDNA data visualize the habitat status regarding detection (presence) / no detection (presumed absence) of the alien couple (*A. astaci* & signal crayfish) versus noble crayfish. Notably, "no detection" is not a proof of absence, and a likely result when population density in crayfish or prevalence of the pathogen is very low. Here, increased sample effort increases the likelihood of detection or strengthens the assumed absence (Johnsen et al. 2020a). Many studies have reported successful eDNA detection of freshwater crayfish at very low densities (Dougherty et al. 2016; Larson et al. 2017; Strand et al. 2019b), but often with low detection frequency. Johnsen et al (2020b) detected noble crayfish eDNA in a number of localities with very low crayfish densities, but the detection frequency was often very low. To achieve a 95% detection likelihood, they found that low-density lakes required an estimated five filter samples of 5L water, corresponding to ~25 L of water. In contrast, high-density lakes had a very high

detection frequency and required only two samples for a 95% detection probability (Johnsen et al. 2020b). Thus, rapid, low-volume sample effort seems insufficient for monitoring low-density crayfish populations. For early detection of alien crayfish, it seems that high sample effort is a key to success.

2.4.3 *Gyrodactylus salaris*

Environmental DNA monitoring has also been developed for the monogenean salmon parasite *Gyrodactylus salaris* (Rusch et al. 2018). The method has later been used to detect *G. salaris* upstream and downstream of migration barriers both in the river Driva and in the River Drammen (Fossøy et al. 2019, Hansen et al. 2021 and 2022a), and in the River Tuloma in northwestern Russia (Hansen et al. 2022b). In both Driva and Drammen rivers, conventional methods (electrofishing and parasitological examination) and eDNA-monitoring were carried out at the same time in all or several locations to compare the two methods. In River Drammen, a fish ladder for salmon in Hellefossen was closed in 2019 to stop the salmon from migrating upstream. This was done to exclude the stretch upstream of Hellefossen in a future eradication measure for *G. salaris*. To document if the closure had the desired reducing effect on the salmon and *G. salaris* population, a monitoring programme was initiated by the Food Safety Authority where eDNA was used alongside conventional monitoring. In this monitoring program (and in Fossøy et al., 2019), another species of *Gyrodactylus*, *G. derjavinoides* and its host, brown trout, were used as positive detection controls. Figure 4 shows the result from the second year of monitoring where there was a complete absence of *G. salaris* on the eDNA samples taken upstream of the closed migration barrier, while the site (control) downstream of the barrier show strong signals both for *G. salaris* and Atlantic salmon. The eDNA monitoring indicates that Atlantic salmon might still be present upstream of Hellefossen, however at a low density. All eDNA samples were positive for both brown trout and *G. derjavinoides*. Electrofishing revealed no Atlantic salmon and thus also no parasites above Hellefossen, while all the salmon caught downstream of Hellefossen were highly infected (more details can be seen in Hansen et al., 2021, 2022a).

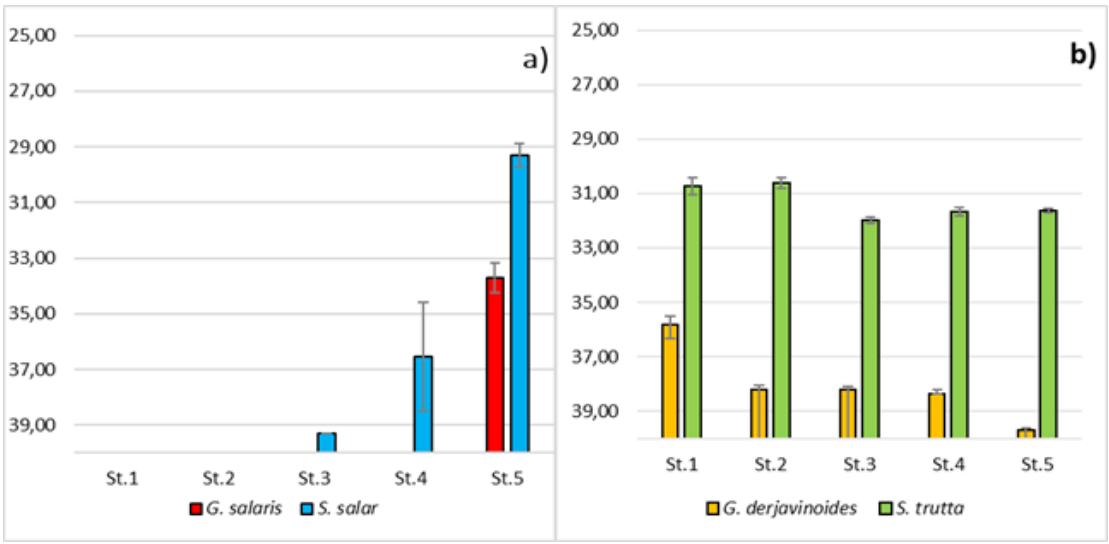


Figure 4. Bar plot showing the average *Cq*-value (±SD) of a) *Gyrodactylus salaris* (red), atlantic salmon, *Salmo salar* (blue) b) *Gyrodactylus derjavinoides* (yellow) and brown trout, *Salmo trutta* (green) per eDNA station. The *Cq*-value reflects the level of target DNA in the sample where lower *Cq*-value indicates higher DNA content in the sample. Station 1 is upstream of the natural migration barrier for salmon in River Drammen and station 5 is below the recently closed migration barrier.

In sum, the results from these studies on eDNA monitoring of *G. salaris* there is good correspondence in detection of both hosts and parasites by conventional methods and environmental DNA, while the assessment of abundance is more difficult. Based on an controlled infection trial with Atlantic salmon and *G. salaris* (Rusch 2021) where eDNA monitoring was assessed, it appears that there is minimal shedding from *G. salaris*. Additionally, it is impossible to know from a strong eDNA signal in a sample, whether the eDNA originates from free eDNA, from fragments or even from a whole parasite caught on the filter, thus making abundance assessment difficult. However, eDNA sampling can give indication of relative abundance provided several samples from each location and statistical modeling (e.g., occupancy modeling). Currently however, the detection probability and limit of detection of eDNA from *G. salaris* in field samples is not established. Therefore, the lack of eDNA detection of *G. salaris* cannot be used as proof of absence or used for declaration of freedom after eradication measures for *G. salaris* have been carried out. However, eDNA surveillance of *G. salaris* can supplement the conventional surveillance and potentially reduce the number of fish needed for conventional parasitology examination. The World Organisation for Animal Health, WOAH, has recently included eDNA as a method for non-lethal sampling of *Gyrodactylus salaris* in the Manual of Diagnostic Tests (WOAH, 2021). It is important to note that the assays in use today can neither differentiate between pathogenic and non-pathogenic strains of *G. salaris*, nor between *G. salaris* and the benign grayling, *Thymallus thymallus*, parasite *G. thymalli*. The use of eDNA methods in a river with presence of *G. thymalli* can yield positive results for *G. salaris* even if the parasite is not present, and this can be a real problem in several rivers in Norway.

2.4.4 Pink salmon - *Oncorhynchus gorbuscha*

A particular example of stream-inhabiting invasive species is the anadromous salmonid Pink salmon. This species is mainly obligate anadromous and is the only such invasive fish species in Norway. In recent years this species has increased substantially in Norwegian rivers, and there is a concern that it will affect native wild Atlantic salmon, both by direct competition and by the spread of salmonid pathogens and parasites (Hindar et al. 2020, VKM report 2020:01). Pink salmon is native to the Pacific Ocean, but was introduced to the White Sea outside the Kola peninsula between c. 1950's and the beginning of the 2000's. From there, individuals migrated and established self-sustaining populations in Norway (Hindar et al. 2020, VKM report 2020:01). Initially, its distribution was mostly limited to Northern Norway, but it has in recent years spread further south. In 2021, spawning pink salmon was observed in rivers all along the Norwegian coastline, as far south-east as Akerselva, Oslo. In Grense Jakobselv, Finnmark, there was an 8-fold increase in spawning pink Salmon from 2019 to 2021 (Berntsen et al. 2022). However, it was unclear how far up in the river the Pink salmon migrated. Quantification of Pink Salmon eDNA

from 6 stations along the river showed that eDNA concentrations decrease exponentially with distance from land (Engesmo et al. 2022). Furthermore, Pink Salmon eDNA was detected at the station furthest inland, where the fish was not observed. However, there are no barriers to migration to this station, it is thus not unlikely that the fish migrates further than observed. In the river Tana, eDNA was used to monitor the pink salmon invasions in both 2019 and 2021 (Fossøy et al. 2022b). Also here, eDNA found traces of pink salmon further inland than observed by other methods. Moreover, the relatively simple field method combined with low total costs allowed surveying many sites, and 21 and 24 tributaries were analyzed in 2019 and 2021 using eDNA, whereas only one or a few sites were surveyed by other methods. This suggests that for this species in this area, eDNA may be a more sensitive detection tool than sightings alone, and more cost-effective than conventional methods (Engesmo et al. 2021, 2022, Fossøy et al. 2022b).

2.4.5 Canadian pondweed - *Elodea canadensis*

There are not many examples of eDNA being used to track invasive, aquatic macrophytes. But it has been successfully done for the canadian pondweed *(Elodea canadensis).* This is an invasive aquatic plant which originates from North America and has colonized Europe at least since it was first recorded in Ireland in 1836 and Britain in 1842 (Simpson 1984). The species was first observed in Norway in 1925, and has now spread to more than 100 southern Norwegian water bodies (Mjelde et al. 2012) and has become the most widespread aquatic invasive macrophyte in Europe (Hussner 2012). The Canadian pondweed is a rooted submerged flowering plant which grows mostly in standing waters (canal, ditches, ponds, lakes). It is dioecious, i.e. individual plants have only male or female flowers. Male flowers are rarely seen in Europe, suggesting that the plant reproduces mainly vegetative with overwintering buds and stem fragments (Spicer & Catling 1988). Vegetative propagules can spread rapidly within lakes and downstream watercourses. Other vectors of dispersion can be by birds, but also most likely people through recreational boating, fish farming or angling (Mjelde et al. 2012, Anderson et al. 2014).

There has been developed a qPCR assay for Canadian pondweed, which was tested on spatial transect field study in the river Leira (Viken) as well as a time series field study in lake Steinsfjorden (Viken) (Anglès d'Auriac et al. 2019). The autumn (October) appeared to be the best period for sampling as plant biomass was at its peak with onset of decay. The samples from October detected eDNA quantities about 1000 times higher than the lowest point observed which was in June. Turbidity due to clay particles did not hamper eDNA detection and the rate of disappearance was in the range of one Log10 eDNA per km in the stream (Anglès d'Auriac et al. 2019).

2.4.6 American lobster - *Homarus gammarus*

Even if it has been demonstrated that eDNA analyses can be used to detect crustaceans with an exoskeleton including invasive/alien species (Forsström & Vasemägi 2016; Dunn et al. 2017; Geerts et al. 2018; Crane et al. 2021; Danziger & Frederich 2022) it has been noted that decapod reads in sediment metabarcoding data are generally low (Lanzén pers. comm.). Several factors may impact the likelihood of detecting eDNA from these animals and should be considered when future monitoring programs are planned. Water temperature influences target species activity and thus also eDNA shedding rates. Life cycle stages do affect the DNA release from crustaceans,

with moulting and spawning likely to increase it (Dunn et al 2017, Crane et al 2021, Troth et al. 2021). There could also be differences with regards to whether the invasive species in question is day or night active.

The American lobster (*Homarus gammarus*) is native to the Northeast Atlantic and it has traditionally been isolated geographically from its European counterpart by the Atlantic Ocean. Lobster import to European markets has, however, caused spreading to European waters, including the Norwegian coast. Based on morphology, American lobster individuals have been observed at several locations along the coast as far North as Ålesund (Jørstad et al. 2011), but since it may be difficult to distinguish *H. americanus* from *H. gammarus* (Agnalt pers. comm) the reported incidents likely underestimate reality. Between 2000-2017, 35 lobsters delivered to the Institute of Marine Research (IMR) were identified as *H. americanus*, using microsatellite markers (Jørstad et al. 2007). To aid detection of and distinguishing between the two lobster species, Sundt (2021) applied digital droplet (dd) PCR assays developed for detection of European (*Homarus gammarus*) and American lobster (*H. americanus*). Both assays include primers and probes targeting fragments of cytb genes. *Homarus americanus* primers and probes had previously been tested *in vitro* by Knudsen et al. (2020) whereas the *H. gammarus* primer/probe was developed in silico using GeneBank and tested by Sundt (2021). Oligos developed for the 2 lobster species are given in Supplement 2. (F: Forward primer, R: Reverse primer, P: Probe)

The assays were optimized with regards to concentration of primer/probe, type of fluorescent dye (ddPCR EvaGreen Supermix or ddPCR Supermix for Probes), annealing- and stabilization temperature and number of amplification cycles. Under controlled environments, using indoor aquariums, Sundt (2021) tested the specificity of the *H. americanus* assay, the amount of DNA shed from lobsters during 24 hrs, and the rate at which DNA degraded in closed tanks and stable temperatures. She finally applied both assays on samples collected from two different natural locations, one at which a *H. gammarus* population inhabits and one where a *H. americanus* female with remains of hybrid eggs was observed in 2016. The ddPCR assay worked well for both species, and the study showed that low, but amplifiable amounts of *H. americanus* eDNA were present in all samples collected from laboratory experiment, with a half-life of target DNA estimated to 27 hrs. No eDNA from either *H. americanus* or *H. gammarus* was detected in the field samples. Samples were collected from an area where a *H. gammarus* population is known to be present indicating that the eDNA approach as applied in current study is not suitable for detection of lobsters.

Danziger & Frederich 2022 argues that to avoid false positives of species using eDNA one needs to check primer specificity for each population in question. Filtration and DNA extraction methods are additional aspects to consider carefully (Geerts et al. 2018; Eichmiller et al. 2016; Kumar et al. 2020). The DNeasy Blood and Tissue kit used by Sundt (2021) successfully extracted DNA from the tissue samples used for positive controls with the chosen primers and probe for both lobster species. We are thus inclined to interpret lack of signal from water samples collected in the field, as a result of sub optimal filter type or sampling strategy such as amount of water filtered for DNA extraction, collecting water at different seasons and/or time of day to ensure collecting at time points of the life cycle with highest shedding rates (Crane et al. 2021), highest activity levels (Moland et al. 2011) (and hence more likely higher shedding rates), and also

collecting samples from sediments and water slurry as well as from the water column (only the latter was done in Sundt´s (2021) study).

3. Decision diagram for detection and monitoring of invasive alien species

Even when using the strictest sample and laboratory operating procedures and most robust validation procedures for molecular assays, we must acknowledge the fact that what a positive molecular detection represents is the almost certain presence of the target molecule and not necessarily the presence of the organism or a viable population of the species (Morisette et al. 2021). As a result, uncertainties, false positives and sometime inconclusive results are associated with eDNA detection, and missteps in early adoption of eDNA tools combined with imperfect communication can lead to incomprehension or distrust of eDNA by managing authorities and other stakeholders (Amberg et al. 2015; Jerde 2021; Sepulveda et al. 2020; Morisette et al. 2021). This is a conceptual departure from traditional techniques where the confirmed presence of IAS, and highly improbable chance of false positive detection, has been the gold standard. Of course, traditional approaches are also generally less sensitive and thus prone to relatively high false negative rates. Since higher, lower cost, scalability and amenity of the molecular approaches make them a key tool for IAS management, a decision support approach that accounts for the nuances of imperfect detection and the difference between detecting molecules and organisms is required.

Following examples in the literature (Sepulveda et al. 2020; Morisette et al. 2021), the decision support process should involve certain considerations at the earliest conceptual stage of IAS monitoring, through to the point of management interventions based on molecular data. Initially, these include defining goals, formulating clear and robust operating standards/procedures, understanding risk tolerance, formulating proactive and reactive management approaches with incorporation of stakeholder perspectives and clearly communicating these (Mosher et al. 2020). The fundamental goal of the monitoring program may be early detection, monitoring of established species (for example, their range expansion) or the effectiveness of eradication programs. Monitoring goals are inherently tied to whether molecular approaches are the most suitable. For example, in certain environments such as enclosed water bodies, monitoring of eradication efficacy with molecular tools may not be suitable due to eDNA shedding from dead organisms and/or other mechanisms of molecule transport/persistence. Furthermore, the method limitation, seasonality or species ecology could hamper eDNA based IAS detection, compared to results obtained with traditional monitoring tools.

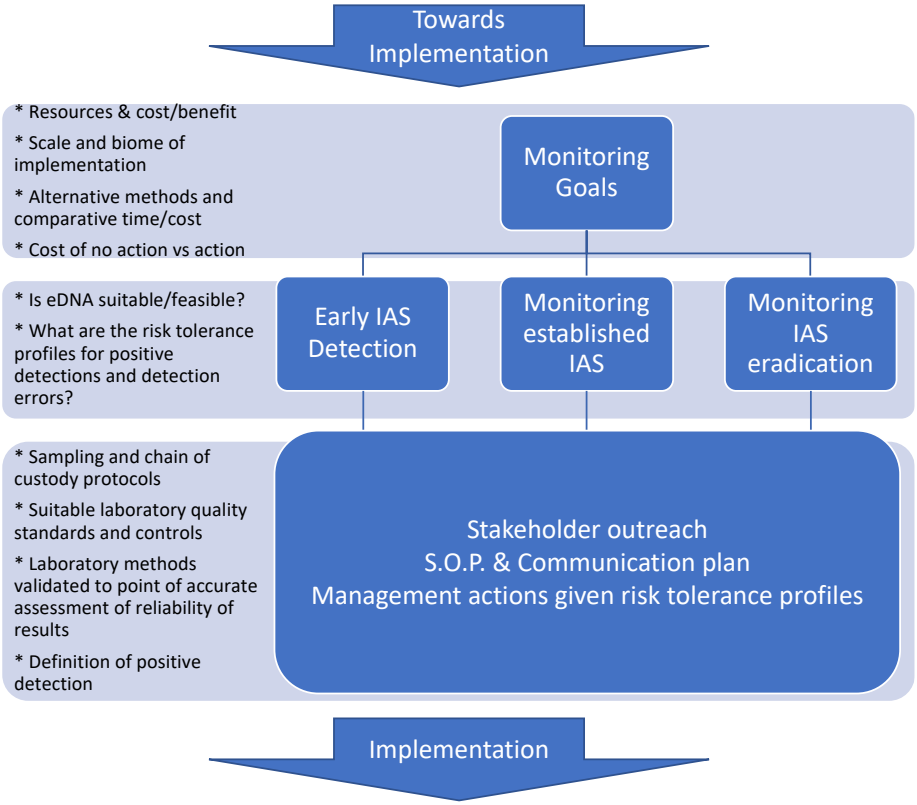
Morisette et al. (2021) highlighted four critical points that should be considered for the use of eDNA monitoring for IAS management: (i) why an invasive species management should consider using eDNA, (ii) can the use of eDNA help in the surveillance needs, (iii) what are the important components to operational implementation, and (iv) how should eDNA tools be used in species surveillance? Furthermore, it is important to assess how eDNA based detection can be

incorporated into already existing monitoring programs, and used together with traditional employed methods, before considering an eventual method replacement. A further early consideration that has ramifications all the way through the planning, implementation and decision support process is the risk tolerance profiles of uncovering the presence of target IAS taxa and the ramifications of imperfect detection (i.e., false negatives/positives). Risk tolerance refers to placing molecular results in context to guide managers as to the ramifications of a detection (and detection error) and how quickly a decision should be made (Sepulveda et al. 2020). Here it is difficult to generalize because these parameters are not only dependent on the monitoring goal/context and the biology and ecology of the IAS, but also what level of 'damage' a focal IAS detection represents and in what dimension (e.g., ecologically, economically, socially). For example, risk tolerance may be high where a slight range extension of an established and/or relatively benign IAS is documented, but extremely low where an early detection of a highly destructive doorknocker species is documented.

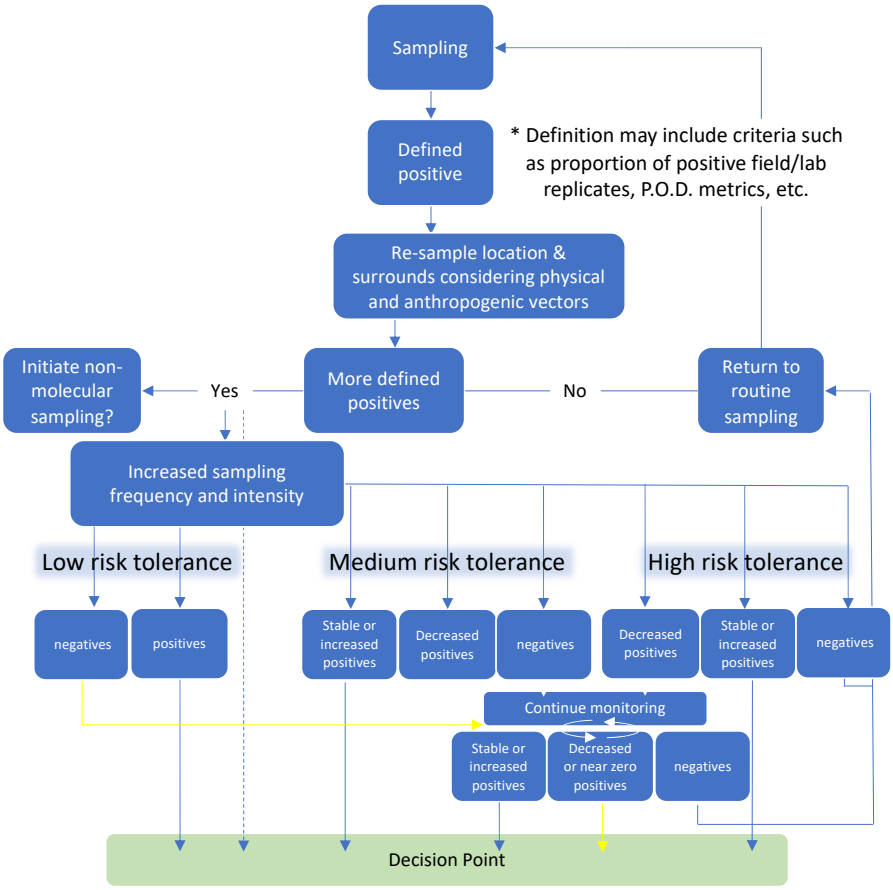
An example of workflow towards implementing an IAS monitoring program and a decision support tree is presented in Fig 5. It should be noted that specific levels of support for particular risk tolerances are not provided, as above, these are necessarily context specific.

Figure 5 (over page). Workflow towards implementation of IAS monitoring using molecular methods, after Sepulveda et al. (2020) and Morisette et al. (2021). Early considerations consist of major tasks (blue) and key considerations within each (black text). Once implementation is underway decision support is needed. Here arrows represent the flow of decision from results, dotted blue and yellow arrows represent potential flows under low risk tolerance. Note that the actual values in relation to positive detections will be dependent on (1) the context of the validation state of the molecular assays, (2) the sampling and replication protocol implemented and (3) the ecological, economical and social risk tolerance profiles in light of (1) and (2). Abbreviations: S.O.D = Standard operating procedure, P.O.D. = Probability of detection, IAS = Invasive alien species.

Early considerations



Decision Support Example – Post-Implementation



4. Recommendations

As for all species-specific assessment programs, the use of eDNA in detection and monitoring of alien and doorknocker species relies on background knowledge of the organism's biology and ecology. For alien and doorknocker species, information on the paths of distribution and invasion also is required to establish effective monitoring programs. Furthermore, knowledge of the ecology and fate of eDNA in the actual study system is of relevance when considering sampling strategy (e.g., locality, frequency, methodology, etc.) and suitable protocols and assays for the target organism must have been rigorously tested.

In this report we point to the many aspects that must be considered when establishing monitoring programs of alien or doorknocker species using eDNA, and give some specific examples of existing protocols and programs. We recommend that:

- The requisite reference sequences of target IAS are available and specific effort is made to sample and sequence IAS in the geographical region as well as closely related taxa. If these data are not currently available, we would recommend this as a top priority as these are used for both construction of barcode reference libraries and design of species-specific assays.
- That rigorous minimum operational and quality assurance standards are applied for field sampling and laboratory workflows. These should include negative controls throughout the entire sample collection and laboratory analysis process, including blank field samples, blank DNA extractions, no-template control PCRs, and index blanks (if appropriate). Exogenous positive controls should also be used to indicate PCR efficacy.
- Minimum reporting requirements for all the different laboratory approaches in section 1.4.2 should be adhered to.
- That incidental detections using general metabarcoding approaches are thoroughly vetted and if deemed robust, considered a qualitative indication that requires further investigation.
- That standard operating procedures, management actions and communication plans are formulated considering the points in Fig. 5 before undertaking eDNA monitoring programs, rather than *ex post facto.*
- In consideration of the molecular approach and its stage of validation, defining what constitutes a positive detection and what that represents (along with associated imperfect detection) as it relates risk tolerance for the specific IAS scenario in question. This is an important step that then facilitates formulation of management decision support thresholds.
- The provided decision support diagram is used on an individual case-by-case basis when considering eDNA use in relevant monitoring programs.

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6. Attachments

6.1 Norsk sammendrag av kapittel 3

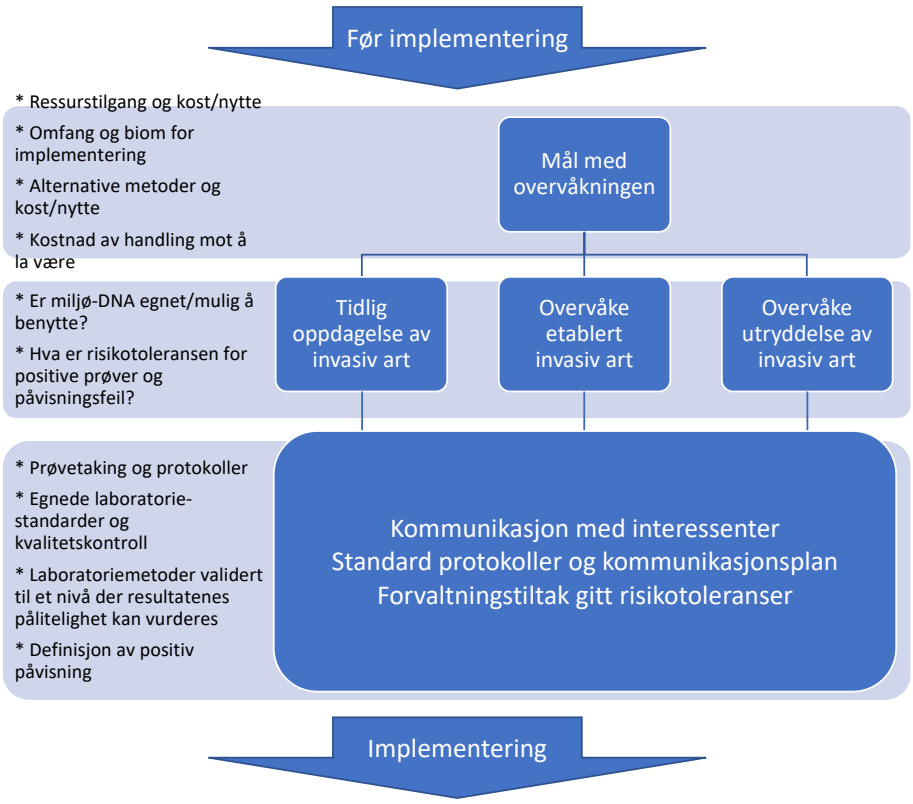
En molekylær påvisning av en art representerer kun tilstedeværelsen av målmolekylet og ikke nødvendigvis selve organismen. Falske positive funn, usikre resultater, feilsteg i tidlig bruk av miljø-DNA og dårlig kommunikasjon kan medføre mistillit til bruk av miljø-DNA basert metodikk hos forvaltningsmyndigheter og andre interessenter. Tradisjonelle metoder bekrefter tilstedeværelse gjennom funn av organismen og falske positive funn er usannsynlige, men metodene er ofte mindre følsomme og har større sannsynlighet for falske negative funn enn metoder som benytter miljø-DNA. Høyere følsomhet, lavere kostnader, bedre skalerbarhet og bekvemmelighet gjør metoder som benytter miljø-DNA til nøkkelverktøy for forvaltning av invasive fremmede arter. For at bruken av disse metodene skal fungere tilfredsstillende, er beslutningsstøtte som tar høyde for nyansene og utfordringene knyttet til miljø-DNA viktig.

Beslutningsstøtte bør ivareta alle steg fra tidlig planlegging av fremmedartsovervåking til forvaltningsmessige grep er bestemt. Dette inkluderer definisjon av mål og prosedyrer, forståelse av risikotoleranse, formulering av proaktive og reaktive styringstilnærminger, og inkorporering av interessenters behov og synspunkter. Målet for et overvåkingsprogram kan være tidlig oppdagelse, overvåking av etablerte arter (f.eks. for å hindre utvidelse av utbredelsen), eller å måle effektiviteten til et utryddingsprogram. I alle tilfeller er det viktig å vurdere om molekylære tilnærminger er de best egnede for å nå overvåkningsmålet. I lukkede vann kan for eksempel bruk av miljø-DNA i overvåking av effekter i et utryddingsprogram være dårligere egnet enn tradisjonelle metoder fordi miljø-DNA frigjøres fra døde organismer, eller kan forbli lenge i miljøet av andre grunner. Av og til kan også sesong eller en arts økologi gjøre miljø-DNA basert påvisning av fremmede arter lite anvendelig sammenlignet med tradisjonelle overvåkingsverktøy. I litteraturen er det fremhevet fire viktige spørsmål for bruk av miljø-DNA i overvåking av invasive fremmede arter: (i) hvorfor forvaltningen bør vurdere å bruke miljø-DNA, (ii) om bruk av miljø-DNA kan hjelpe forvaltningens overvåkingsbehov, (iii) hvilke komponenter er viktige for operativ gjennomføring, og (iv) hvordan bruke miljø-DNA verktøy i overvåkning av arter.

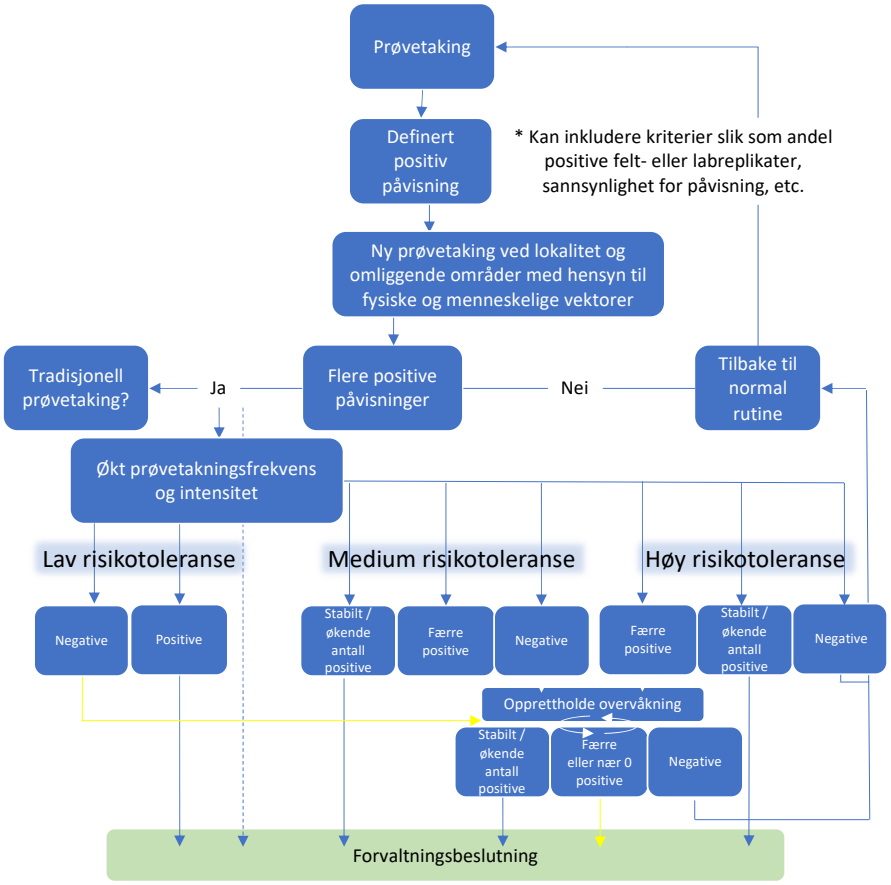
Det er viktig å vurdere hvordan miljø-DNA basert påvisning kan inkorporeres i allerede eksisterende overvåkingsprogram og brukes sammen med tradisjonelle metoder før man vurderer erstatning av metode. Tidlige vurderinger av risikotoleranse er står sentralt i hele prosessen. Risikotoleranse setter resultatene fra molekylær-basert overvåkning i kontekst, og veileder forvaltningen i forhold til konsekvensene av en påvisning (og påvisningsfeil) og hvor raskt en beslutning bør tas. Generalisering er vanskelig her, da parameterne er avhengig av målet med overvåkingen, sammenhengen, biologien og økologien til den fremmede arten, samt skadenivået en påvisning av en invasiv fremmed art vil være for samfunnet (økologisk, økonomisk eller sosialt). Risikotoleransen kan være høy om det dokumenteres en liten utvidelse i utbredelsen av en etablert og/eller relativt godartet invasiv fremmed art, men ekstremt lav der det dokumenteres en tidlig påvisning av en svært destruktiv dørstokkart.

Figur 5 (neste side) viser et eksempel på arbeidsflyt for implementering av et overvåkingsprogram for en invasiv fremmed art og et tre for beslutningsstøtte. Vi foreslår ikke beslutningsstøtte for de enkelte risikotoleransene da disse er avhening av hver enkelt situasjon og sammenheng. Etter Sepulveda et al. (2020) og Morisette et al. (2021). Tidlige vurderinger består av hovedoppgaver (blå) og sentrale vurderinger for hver av disse (sort tekst). Når implementeringen er igangsatt, er det behov for beslutningsstøtte. Her representerer pilene beslutningsflyten fra resultater til beslutning, stiplede blå og gule piler viser mulige veier under lav risikotoleranse. Merk at de faktiske verdiene av positive påvisninger vil være avhengig av (1) valideringstilstand til den molekylære analysemetoden/protokollen som er brukt, (2) hvilken prøvetakingsprotokoll som er benyttet (f.eks. antall replikater) and (3) den økologiske, økonomiske og samfunnsmessige riskotoleransen gitt (1) og (2).

Tidlige vurderinger



Eksempel på beslutningsstøtte etter implementering



6.2 Examples of invasive and doorknocker species of concern in Norway with species specific assays for detection

Scientific name	English name	Norwegian name	Invasive/ doorknocker	Reference	Gene target, primer/probe (5' - 3')	In list of Union concern*
Aphanomyces astaci	Crayfish plague	Krepsepest	Invasive fungus	Vrålstad et al. (2009)	ITS AphAstITS-39F: AAGGCTTGTGCTGGGATGTT AphAstITS-97R: CTTCTTGCGAAACCTTCTGCTA AphAstITS-60T: 6FAM-TTCGGGACGACCC-MGBNFQ	
Batrachochytrium dendrobatidis	Chytrid fungus	Bd	Invasive fungus	Boyle et al. (2004) used in Taugbøl et al. (2021)	ITS ITS1-3: CCT TGA TAT AAT ACA GTG TGC CAT ATG TC 5.8S Chytr: AGC CAA GAG ATC CGT TGT CAA A Chytr MGB2: CGA GTC GAA CAA AAT	
Chionoecetes opilio	Snow crab	Snøkrabbe	Invasive crayfish	Kang (2019)	COI CO-F: GTATAAGCCTAGATCAAATACCA CO-R: AAAGTATGGTAATTGCTCCAGC	
Crassostrea gigas	Pacific oyster	Stillehavsøsters	Invasive mollusk	Bott & Giblot-Ducray (2015)	COI ForwP: TCTTATTCGTTGGAGACTTTATAACCCT RevP: ATAACCAACGCATGCCTAGTTAC TaqMan MGB probe: CCC CGT GAC TTA TAA TG	
Didemnum vexillum	Sea vomit	Japansk sjøpung (havnespy)	Invasive tunicate	Matejusova et al. (2021)	COI DvexFP: CGACTAATCATAAAGATATTAGAACA DVexRP: TTCTTGTAGAACTTAATTCTATTCG-3 DvexProbe: FAM-ATAGT{T}{A}GAGCT{A}G{A}TTTAGT{A}TA{A}-BHQ1	
Didemnum vexillum	Sea vomit	Japansk sjøpung (havnespy)	Invasive tunicate	Gargan et al. (2022b) used in Fossøy et al. (2022a)	COI: Dvex-F1: TGAGCTGCTATAGTTMGAGCTAGATTTAGT Dvex-R1: TTCAAACGRGGAAAAGCTATATC Dvex-PR: ATAATTTTGTTATCACGGCTCAT	
Elodea canadensis	Canadian Waterweed	Vasspest	Invasive plant	Anglès d'Auriac et al. (2019)	trnL-trnF spacer EctrnL_F: TTTCTCCTTCATTGTATTCTTTCACA EctrnL_R: TGTTGATTTCTATCTGTATTGTAGAC EctrnL_P: FAM-TCCGAACAGAAATGCCTCTCTCTTATCC-BHQ1	
Eriocheir sinensis	Chinese mitten crab	Kinaullhåndskrabbe	Doorknocker crayfish	Andersen et al. (2018)	Cyt-B Erisin_cytb_F02: ACCCCTCCTCATATCCAACCA Erisin_cytb_R02: AAGAATGGCCACTGAAGCGG Erisin_cytb_P02: FAM-TTTGCTTACGCTATTTTACGATCAATTCCT-BHQ1	yes

Scientific name	English name	Norwegian name	Invasive/ doorknocker	Reference	Gene target, primer/probe (5' - 3')	In list of Union concern*
Eosx lucius	Northern pike	Gjedde	Regional invasive freshwater fish	Fossøy et al. (2017)	CytB El_CytB_177-199_F: CTCCACAGCCTTCTCATCAGTCT El_CytB_218-241_R: TTCGGATAAGTCAGCCGTAGTTAA El_CytB_201-216_P: CCACATCTGCCGGGAC	
Faxonius limosus	Spiny-cheek crayfish		Doorknocker crayfish	Mauvisseau et al. (2018)	COI CO1-Ol-01-Forward: CCTCCTCTCGCTTCTGCAAT CO1-Ol-01-Reverse: AACCCCTGCTAAATGCAACG Probe: CTCATGCAGGGGCATCAGTGG	yes
Faxonius rusticus	Rusty crayfish		Doorknocker crayfish	Dougherty et al. (2016)	COI Orusticus_COI_5F: CAGGGGCGTCAGTAGATTTAGGTAT Orusticus_COI_5R: CATTCGATCTATAGTCATTCCCGTAG	yes
Faxonius virilis	Virile crayfish		Doorknocker crayfish	Knudsen et al. 2019	COI Faxvir_co1_F05: CAGGAAGATTGATTGGGGACGA Faxvir_co1_R01: GTTATCCCTGCAGCCCGTAT Faxvir_co1_P01: FAM-TTGGAGGTTTCGGGAACTGGCTGATTC-BHQ1	yes
Gyrodactylus salaris			Invasive pathogen	Rusch et al. (2018)	ITS G.sal208F: GGTGGTGGCGCACCTATTC G.sal149R: ACGATCGTCACTCGGAATCGAT G.sal188P: FAM-CAAGCAGAACTGGTTAAT-MGBNFQ	
Gyrodactylus salaris			Invasive pathogen	Collins et al. (2010), used in Fossøy et al. (2019)	ITS Gsal2_F: CGATCGTCACTCGGAATCG Gsal2_R: GGTGGCGCACCTATTCTACA Gsal2_P: FAM-TCTTATTAACCAGTTCTGC	
Hemigrapsus sanguineus	Japanese shore crab		Doorknocker crayfish	Knudsen et al. (2020)	COI Hemsan_COI_F01: CCTGGGCCGGTATAGTAGGT Hemsan_COI_R01: GGGGCTCCGAGTATAAGTGG Hemsan_COI_P01: FAM-CGAGCAGAATTAAGACAACCAGGAAGC-BHQ1	
Homarus americanus	American lobster	Amerikahummer	Invasive crayfish	Andersen et al. (2018)	Cyt-B Homame_cytb_F02: TTTTAGTAGCAGCAGCGACTCTT Homame_cytb_R14: CCAAGAAGGTAGGGATTTAGAAGA Homame_cytb_P12: FAM-TGCAAGACATATTGATAAAGTTCCATTCCA-BHQ1	
Lepomis gibbosus	Pumpkinseed	Rødgjellet solabbor	Invasive freshwater fish	Clusa & García-Vázquez (2018)	16S LeGi-16S-F: GGACACGGGGCTAAACCAAAT LeGi-16S-R: GGGCTCTTAGTTGTGGAATTGCA	yes

Scientific name	English name	Norwegian name	Invasive/ doorknocker	Reference	Gene target, primer/probe (5' - 3')	In list of Union concern*
Lepomis gibbosus	Pumpkinseed	Rødgjellet solabbor	Invasive freshwater fish	Engesmo et al. (2020)	CytB Pumpkinseed:CytB-F: GCCGCCACTGTAATTCACC Pumpkinseed:CytB-R: TGCGTCCGAGTTTAAGCCTA Pumpkinseed:CytB-P: CACGAAACAGGCTCCAACAACCC	
Neogobius melanostomus	Round goby	Svartmunnet kutling	Doorknocker fish	Nathan et al. (2015), Nevers et al. (2018)	COI GobyCOI-F2d: CTTCTGGCCTCCTCTGGTGTTG GobyCOI-R2d: CCCTAGAATTGAGGAAATGCCGG GobyCOI-Pr: 6FAM-CAGGCAACTTGGCACATGCAG-BHQ3	
Oncorhynchus gorbuscha	Pink salmon	Pukkellaks	Invasive anadromous fish	Gargan et al. (2022a), used in Fossøy et al. (2022b)	COI PinkF: CACCGCCMTAAGCCTACTAA PinkR: AGGCATGGGCTGTAACGATT PinkPr: CGCTCTTCTAGGGAATGACCA	
Oncorhynchus gorbuscha	Pink salmon	Pukkellaks	Invasive anadromous fish	Knudsen et al. (2022)	COI Oncgor_CO1_F09: TCCTTCCTCCTCCTCCTTTC Oncgor_CO1_R06: TGGCCCCTAAAATTGATGAG Oncgor_CO1_P06: FAM-CAGGGGCATCCGTCGACTTAACTAT-BHQ1	
Pacifastacus leniusculus	Signal crayfish	Signalkreps	Invasive crayfish	Rusch et al. (2020)	COI Paclen_CO1_F: GAGTGGGTACTGGATGAACTG Paclen_CO1_R: GAAGAAACACCCGCTAAATGAAG Paclen_CO1_P: VIC-CAGCGGCTATTGCT-MGBFNQ	yes
Paralithodes camtschaticus	Red king crab	Kongekrabbe	Invasive crayfish	Jensen et al. (2012)	COI Paca3F: GCAGTAATAAATACGGATCACACAAATAA Paca3R: GTCTAAGGTTATTCCTTGTGGACGT Paca3T: TGGCTGGAGTATCTTCTATTTTAGGGG	
Perccottus glenii	Amur sleeper		Doorknocker fish	Roy et al. (2018)	COI 279F: CTTTTGACTTCTTCCTCCTTCACTA 365R: GGATAAACAGTTCAACCTGTACCC Pr309F: ACTCTTATCCTCCTCAGGAG	yes
Phoxinus phoxinus	Eurasian minnow	Ørekyt	Regional invasive fish species	Fossøy et al. (2017)	CTRL Orekyt_CTRL_19-42_F: GGATGGCTAACCCATATCTCAACT Orekyt_CTRL_68-88_R: GTCAAACCCCAAAAGCAAGGA Orekyt_CTRL_51-64_P: CGCACGCTCTCGAA	

Scientific name	English name	Norwegian name	Invasive/ doorknocker	Reference	Gene target, primer/probe (5' - 3')	In list of Union concern*
Phoxinus phoxinus	Eurasian minnow	Ørekyt	Regional invasive fish species	Engesmo et al.(2022)	CytB Phopho_cytb_F07: CGT CAC CCC ACC CCA TAT TC Phopho_cytb_R09: GGG TGT TCT ACG GGT ATG CC Phopho_cytb_P07: -TGC CTA TGC TAT CTT ACG GTC TAT CCC	
Procambarus clarkii	Red swamp crayfish		Doorknocker crayfish	Mauvisseau et al. (2018)	COI CO1-Pc-03-Forward: GGAGTTGGAACAGGATGGACT CO1-Pc-03-Reverse: AATCTACAGATGCTCCCGCA Probe: CCTCCTTTAGCTTCTGCTATTGCTC	yes
Procambarus fallax f. virginalis	Marbled crayfish	Marmorkreps	Doorknocker crayfish	Mauvisseau et al. (2019b)	COI Pv-COI-Forward: GTATAGTTGAGAGGGGAGTA Pv-COI-Reverse: CCATAGTTATACCAGCTGCC Probe: 6FAM-AGGTATTTTTTCCTTGCA-BHQ1	yes
Rithropanopeus harrisii	Harris mud crab		Doorknocker crayfish	Knudsen et al. (2022)	COI Rhihar_co1_F03: GTCAACCTGGTACTCTCATTGGT Rhihar_co1_R03: ACGAGGAAATGCTATATCAGGGG Rhihar_co1_P03: FAM-TGTTGTAGTAACAGCTCACGCCTTTGT-BHQ1	
Rutilus rutilus	Roach	Mort	Regional invasive freshwater fish	Fossøy et al. (2017)	16S Mort_16S_312-331_F: TCCGAGTGGACTGGGCTAAA Mort_16S_351-374_R: CAGATGTTCTGCGGCTTATAGATG Mort_16S_334-348_P: CCCAAAGCCAAGAGA	
Scardinius erythrophthalmus	Common rudd	Sørv	Regional invasive fish species	Engesmo et al. (2021)	CytB Scaery_cytb_F14: TCG CAT TCC ACT TCC TCC TG Scaery_cytb_R07: AAG CTG TAA GGG CAA GCA GT Scaery_cytb_P08: TAC ACG AAA CAG GAT CGA ACA ACC CGG	
Tinca tinca	Tench	Suter	Invasive freshwater fish	Engesmo et al. (2022)	COI Suter-83bp-372F20: CCTCAGTAGACCTAACAATT Suter-83bp-434R21: AGTTGTGGTGATAAAATTGAT Suter-83bp-391L19: TGCTAGGTGAAGTGAGAAA	

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