Chapter 8

Using Environmental DNA for Invasive Species Surveillance and Monitoring

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Abstract

The method employed for environmental DNA (eDNA) surveillance for detection and monitoring of rare species in aquatic systems has evolved dramatically since its first large-scale applications. Both active (targeted) and passive (total diversity) surveillance methods provide helpful information for management groups, but each has a suite of techniques that necessitate proper equipment training and use. The protocols described in this chapter represent some of the latest iterations in eDNA surveillance being applied in aquatic and marine systems.

Key words eDNA, Noninvasive sampling, Detection, Metagenomics

1 Introduction

Indirect genetic detection of species from environmental samples is an emerging field in natural resource management and conservation biology [1–3]. While the general approach has been used in terrestrial studies for many years, applications of environmental DNA (eDNA) screening in aquatic environments have only recently been appreciated for their insights into the presence of incipient invasive species [4, 5] or threatened and endangered species [6, 7]. The general approach in aquatic systems is to collect a water sample, extract all the DNA from the sample, and then either screen for individual species using targeted, species-specific molecular markers [8] or high-throughput sequencing to reveal communities of species [9–11]. With the limitations in traditional aquatic sampling techniques, such as electrofishing and gill nets where some species are notably undetected due to low abundance or low probability of capture [12], there is growing interest in genetic and genomic applications for improved detection and monitoring of rare species, in particular for nonnative or invasive species [13, 14]. However, the same criticisms applied to traditional aquatic sampling techniques are also applicable to eDNA

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screening of species presence, and consequently, quantifying the accuracy and reliability of molecular surveillance results is essential to advance natural resource management based on eDNA detection of targeted species and total communities [15, 16].

Most eDNA surveillance studies on aquatic invasive species have focused on developing and implementing active surveillance that targets a single species [8, 17, 18]. Passive surveillance approaches, like high-throughput sequencing (HTS) applications, can potentially detect unexpected invasive species by screening all of the DNA in a given sample [9, 14] and identifying a community of species [10, 11]. In initial uses of eDNA for invasive species surveillance, methods were developed to provide rapid answers for management groups, leaving in-depth development of the tools by the wayside. Thus, the goals of this chapter are to not only describe the methods being implemented for eDNA surveillance of invasive species, but also to provide updates on where the methods in field are evolving and which methods are appropriate for specific situations.

To utilize eDNA as a surveillance platform and to choose a method to apply to the work, one must first consider active surveillance vs. passive surveillance techniques. Active surveillance, the most utilized route for eDNA surveillance to date, involves analyzing samples in a targeted fashion where samples are screened for a single species or group of species. Passive surveillance uses high-throughput sequencing platforms (HTS; i.e., next-generation sequencing methods) to screen for all species in a sample. The overall process for both active and passive eDNA surveillance involves initial sample collection, followed by DNA extraction and subsequent sample analysis (Fig. 1).

2 Materials

2.1 Sample Collection and Filtration

Sample filtration and preservation:

1. Sterile collection containers (250 ml volume or greater).
2. Whatman filter, 25 mm diameter, 5 μm pore size (GE Healthcare).
3. Whatman Swin-Lok filter holder (25 mm) or similar filter holder.
4. Plastic tubing.
5. Vacuum pump (either a hand pump or powered device similar to Pegasus Athena peristaltic pump will suffice).
6. Side-arm collection flask.
7. Plastic tubing.
8. Longmire's lysis buffer solution: 1 M Tris–HCl, pH 8.0, 0.5 M EDTA, pH 8.0, 5 M NaCl, Double-distilled (sterile) water, 25 ml of 20 % SDS per liter. Using a calibrated and decontaminated pipette, add 700 μl of Longmire's preservation buffer to

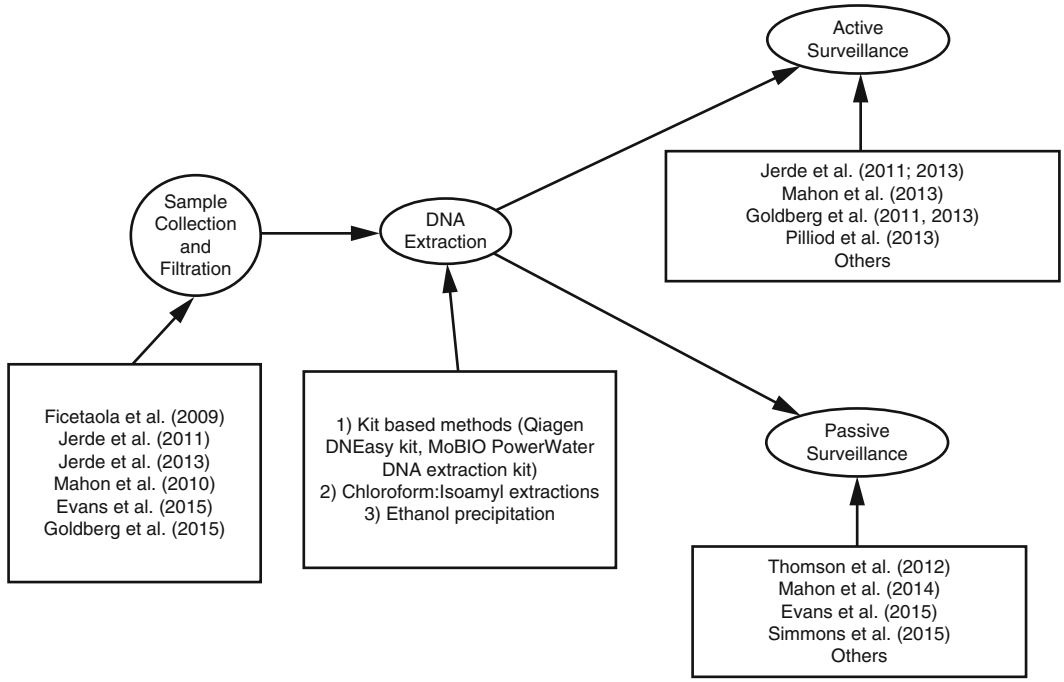

Fig. 1 Schematic flow chart for general eDNA surveillance methods and examples or published references for each associated step

each of the requisite number of 2 ml microcentrifuge tubes (i.e., 2× the number of 2 l water samples).

9. 2 ml microcentrifuge tubes.

2.2 DNA Extraction

1. Water bath (capable of 65 °C).
2. 24:1 chloroform:isoamyl alcohol.
3. Centrifuge for 2 ml tubes capable of 15,000 × *g*.
4. Micropipettes.
5. Ice cold isopropanol.
6. 5 M NaCl.
7. −20 °C freezer.
8. 70% ethanol.
9. TE buffer: 10 mM Tris, bring to pH 8.0 with HCl, 1 mM EDTA.
10. Optional Vacuum Centrifuge (e.g., Eppendorf™ Vacufuge™ Concentrator or equivalent).

2.3 Active Surveillance

1. For quantitative digital droplet Polymerase Chain Reaction (ddPCR): QX200™ AutoDG™ Droplet Digital™ PCR system and all associated consumables related to utilization of this system, available at http://bio-rad.com.

2. Micropipettes and tips for handling samples.
3. Species-specific PCR oligonucleotide primers and hydrolysis probe (project specific, related to intended target organism (s) of interest).
4. Microcentrifuge tubes.
5. Double-distilled (sterile) water.

2.4 Passive Surveillance

1. PCR thermocycler.
2. Micropipettes and tips for processing samples.
3. Microcentrifuge tubes.
4. PCR primers targeting amplicon that is general to the group of organisms under investigation (e.g., fish, invertebrates, etc.).
5. QIAquick Gel Extraction Kit (Qiagen Inc.).
6. Double-distilled (sterile) water.
7. TruSeq Nano DNA Library Preparation kit (Illumina).
8. Illumina MiSeq high-throughput DNA sequencer and associated flow cell (Illumina).
9. Unix-based computer for bioinformatics processing.

3 Methods

3.1 Sample Collection and Filtration

Routine sample collection involved taking a number of water samples from field locations to be screened. Early application of active surveillance using eDNA varied in the amount of water collected. Ficetola et al. used 15 ml samples in ponds [19]. Jerde et al. used two-liter water samples as a part of a broad-scale surveillance program for the invasive bighead and silver Asian carp (*Hypophthalmichthys* sp.) in the Laurentian Great Lakes [5, 17]. Recent work has moved to 250 ml samples being collected and filtered [10, 20]. Samples are collected and filtered in the field.

Using sterile collection containers, 250 ml water samples are collected from field locations and vacuum filtered through the Whatman Swin-lok filter holder onto the 5 μm PCTE filter paper. After filtration, the individual pieces of filter paper are then placed into 2 ml microcentrifuge tubes containing premade Longmire's solution [21]. In this solution, the genetic material collected is stable for at least 150 days at ambient temperature [22].

3.2 DNA Extraction

1. Heat the tube containing Longmire's solution and the collected sample on the PCTE filter at 65 °C in a water bath for 10 min.
2. Briefly cool the sample prior to adding 0.7 ml of 24:1 chloroform:isoamyl alcohol in a fume hood.
3. Mix the samples on a vortexer vertically for 5 min, dissolving the filter papers and lysing any cells in the sample.

4. Centrifuge the samples for 15 min at 15,000 × *g* at room temperature.
5. Pipette the supernatant (~500 μl) to a new microcentrifuge tube.
6. Precipitate the DNA from the supernatant solution by adding an equal volume of ice-cold isopropanol and a half volume of 5 M NaCl, incubating this solution for ~1 h at −20 °C.
7. Pellet the DNA by centrifuging the sample at 15,000 × *g* for 15 min at room temperature.
8. Pour off the supernatant and add 150 μl of 70% ethanol, washing down the inner walls of the tube.
9. Repeat the 70% ethanol wash and centrifugation.
10. Remove residual ethanol by air drying or use a vacuum centrifuge (45 °C, 5–10 min).
11. Resuspend the DNA by adding 100 μl of TE buffer and vortexing gently. If necessary, heat the solution for 10 min at 55 °C and remix via vortexing gently.
12. DNA extractions can be stored in the refrigerator (~4 °C) or at −20 to −80 °C until downstream analyses can be performed. Repeated freeze–thaw cycles should be avoided.

3.3 Active Surveillance

The goal of eDNA monitoring methods for many studies has been to develop a rapid, accurate, and relatively inexpensive surveillance tool that can be applied to the system in question. The large majority of studies to date have used routine equipment for sample analyses that most standard molecular ecology laboratories have on hand. Largely, active, targeted surveillance has necessitated using a detection platform such as polymerase chain reaction (PCR) for qualitative analyses (presence/absence of target DNA in a given sample). More recent studies have moved to quantitative measures, even beyond traditional qPCR, such as digital droplet PCR (ddPCR) that can calculate concentrations of target species DNA collected in a sample [20, 23–25].

Absolute concentrations of target species DNA can be measured using a BioRad© QX200 Droplet Digital PCR system and primers and hydrolysis probes developed for quantitative PCR (*see* [23], for example, of setup). Hydrolysis probes necessitate tagging, and in our prior work we have utilized a dual-labeled probe with a 5′ 6-FAM fluorescent tag and a 3′ Black Hole Quencher [20, 23], Simmons et al. [20]. The instrument can also utilize EvaGreen fluorescent chemistry, leaving out the need for probes. A routine ddPCR reaction mixture consists of 1000 nM of each primer and probe, 1× BioRad© ddPCR Supermix for probes, 2.5 μl DNA and sterile water for a total reaction volume of 25 μl. The BioRad© QX200 droplet generator partitions the reaction mixture into nanodroplets, combining 20 μl of the reaction mixture with 70 μl of droplet oil. This results in a total sample volume

of 40 μl (>20,000 individual nanodroplets) containing sample, primers, probe, and mastermix, which is then transferred to a PCR plate for amplification and then screened and analyzed on the QX200 instrument. For each ddPCR plate run, negative and positive controls are necessary to evaluate potential contamination and success of the reaction chemistry, respectively.

3.4 Passive Surveillance

A number of preparatory steps are needed for using eDNA in passive surveillance, i.e., high-throughput sequencing screenings. Prior to sequencing, DNA extractions from field sites can be pooled by sample location into composite sample(s) [11, 20]. Individual or pooled samples are then PCR amplified using a targeted or universal vertebrate primer set [10, 26, 27]. Amplification and purification procedures vary by amplicon. Our approach described here follows methods developed in Evans et al. [10] for fish community analysis. For metabarcoding studies of marine invertebrates, *see* also Chapters 12 by Fonseca and Lallias, 13 by Bourlat et al., and 14 by Leray et al.

Library preparations are then performed on amplified PCR products for each sample using the Illumina TruSeq Nano DNA Library Preparation Kit, omitting the DNA fragmentation step due to the small, discrete size of the amplicons, typically less than 250 bp. Samples are then loaded onto a MiSeq v2 flow cell in equimolar amounts for sequencing using a 500 cycle (Paired end 250 bp; PE250) v2 reagent kit. Following sequencing, base calling is performed by Illumina Real Time Analysis (RTA) v1.18.54. The output of RTA is demultiplexed and converted to FastQ format with Illumina Bcl2fastq v1.8.4.

3.4.1 Bioinformatic and Statistical Analyses

To analyze the data after MiSeq sequencing, the resulting FastQ files are filtered to remove exact sequence duplicates, singletons, sequences with more than five ambiguous bases, and sequences with less than 100 bp using PRINSEQ v0.20.4 [28]. Custom databases can be generated to screen the resulting data using a script available at http://www.auburn.edu/~santosr/scripts/NCBI_retrieval.prl and NCBI's Genbank database. The NCBI's Basic Local Alignment Search Tool (BLAST) can then be used to analyze sequences at identity thresholds (>98% and with over 100 bp matches and expect values of less than 1e−5).

Evaluation of species distribution as inferred by eDNA detection in georeferenced water samples can be accomplished by using Species Occupancy Models (SOMs) [29, 30]. These models, in general, attempt to provide a probability of species occupancy at a location based on the occurrence record of species coupled to habitat covariates when the detection probability of the species is less than one [31]. These SOMs can incorporate replication at multiple levels (within sample and within locations), account for detection errors, and ultimate quantify incidences of false positives and

negatives [32]. However, without SOM connections to the underlying hydrology, which would describe how DNA is transported in a system, occupancy in lotic systems may be limited [16].

4 Quality Assurance and Quality Control (QAQC)

Species distribution modeling has been used for conservation management and planning for many decades [33], but the use of eDNA has posed a number of challenges largely due to the inherently indirect nature of the detection method [15]. What has been coined the 'molecular revolution' for recording of biodiversity and species occupancy [34] is a rapidly evolving approach with changes in collection, extraction, amplification, and screening of DNA (Fig. 1). Each of these steps may introduce unwanted errors in the form of false negatives and false presences [15] that can be largely controlled by improved protocols and procedures [35], quantification of detection errors [30], and assessing the appropriate level of sample replication [32].

4.1 General QAQC

There are multiple QAQC components that are common to all eDNA methods and analysis platforms. One of the most important QAQC components of eDNA studies is instituting protocols that minimize the probability of contamination of eDNA extractions and downstream analyses. This can be achieved through a number of different steps. First, it is important to conduct all DNA extraction and amplifications in a room or location dedicated to low-quantity DNA sources. Concentrated DNA of the eDNA target species, in the form of high concentration DNA extracts and more importantly PCR products, should not be handled or opened in this dedicated space. Researchers also need to restrict the flow of items and individuals between high concentration and low concentration DNA working spaces. To minimize the possibility of contamination among eDNA samples and reagents within the low quantity DNA working space, a number of steps can be taken including the use of filter tips, regular changing of gloves, plus frequent sterilization of pipettes, sample trays, and extraction surfaces. Finally, negative controls should be included in all DNA extractions and PCRs to monitor for contamination events.

4.2 Active Surveillance QAQC

Digital droplet PCR: Multiple research groups have abandoned the traditional endpoint PCR systems to utilize quantitative amplification methods, including quantitative PCR (qPCR) and digital droplet PCR (ddPCR) [9, 14, 36, 37]; and others). These platforms analyze samples for targeted species, either individually or multiplexed (multiple target species at one time), and provide either semiquantitative (qPCR) or total (ddPCR) concentrations (copies of target sequence per microliter) of the targeted DNA

fragments from species of interest. This approach and these platforms are reported to be more sensitive [38] and less prone to false positives than traditional endpoint PCR methods [39]. Levels of detection for qPCR and ddPCR have been demonstrated at extremely low levels, in some instances to less than one copy of target DNA per microliter of sample [38–40]. Additionally, even within the quantitative methods, ddPCR may provide substantial advantages over qPCR, particularly because ddPCR does not necessitate production of a standard curve for each sample run, removing a step that both reduces costs of analysis and also lowers the potential for calibration error in the study [23–25]. Additionally, ddPCR has to be a more precise method and has shown reduced susceptibility to reaction inhibitors over traditional qPCR [41].

Quantitative PCR and ddPCR methods require stringent quality assurance procedures, particularly in primer/marker design. When designing the surveillance or monitoring assay, if using a probe-based qPCR and digital droplet PCR methodology, base pair mismatches on both primer and probe, particularly at the 3′ end of the qPCR primers need to be accounted for to avoid false positives [40]. Along with this, false negative problems need to be considered when target DNA is rare and potentially swamped out by nontarget DNA in a sample [40].

The increased sensitivity for qPCR and ddPCR should necessitate additional precautions to help prevent contamination and false positives. This would include additional sterilization of equipment in the laboratory, positive pressure UV capable PCR hoods, procedure room separation, etc., as others have done in the ancient DNA field (*see* Goldberg et al. [35]).

In general, standard qPCR quality control guidelines developed for other methods and procedures should be followed as appropriate (*see* [42]). These include methods to ensure the reliability of results in quantitative eDNA analyses to promote interlaboratory repeatability and to increase experimental transparency [42]. Following the digital guidelines for qPCR, a series of best practice ddPCR guidelines has been developed [41]. These include best practice suggestions ranging from experimental design through ddPCR assay validation [41].

4.3 Passive Surveillance QAQC

HTS analyses of eDNA samples: The advent of high-throughput metagenomic sequencing platforms has the potential to revolutionize the use of DNA for surveillance and monitoring. With these platforms, we have the ability to not only screen eDNA samples for targeted rare species of interest, but we can also screen and analyze samples for overall biodiversity in the system being investigated. Although there are a number of different chemistries and processing methods, high-throughput platforms function by sequencing all fragments of DNA in a sample rather than targeting individual DNA fragments using species-specific amplification.

Additionally, some protocols use a preplatform target enrichment or PCR amplification, providing the ability to target certain groups (e.g., all fish) in a given sample. Depending on the platform utilized, thousands to multiple millions of sequence reads can be generated in a given run, providing depth of coverage of each individual sequence read. Previous eDNA studies utilizing this platform have demonstrated successful application to determine total aquatic biodiversity in real-world systems [10, 11, 20, 43]. To ensure accuracy of matches to target species, particularly if management actions are to be taken based on the data collected, data analyses and stringency of matches to available genetic databases such as NCBI's Genbank should be explicitly stated (e.g., [43]). Consistency of analyses, thresholds for matches to target species, and continued expansion of available genetic barcode-type data within available databases should all be considered when developing the metagenomic assay. Additionally, type of genomic platform utilized should be carefully considered (e.g., Illumina MiSeq or HiSeq or 454, Oxford Nanopore MinION, etc.) as the data each produces differs and can provide different, yet still revealing results.

4.4 Choosing Active vs. Passive Surveillance Methods and Platforms

The standard PCR approach (e.g., [4, 44]) for target eDNA surveillance has the distinct advantage of using technology and techniques found in many molecular genetics labs and can be performed relatively cheaply, assuming a marker exists for the species targeted for surveillance. Progressing up the technological ladder to qPCR, ddPCR, and HTS, the infrastructure necessary to perform the assays on different platforms becomes more costly and with fewer laboratories available to conduct such assays. In many studies, the choice of active vs. passive surveillance methods may be pragmatic. However, it is clear that for at least some platforms, there are issues of detection sensitivity that may drive assay choice. Nathan et al. showed that qPCR and ddPCR were much more sensitive to detecting a target species compared to traditional PCR [23]. Similarly, Doi et al. showed that when eDNA is at very low copy number (<100 per sample) ddPCR outperforms qPCR [25]. A comparison of HTS detection sensitivity to target approaches is an area of ongoing research [20].

While the upfront cost for any platform can be relatively expensive, >$250,000 for some HTS approaches, a per sample cost may also weigh on the decision to choose active or passive surveillance techniques. Nathan et al. estimated a $4.27, $8.87, and $4.02 cost (US$) per sample for PCR, qPCR, and ddPCR platforms, respectively [23]. It should be noted that the uptick in qPCR cost was largely for production of a calibration curve to estimate the amount of DNA in the sample, and with the PCR approach there was no quantification of the amount of DNA.

Ultimately, the choice of using active or passive surveillance will be largely driven by the question needing to be answered [14, 20].

For now, if presence of only one or two species is needed, then it appear PCR, qPCR, or ddPCR platforms are more cost effective, accessible, and reliable. However, if the ecological question of interest is about estimated biodiversity or species richness [10, 11], then HTS to screen and identify suites of species will likely be the best approach. This will particularly be the case as costs for HTS methods, either in house or at commercial facilities, continue to drop and technologies continue to improve [45].

As active and passive approaches to eDNA surveillance advance, data quality will continue to improve and both scientists and management agencies will have the opportunity to more confidently take action to address questions regarding rare species, whether threatened or endangered or invasive, in aquatic environments. These responses can then begin to protect native systems, either through documentation of current biodiversity and potential habitat protection for rare species or instigating early detection and rapid response actions for invasive species. While both active and passive eDNA methods are excellent additions to monitoring science, neither is a 'silver bullet' for surveillance. However, they are both extremely valuable tools and should continue to be developed and supported by scientists and management groups.

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