Environmental DNA Advancing Our Understanding and Conservation of Inland Waters☆

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Environmental DNA (eDNA)

Environmental DNA (eDNA) is a new technique and field of study with far reaching implications for environmental and biodiversity assessment within the fields of ecology, evolutionary biology, agriculture, disease control and beyond. Environmental DNA is defined as DNA extracted from an environmental sample that does not target a particular organism or set of organisms (**Bohmann et al., 2014**; **Seymour, 2019**). The sources of eDNA are expected to be predominantly free-floating DNA that has recently left its species of origin through natural body secretion (e.g., shedding, molting, feces, urine, gametes), or as fragments released from decaying bodies (**Jo et al., 2019**; **Moushomi et al., 2019**). A major breakthrough in the utilization of eDNA was the discovery that once eDNA has been isolated from a sample it can be used to identify the species of origin using molecular methods (**Ficetola et al., 2008**). Since eDNA can be sampled from practically any environment imaginable, including water, soil, air, ice; the general concepts and methods regarding eDNA-based research can be broadly applied.

It is important to clarify that the definition of what constitutes an eDNA sample is debatable among researchers. In very broad terms, eDNA is often used to describe any DNA captured from environmental sampling, including bacteria and single cell eukaryotes or homogenization of bulk samples (e.g., insects from a trap blended together). However, it is important from a methodological standpoint to be aware of the environmental source and intended target because methods that work to identify bulk samples or bacteria communities are not always optimal for identifying non-target eukaryotes from water or sediment samples. To avoid confusion, this chapter will restrict the definition of environmental sampling to non-bulk sampling (e.g., water and sediment) and eDNA to eukaryote-derived DNA from environmental samples. Overall, this chapter provides a concise summary of eDNA concepts and applications in freshwater ecosystems, outlines the use of eDNA-based biodiversity assessment and the standard approach in using eDNA to sample freshwater environments, and offers insights into current pursuits using eDNA.

Biodiversity assessment in freshwater environments encompasses a diverse set of methodologies depending on the community or species of interest, sampling environment and the level of taxonomic identification required for a given study. Often methods are very specific to a set of species or environmental conditions, which can limit the ability, or effectiveness, of using the method across different sampling sites or time points. For example, sampling macroinvertebrates from streams is often conducted with kick-net sampling, which is only fully feasible if the depth of the rivers are shallow enough to traverse, to sample the middle of the river and both edges, and the substrate is suitable for adequately disturbing the riverbed. If the substrate is too coarse (e.g., boulders or bedrock) or fine (mud or silt), the method will often miss individuals within the environment. Additionally, seasonal conditions may alter a sampling site's physical characteristics (e.g., flooding, freezing or drought), thereby preventing subsequent or regular sampling.

In contrast, sampling for eDNA is more standardized for the type of environmental sample, be it water, soil or air, and can be consistently sampled regardless of the ecosystem type, season, or taxa of interest. The reliability of eDNA to detect communities has repeatedly been shown to be on par or better than traditional sampling methods (**Valentini et al., 2016**; **Sales et al., 2020**; **Seymour et al., 2020**). In addition, the standardized molecular and bioinformatics approaches used to process and analyze eDNA data are often faster and cheaper to routinely conduct compared to traditional taxonomic identification methods (**Davy et al., 2015**) and avoid the potential bias induced by using multiple or inexperienced taxonomic identifiers (**Deiner et al., 2017**). However, there are still several limitations, ongoing developments and potential pitfalls associated with the use of eDNA. For example, it is important that the environment is sampled in a structured manner, such as depth profile sampling within a lake or a sampling transect along the distance of a river, to account for eDNA movement within the environment (e.g., distribution/transport) (**Li et al., 2019**). This chapter serves as an overview to the understanding and practice of eDNA-based research in freshwater environments, particularly for new and interested researchers. It is not intended as a definitive statement on the subject, as the field is rapidly changing and many views and discussions are ongoing regarding the topic, some of which are highlighted below.

Freshwater environments

This section describes three important aspects of the use of eDNA in freshwater environments: The background and utilization of eDNA, how eDNA accumulates in freshwater environments and the processes that remove eDNA from the environment. **Fig. 1** illustrates the factors that influence eDNA detection in natural environments.

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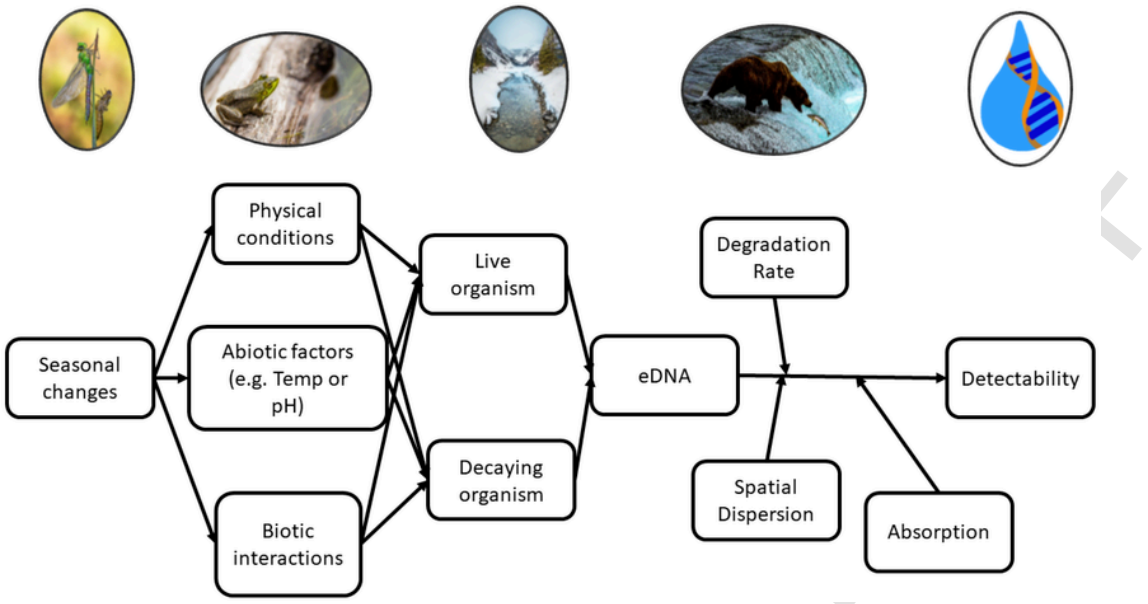


Fig. 1 Diagram showing the ecology of eDNA and how environmental, physical and biotic factors can influence eDNA detection. As eDNA is released into the environment, the tissues, cells and cell particles that are needed for sampling and detection are readily broken down, or absorbed, over time, meaning the detection of eDNA is time dependent. Many factors influence the detection of eDNA, such as seasonal changes to species activities or life stages, which will affect eDNA production, or differences in environmental conditions, such as chemical composition, which can affect degradation of eDNA. Environmental DNA may also readily traverse through waterways, meaning the detection of eDNA may not represent the specific location of the sample. Substrates may also bind eDNA particles making them undetectable, unless the substrate itself is sampled.

Background of eDNA research and utilization in freshwaters

The methodological background for eDNA originated in the late 1970s and early 1980s with the establishment of the polymerase chain reaction (PCR) and DNA sequencing (**Thomsen and Willerslev, 2015**). PCR allows for the replication of targeted genetic sequences, which is important for eDNA since the concentration of target DNA in environmental samples is generally too low to be detected using visual or sequencing methods, unless PCR is first used. The development of quantitative PCR (qPCR) in the 1980s, which allows for real-time observation of a PCR reaction via fluorescence, provided a more accurate means to detect single species from eDNA samples. qPCR is still widely used today for population-level assessments and has well-established reporting guidelines and practices, referred to as the Minimum Information for Publication of Quantitative Real-Time PCR Experiments MIQE guidelines (**Bustin et al., 2009**).

Community-based assessments from eDNA, which are reliant on the development of DNA sequencing, have taken longer to develop methods and guidelines. Modern DNA sequencing allows us to determine the nucleotide order of a genetic sequence without the use of a reference genetic sequence. The development of DNA sequencing led to the development of DNA barcoding in the early 2000s (**Hebert et al., 2003**). DNA barcoding is a method of using a specific genetic sequence, often a specific gene or region within a gene, to identify species. Several DNA barcode reference libraries have been created, with the intent to catalog barcode sequences of all life on the planet, and are essential for eDNA studies that use high throughput sequence methods because we use these reference databases to link sequences detected in environmental samples to actual species identifications.

Using molecular methods to detect bacterial and fungal communities from environmental samples predates eDNA research, including using soil and water samples to detect microbial strains (**Ogram et al., 1987**; **Fisher and Triplett, 1999**) and permafrost to assess ancient fungal and pollen diversity (**Eske and Alan, 2005**). It is important to distinguish these earlier microbial and fungal studies from eDNA here, as eDNA entails detecting species and communities from genetic material that is not part of the target organism(s). Additionally, the concentration of targeted genetic material in eDNA is much smaller, compared to the high concentration of bacterial or fungal DNA found in environmental samples that require different methodologies (e.g., primer selection to avoid sequencing non-targeted species). The closely related field of ancient DNA (aDNA) uses a combination of museum and environmental samples (ice or deep sediment cores) to assess ancient communities and populations (**Hofreiter et al., 2001**). Apart from similarity in molecular methodological approaches, aDNA research has greatly influenced eDNA clean room and sample processing protocols, which are essential for ensuring data generated from eDNA samples is viable.

Interest in using eDNA for monitoring programs received a lot of attention, but was initially slow to take hold, as the method was largely untested. The first studies showing the application of eDNA, specifically multi-cellular eukaryotes, used water samples to detect American bullfrogs (*Lithobates catesbeianus*) in French ponds (**Ficetola et al., 2008**). The establishment of the silver carp (*Cyprinus*

carpio) eDNA monitoring program in the United States is perhaps one of the most important eDNA applications to occur, as it established an important baseline for using eDNA as an established biomonitoring tool (**Jerde et al., 2013**). Additionally, the UK monitoring of the current great crested newt (*Triturus cristatus*), an important conservation species in Europe, was one of the first programs specifically designed to utilize eDNA detection (**Biggs et al., 2015**). With these and other subsequent successes, there is an ever-growing list of single species (i.e., population-based) eDNA programs across several countries and organization. Community-based eDNA assessments that rely on reference databases are less common but have been increasing since about 2014. Seminal works for key taxonomic groups include fish (**Valentini et al., 2016**; **Hänfling et al., 2016**), macroinvertebrates (**Bista et al., 2017**), and diatoms (**Vasselon et al., 2017**) and provide proof of concept matching eDNA biodiversity to traditional sampling methodologies. Presently many government bodies are planning to or have established community-based eDNA biodiversity assessment protocols for a wide range of species and biomonitoring communities.

An understanding of how eDNA is generated and remains in the environment is essential for utilizing eDNA-derived population and community information (**Barnes and Turner, 2016**). There are two important processes that affect eDNA in freshwaters: accumulation (or production) and removal (via transport and degradation). Environmental DNA is constantly being produced and subsequently degraded through biological and physical processes. The amount of eDNA present in the environment at any time varies depending on the abundance of the individuals and the intraspecific rate at which each species sheds or releases eDNA, either through bodily functions or via decomposition (**Jo et al., 2017**; **Moushomi et al., 2019**). While some species routinely shed eDNA via skin cell loss or excrement, other species may be poor shedders due to limited or timed molting or slow-decaying exoskeleton and can be difficult to detect at low population abundances, such as the American signal crayfish (*Pacifastacus leniusculus*) (**Dunn et al., 2017**), and some invasive aquatic plants (**Kuehne et al., 2020**). Additionally, eDNA accumulation is likely to be affected by seasonal activity of individual species. For example, spawning times for fish can result in large volumes of gametes released into the environment, which may dominate other species eDNA detection and not be directly related to population sizes. Likewise, algal blooms can dominate samples and affect detection of other species such as diatoms and other primary producers. The timing of macroinvertebrate larval emergence may also affect the lateral transfer of biomass in lakes and streams, whereby pupae move from the benthos to the water surface, thereby affecting detection at different time points if not accounted for in the sampling design.

Once eDNA is produced, it can be transported away, particularly in rivers, or degraded. Degradation times differ depending on the environmental conditions; eDNA detection in lentic environments (e.g. lakes and ponds) can be up to 2 months, whereas in sediments it can be for several years (**Barnes and Turner, 2016**). Lotic eDNA will readily travel downstream once produced and can be detected several kilometers downstream in highly channelized settings (**Deiner and Altermatt, 2014**; **Seymour et al., 2018**), though downstream detection is much shorter in natural settings due to effects of dilution from river confluences and absorption from clay based substrates (**Shogren et al., 2017**). The rapid degradation of eDNA in water environments is thought to be due to hydrolysis as well as microbial activity (**Barnes and Turner, 2016**; **Jo et al., 2019**; **Moushomi et al., 2019**), although the exact processes are still unclear. Environmental DNA can last for several years in freshwater soil environments once it settles into the anoxic zone, as DNA can maintain its structure more easily under low oxygen conditions. Abiotic factors can also increase the degradation rate of eDNA in the environment (**Strickler et al., 2015**; **Seymour et al., 2018**) including low pH, high temperature, and potentially high UV exposure, although natural levels of UV are not expected to have significant effects on eDNA degradation rates (**Andruszkiewicz et al., 2017**; **Mächler et al., 2018**). The short term detectability of eDNA in water samples makes freshwater eDNA a measure of recent species presence, as opposed to long-term historical measurements from sampling sediments (**Deiner et al., 2017**). As such, water eDNA sampling is preferred for studies looking to assess the current community and populations inhabiting a site. However, sediment sampling may be more appropriate for assessing species that predominantly live within the sediment as their eDNA may not be visible or obtainable via water eDNA.

Using eDNA for biomonitoring and conservation

Freshwater biomonitoring generally focuses on routine monitoring of three main groups that are linked to environmental status: fish, macroinvertebrates, and diatoms. Detection and monitoring of these three groups can be labor and time intensive, which has led to increased interest in using developing eDNA-based biomonitoring methods and applications. Traditionally the monitoring of these groups differs substantially, with different research groups generally specializing on one group or another. Similarly, the adaption of eDNA for monitoring these groups, while more similar than the differences in traditional methods, has differed in their utilization and methods employed.

Fish biomonitoring

Fish biodiversity is, by far, the most studied within the eDNA research sphere, due to the economic importance of fisheries and relatively low diversity of fish communities compared to macroinvertebrates or diatoms, making validation of studies relatively easy. In addition, there is general widespread consensus that eDNA-based fish biomonitoring outperforms traditional sampling methodology, with the majority of studies occuringwithin lentic environments (**Jerde et al., 2013**; **Valentini et al., 2016**; **Hänfling et al., 2016**). From a conservation standpoint, eDNA sampling is advantageous over other methods because it is non-invasive, and no animals are harmed, compared to the several traditional sampling methods, such as electro-fishing, gill-netting, fin clipping that harm or kill fish. Combined with other non-invasive monitoring methods, such as hydro-acoustic monitoring, eDNA sampling offers a rapid and routine means to monitor fish population dynamics across the globe (**Berger et al., 2020**). A limitation of eDNA-based fish biodiversity assessment is the

need to ensure that the sampling location (e.g., a lake) is either well-mixed or that the sampling has sufficient spatial coverage for the size and depth of the habitat of interest (**Li et al., 2019**).

Freshwater macroinvertebrate biomonitoring

Macroinvertebrates are a group of major interest for biomonitoring, particularly in river ecosystems where they are an important component of environmental assessment (**Seymour et al., 2016**) and indicators of functional change (**Heino et al., 2004**). Several recent studies have shown stark differences in data from eDNA methods and traditional macroinvertebrate sampling; however, the general biodiversity assessment patterns (e.g., assessment scores, inter-community variation) are often found to be similar (**Macher et al., 2016**). A major advantage of eDNA sampling over traditional macroinvertebrate sampling is the ability to sample water from any type of freshwater environment, including lakes, as well as both deep and fast-flowing rivers. Conversely, traditional kick-netting used for macroinvertebrate sampling is largely restricted to rivers that are traversable by foot and where the substrate is gravel or small-stoned. Macroinvertebrate communities can be assessed rapidly, compared to traditional taxonomic identification, by extracting and sequencing DNA from a kick-net sample (i.e., bulk sampling), though this should not be directly compared with eDNA sampling given the targeted approach of the bulk sampling method. It is suggested that the bulk sampling approach produces more reliable detection compared to sampling of aquatic eDNA, although community dynamics are equally well observed (**Macher et al., 2018**). Moreover, collection of aquatic eDNA that utilizes a spatially structured sampling design results in a more representative biodiversity being sampled compared to benthic kick net samples, including increased detection of biomonitoring species that are traditionally difficult to assess, including Chironomidae (**Seymour et al., 2021**).

Diatom biomonitoring

Diatom communities are highly sensitive to changes in abiotic conditions, are routinely used for biomonitoring purposes in both lotic and lentic environments, and are generally the most sensitive to environmental change compared to fish and macroinvertebrates (**Pandey et al., 2017**). The importance of diatoms for biomonitoring are highlighted by their use in the European Union's Water Framework Directive (WFD) (**Valentin et al., 2019**), with similar efforts ongoing in Canada (**Maitland et al., 2020**). However, diatom identification is notoriously difficult compared to fish or even macroinvertebrates, which prompted a faster adaptation of molecular methods for diatom biomonitoring purposes compared to other biomonitoring groups. Single species assays are possible for some species; however, community spatial and temporal dynamics have received the most interest, particularly for biomonitoring purposes (**Tapolczai et al., 2019**). While some studies have used surface water for collection of diatom eDNA (**Seymour et al., 2020**), this might not be optimal over standard field sampling approaches that directly sample diatom by scrapping stones collected from river beds (e.g., bulk sampling) (**Vasselon et al., 2017**).

Detection of rare species and conservation efforts

There is an ever-increasing list of eDNA conservation efforts under development for several freshwater species (**Mauvisseau et al., 2019**; **Belle et al., 2019**). Perhaps the oldest example of an established eDNA monitoring program is the endangered great crested newt (*T. cristatus*) monitoring program in the United Kingdom (**Biggs et al., 2015**). Great crested newts require a network of ponds to form their home ranges, which can make it difficult to identify which ponds harbor newts at any given time. Given the endangered status of the great crested newt, land use changes that might affect great crested newt habitat require extensive habitat surveying. Given the high cost and difficulty in determining great crested newt presence using traditional methods (e.g., aquatic funnel traps, netting, torchlight, and egg counts), an eDNA-based monitoring program was established by Natural England (a UK environmental agency) to provide a more rapid and efficient monitoring protocol (**Rees et al., 2014**). Other notable conservation studies, for which eDNA detection is of interest, include Eastern hellbenders (*Cryptobranchus alleganiensis*) (**Santas et al., 2013**), freshwater pearl mussel (*Margaritifera margaritifera*) (**Stoeckle et al., 2016**), European eel (*Anguilla anguilla*) (**Weldon et al., 2020**), and wetland amphibian and fish communities (**Kačergytė et al., 2021**).

Detection of invasive species

Invasive species pose a threat to endemic biodiversity, as their establishment can lead to dislocation or extirpation of local, often rare, species. Early detection is crucial to effectively manage invasive species eradication and to prevent establishment (**Mehta et al., 2007**; **Sepulveda et al., 2020**). If invasive species become established, they are often very difficult to eradicate and can have negative effects on local biodiversity, threaten endemic species and incur huge economic cost (**Sepulveda et al., 2020**). The high sensitivity of eDNA-based assays can offer successful early detection of invasive species, even preceding early stages of invasions (**Sepulveda et al., 2020**). The standards and methods of invasive species eDNA development and implementation are very similar and are closely linked to those used in conservation biology. Some examples of invasive species that are routinely surveyed for using eDNA include the bluegill sunfish (*Lepomis macrochirus*) (**Takahara et al., 2013**), the New Zealand mudsnail (*Potamopyrgus antipodarum*) (**Goldberg et al., 2013**), and the red swamp crayfish (*Procambarus clarkii*) (**Tréguier et al., 2014**).

Detection and tracking of disease

An emerging aspect of eDNA research is the use of environmental samples to monitor and track the spread of disease vectors, including recent interest in using water samples to survey human coronavirus in waste water (**Farkas et al., 2018**; **Randazzo et al., 2020**). Additionally, eDNA is currently being used to assess the spread of polycystic kidney disease (PKD) (**Carraro et al., 2018**), an economically important disease in salmonids, and to help develop better spatial models for human pathogens such as cholera (**Vezzulli et al., 2017**) and schistosomiasis (**Sengupta et al., 2019**).

eDNA methodological workflow: From sampling to data

Sampling the environment and extracting eDNA

Detection of eDNA is done by sampling the environment of interest, particularly the material that the species or community of interest regularly interacts with. Sampling itself can be conducted by taking a sample of the environment such as using a digging device to sample the soil (e.g., shovel, sediment corer) or using a bottle to take a water sample. However, one must be aware that it is very easy to contaminate eDNA samples, with other eDNA sources being transferred by the sampling person or from unclean or reused sampling equipment. For example, if a person sampling for fish eDNA in a lake was fishing in a neighboring lake the day prior, they could contaminate the samples they are taking as particles acquired the day before could be transferred from their person to the sampling container. It is therefore important to develop stringent sampling protocols for cleaning of persons and equipment and using disposable or single-use sampling equipment (e.g., gloves and sampling containers). The use of field controls/blanks to help identify any potential false positives that may arise from contamination are also strongly advocated. Field controls are previously prepared samples that resemble the field samples, such as a sampling bottle of autoclaved water, that should be brought into the field and processed in the same manner as the field samples. The results from the field controls can then be crosschecked with the field samples later to assess any potential sources of contamination.

Processing of the samples as soon as possible after collection is important to avoid further eDNA degradation. Water samples should be filtered for eDNA as soon as possible and the filters kept dry, preserved with lysis or fixing agents or frozen to avoid DNA degradation. Likewise, soil or sediment, once sampled, should be kept in cool and dark conditions for transport, either for laboratory analyzes or to freezers for longer-term storage. Environmental DNA extraction is generally done following modified DNA extraction protocols (**Spens et al., 2017**; **Tsuji et al., 2019**). Extraction of eDNA can be performed with modifications of commercial kits or using "home-brewed" methods (**Spens et al., 2017**; **Lear et al., 2018**). It is important to note that eDNA will be extracted at low concentrations, compared to extractions from bulk or tissue samples, in many cases resulting in only trace amounts of relevant DNA, with much larger concentrations of non-target DNA from other taxa, such as bacteria or single-cell eukaryotes. Additionally, inhibitors of downstream PCR reactions (for qPCR or metabarcoding) might be retained during extraction from environmental samples, such as humic compounds from soil (**Matheson et al., 2010**). Therefore, it is important to check for sample inhibition and consider using an inhibition cleanup step post-DNA extraction.

Generating data from eDNA samples

Once eDNA is isolated from the environmental sample, the DNA fragments need to be associated with the species or group of species (i.e., community) of interest. Currently, two molecular approaches are generally used to identify the eDNA fragments (Fig. 2): quantitative PCR (qPCR), also called real-time PCR, and metabarcoding. qPCR allows for a species-specific detection method using species-specific primers. Metabarcoding is a high throughput sequencing (HTS) method that allows multi-parallel sequencing within a single sample, allowing for community-based (e.g., multiple species) assessments from a single eDNA sample. The qPCR workflow is currently more standardized, cheaper and faster compared to metabarcoding, making it the preferred method for monitoring of rare species, detection of invasive species, or disease monitoring (**Wood et al., 2019**). Metabarcoding applications are newer, generate more data and allow for community level assessments, although single species can also be extracted from metabarcoding results (**Deiner et al., 2017**).

Using qPCR (single species)

qPCR methods can be used for several different applications, ranging from control laboratory experiments to species-specific detection in complex natural environments (**Goldberg et al., 2015**; **Thalinger et al., 2021**). The qPCR method can detect low levels of DNA in a sample, making it suitable for eDNA-based research. Droplet digital PCR (ddPCR) may also be used, which uses similar molecular chemistry as qPCR with increased sensitivity to detecting low concentrations (**Doi et al., 2015**). Currently, however, ddPCR machines are more costly to set up and implement, leaving qPCR still at the forefront for those looking to do standardized single species detection (**Doi et al., 2015**).

A full breakdown of the steps to create and validate PCR primers for reliable use in biomonitoring has recently been outlined by **MacDonald and Sarre (2017)**. Briefly, special consideration needs to be taken to ensure the primers are species-specific so that false positives are not mistakenly identified during the data processing. Steps for creating qPCR primers should include *in silico* assessment for primer specificity, using software such as NCBI's primer blast (**Ye et al., 2012**), as well as laboratory test of the primers against target and

non-target organisms. Depending on the intended application of the primers, either laboratory only use (controlled experiments) or for government biomonitoring programs, the test of the primers can be quite extensive in order to ensure that the primers are species-specific and avoid false positives. To ensure comparison of results across multiple samples and to quantify the copy numbers for each sample, a set of size standards needs to be included into each set of reaction runs. These size standards should contain known quantities of the target sequence that is being amplified during the qPCR run. Size standards can be created using DNA cloning (e.g., plasmids) or purchased as synthetic sequences from a company. The range of size standards to include into each run should capture the full range of values that is to be amplified from the samples. Particularly for eDNA studies, it is important to assess the limits of detection (LOD), which is the primer's ability to detect target sequences at low concentrations (**Klymus et al., 2020**). It is important to also be aware that some environmental samples may incorporate PCR inhibition, which can be assessed by spiking samples with known quantities of the cloned or synthesized size standard.

While there are many things that need to be considered to quality check and verify qPCR data, the whole process is relatively quick once the primers and protocols are established. The final data is readily accessible after each qPCR run and usable for analyzes immediately, whereas the data generated via high throughput sequencing will generally take several days to reach a similar usage point.

Using metabarcoding (multi-species)

Metabarcoding entails using high throughput sequencing (HTS) to determine the sequence information from a pool of genetic material, which can then be linked to a DNA barcode database, hence the name metabarcoding (**Deiner et al., 2017**). The taxonomic diversity that can be detected from a metabarcoding analyzes is dependent on the specificity of the primers used and the reference database that is used to link genetic sequences to morphological taxonomy. No metabarcoding primer is perfect for all studies, with many different primers designed to capture different taxonomic groups (**Cordier et al., 2019**; **Seymour et al., 2020**). Some primers that work well with bulk sampling (e.g., insects blended together) will not work as well with eDNA samples because the primers will also sequence undesirable species, often protists or bacteria, that are more common (i.e., have more DNA available for sequencing) in the sample. With the increased interest in using eDNA, however, new primers are being developed that take into account the type of environmental sample and the desired taxonomic diversity (**Leese et al., 2021**). Generally, it is advised that primers perform better when they are specifically developed for each project, since they will be tailored to the regional diversity and environmental condition. However, the effort in designing study specific primers can be costly and time-consuming and several metabarcoding primer sets currently exist for a wide range of species that have been used across different studies, which may be suitable for a given project (**Deiner et al., 2017**).

After sequencing, the next steps require bioinformatics pipelines to perform quality checks, removing low quality reads, merging paired-sequence ends and checking for chimeras. The sequence reads are then grouped into clusters called operational taxonomic units (OTUs) or amplicon sequence variants (ASVs), depending on the pipeline and clustering algorithms being used (**Porter and Hajibabaei, 2018**; **Tsuji et al., 2020**). Once sequencing clusters (e.g., OTUs or ASVs) are created they can be analyzed directly, which is commonly done for bacterial or fungal diversity, or they can be linked to taxonomic identifiers using relevant reference databases (**Hebert et al., 2003**).

DNA sequencing reference databases contain the DNA barcode sequences for different species. Most publicly available libraries are incomplete, given the extensive number of species that require sequencing, with efforts ongoing to fill in the gaps (**McGee et al., 2019**). Three such databases are most commonly used. First, and the most iconic reference database, is the Barcode of Life Data System (BOLD), which curates species barcode sequences of the COI gene. Second is the substantial SILVA database for the small and large ribosomal subunits along with several smaller reference databases for other genes, including 12S (Mitofish), cytochrome *b* and rbcL (**Weigand et al., 2019**). Third, the NCBI database has the most diversity across available gene regions but these data are not as stringently curated compared to other reference databases. Despite this, the NCBI is highly accessible, compared to other databases, and is routinely utilized for generating personal reference sequence libraries for different gene regions (**Hänfling et al., 2016**; **Fernández et al., 2018**).

Once the data is formatted as either purely genetic cluster data or taxonomically linked community tables, it can be used similarly to other ecological analyzes. The read counts, which are the number of matching sequences recorded during the sequencing, will be available for each genetic cluster or taxonomic group. Read count numbers may provide some insight into the abundances of each group, however they are also generated due to variation in PCR efficiency. As such, caution should be made when using read counts in analyzes, as they do not represent abundances specifically, but will also vary artificially due to PCR chemistry. The most conservative approach is to convert the read count data to presence/absence, which is similar to how many traditional ecological analyzes proceed. There are alternative methods to convert read count data to semi-quantitative formats, such as relative read counts prior to additional analyzes; however, biases arising from the molecular methods may still persist (**Lamb et al., 2019**).

Future directions using eDNA

Environmental DNA is a tool as well as a field of study, which makes for an exciting and diverse set of future applications and research. Within the context of inland waters research, there are three key directions for eDNA research: network based analyzes, functional diversity assessment and use of alternative molecular methods. The description of these topics below is by no means exhaustive and the emerging foci are likely to change rapidly in the coming years.

Network analyzes

Ecological networks are a way to assess interactions among taxa across deep diversity pools, and have been linked to several aspects of metacommunity dynamics that are directly relatable to biomonitoring principles (**Araújo et al., 2011**). The scale of eDNA-derived data, particularly from metabarcoding, allows for the application of robust standardized analyzes to conduct network analyzes. Much research has been done to incorporate network-based approaches to link sample sites, either through time or space, which enables the assessment of environmental variables on network properties and the underlying biotic interactions, a key aspect for effective biomonitoring (**Bohan et al., 2017**; **Seymour et al., 2020**). In contrast to current biomonitoring assessments, eDNA-derived data has the potential to include a much wider range of taxa and indicator groups, which are currently excluded or simplified due to limitations of traditional taxonomic identification. Network-based eDNA assessment may further add to existing biomonitoring efforts by allowing for a finer-scale assessment, such as exploring differences in community dynamics among neighboring sites or evaluating moderate changes in environmental conditions (**Cordier et al., 2017**).

Although there is a need to ensure continuity between traditional and eDNA-based assessment methods, there should also be a forward-thinking approach that does not restrict eDNA research to simply confirming traditional expectations. Not only can eDNA data be used to assess environmental filtering on individual indicator taxa, allowing for comparable biometric data to traditional sampling, it can also be used to determine interactive qualities that are also linked to the stability and ecological complexity of habitats (**Cadotte and Tucker, 2017**). Additionally, the advancement of machine learning algorithms (**Cordier et al., 2017**) and newer abiotic monitoring systems, such as advanced mass spectrometry (**Collins et al., 2021**), combined with eDNA, potentially allows for a much deeper exploration of environmental samples for linking environmental effects to changes in natural populations and communities.

Functional diversity

Functional diversity has recently received increased focus in ecology, although it has long been recognized as an important factor in understanding the complete structure of an ecosystem (**Young and Collier, 2009**). More often biodiversity is quantified using species richness, which is the number of unique taxonomic units per site or sample. Richness is often positively correlated with heterogeneous environments, which is often attributed to higher levels of functional diversity (**Cardinale et al., 2011**; **Donohue et al., 2013**). Functional diversity directly quantifies the functionally disparate taxa within a community, and is becoming increasingly recognized as an important component of effective and more direct measure for linking community response to environmental change (**Young and Collier, 2009**). By measuring functional diversity, we get a better understanding of the characteristics within a community and can then relate these to environmental variation or to assess changes over time.

Environmental DNA research can further our understanding of functional diversity. For example, we can incorporate existing functional diversity of known species, such as feeding groups for macroinvertebrates (**Moog, 2017**), into eDNA data analyzes (**Seymour et al., 2021**). Attempts are also being made to detect variation in functional genes via transcriptomics directly from environmental samples (**Taberlet et al., 2018**).

Alternative molecular methods

A major interest in current eDNA research is to design methods that allow for estimation of abundances from high throughput sequencing (HTS). However, abundance estimates from HTS that use PCR (e.g., metabarcoding) are currently not reliable due to PCR amplifying sequences at different rates. Efforts are being made to incorporate PCR-free sequencing technologies with eDNA samples, such as shotgun sequencing (**Ji et al., 2020**), LAMP (**Lee, 2017**) and CRISPR (**Williams et al., 2019**). There are limitations to such applications at present, including incomplete reference databases, incompatibilities of some species to specific methods, the need for additional primer development, and the inability of methods to detect lower eDNA concentrations.

Another area of interest is to better understand the health of organisms sampled via eDNA. Currently, there is a lack of knowledge regarding the condition of the species when sampling eDNA, as the source of eDNA could originate from healthy, sick or dead organisms. The condition of the organism is important as different conditions may require different management strategies. As such, there is an increased interest in using eRNA, which refers to environmental RNA. The potential of eRNA may allow for aquatic health assessment via insights into organism functionality (**Veilleux et al., 2021**).

Case studies

Bista et al. (2017) was one of the first to assess temporal dynamics of community diversity using eDNA, showing that eDNA can reliably depict temporal dynamics of Chironomidae communities. Chironomidae, informally known as non-biting midges, are a highly diverse family of flies with over 10,000 species globally. The larvae of Chironomidae are aquatic and form an important component of the benthic community of freshwater ecosystems, particularly in lentic or slow moving lotic systems. Chironomidae larvae predominately feed on algae, or small organisms, while serving as important food sources for fish, amphibians and larger predatory insects. Adult Chironomidae emerge, often en masse, from the surface of lakes or ponds by floating to the surface of the water as pupae and shedding their pupal skin (i.e., exuvia) as they transform into adults. As adults, Chironomidae serve as important food sources for bats and bird species, with some having evolved nesting strategies to coincide with annual cycles of Chironomidae adult emergences. Chironomidae are noted as important indicator species, meaning the presence or absence of key species is indicative of pollution.

However, traditional identification of Chironomidae species is notoriously difficult due to their small size and morphological similarity between species, which requires a high level of expertise to identify individuals to species level. Furthermore, sampling Chironomidae in lakes is mostly conducted by skimming exuvia from the lake surfaces during adult emergences, as sampling the lake bottom for larvae requires additional equipment and effort, and sampling from the edges of the lake will miss most species. Such traditional sampling limits observations to emergence times and does not capture the benthic community, which is essential for regular assessment. By using eDNA and monthly exuvia sampling of lake water **Bista et al. (2017)** compared emergence and biodiversity patterns of Chironomidae communities. Environmental DNA revealed much about the status of emerging species during their larval development times that had not been detected using traditional sampling through the winter months. Overall, these findings helped establish eDNA as a means to capture temporal trends in community biodiversity. Future efforts could look to compare surface and sediment eDNA to determine the overlap in

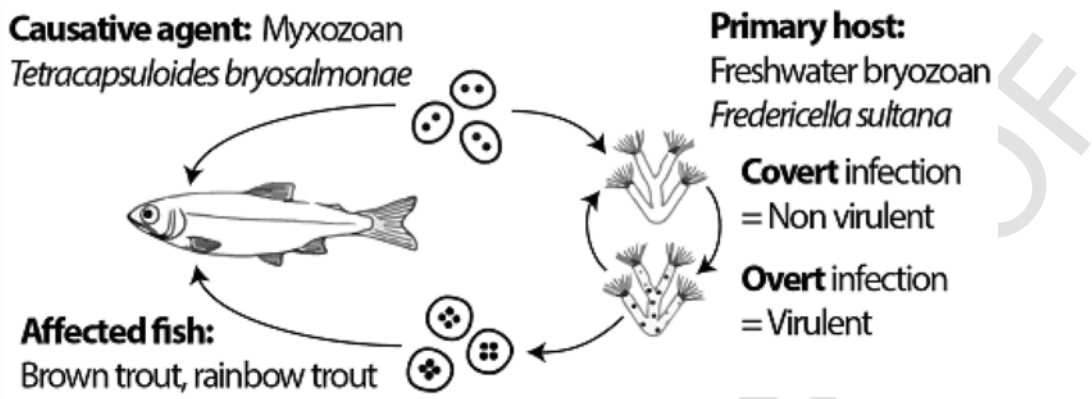
community composition and dynamics.

Hänfling et al. (2016) presented a robust assessment of fish stocks in three large lakes in the United Kingdom and was one of the first studies to show that rank abundance comparisons across temporal eDNA sampling points was an effective means of assessing fish populations. Fisheries management is important for environmental assessment, societal enjoyment and economic stability. It is therefore important for agencies to accurately assess fish populations to apply appropriate management strategies. Fisheries managers use a wide range of assessment methods, which are often destructive or harmful to the fish, prompting many organizations to invest in non-invasive sampling methods.

Hänfling et al. (2016) looked to verify if eDNA metabarcoding was a viable means for routine monitoring of fish populations. The lakes used in the study have a long history of fisheries assessment, so their biodiversity and environmental condition linked to fisheries populations is well known. As such, results from eDNA assessments could be directly compared to current gill netting data as well as historical population structures. **Hänfling et al. (2016)** utilized a self-made reference database (freely available via their publication) that incorporated all fish species expected to live in or near the lake. Using a replicate transect sampling design, they compared the site occupancy of each fish species identified using two gene markers (12S and CytB). They showed that rank abundance of sequence reads reflected the rank abundance of species within the three lakes, suggesting read counts reflected differences in fish abundances. Additionally, they showed spatial preference for eDNA signal, whereby shore vs. off shore sampling affected the detection of different species (**Hänfling et al., 2016**). Overall, the key finding was that eDNA assessment of fisheries outperformed the traditional assessments methods, thereby providing a proof-of-concept for a non-invasive means of environmental assessment.

This study is an excellent example of a well designed and implemented eDNA research effort and has been followed by several other studies that have highlighted the importance of fisheries eDNA (**Jerde et al., 2019**; **Miya et al., 2020**). Additional work will look to improve on the ability to calculate abundances of different fish species from eDNA by incorporating biomass information directly from

individual samples, such as proposed recently by **Yates et al. (2020)**.



Lifecycle of Myxozoan Tetracapsuloides bryosalmonae

Carraro et al. (2018) is one of the first studies to apply river network based approaches to understand the spatial dynamics of eDNA in a river network. The study takes into account the transport time and spatial dynamics induced by flow to observe and model the biological distribution of the bryozoan *Fredericella sultana* and its parasite *Tetracapsuloides bryosalmonae*.

Tetracapsuloides bryosalmonae is of general interest to researchers and managers as it is the agent that causes proliferative kidney disease in salmonid fish. The life cycle of *T. bryosalmonae* involves seasonal cycles whereby the *T. bryosalmonae* persist in their primary host *F. sultana* throughout the year. During the summer, the *F. sultana* releases spores of *T. bryosalmonae* into the water, which infects fish through their gills. *Tetracapsuloides bryosalmonae* will grow and multiply inside the fish and during the autumn will leave the fish via being excreted with their urine to then infect other *F. sultana*. Effectively monitoring the timing and outbreaks of *T. bryosalmonae* life stages can therefore be essential for minimizing loss of fish to PKD.

Carraro et al. (2018) model highlights the importance of understanding the distribution of the target species and the hydrodynamics for effectively using eDNA for biomonitoring. Additional efforts to merge real time environmental data to project expected changes in species distribution will help managers gage what level of distribution is expected in combination with routine monitoring of key sampling sites along a given river stretch.

Concluding remarks

Environmental DNA is a rapidly growing field that is becoming more widely used. It has already made major inroads into freshwater science across academic, government and private sectors. Regardless of whether traditional or eDNA-based sampling is used, it is important to be aware of the use and application of eDNA, as many agencies and employers are rapidly looking to adapt eDNA methods where applicable. Environmental DNA research can also be seen as an opportunity for emerging scientists. The field is very new, which allows for new researchers to rapidly develop and implement novel ideas and studies. Combined with other emerging and developing research fields, eDNA based research offers a valuable doorway into modernized biomonitoring of ecosystems, environmental assessment, conservation biology, and many other potential avenues for understanding and discovering previously unobservable aspects of our natural world .

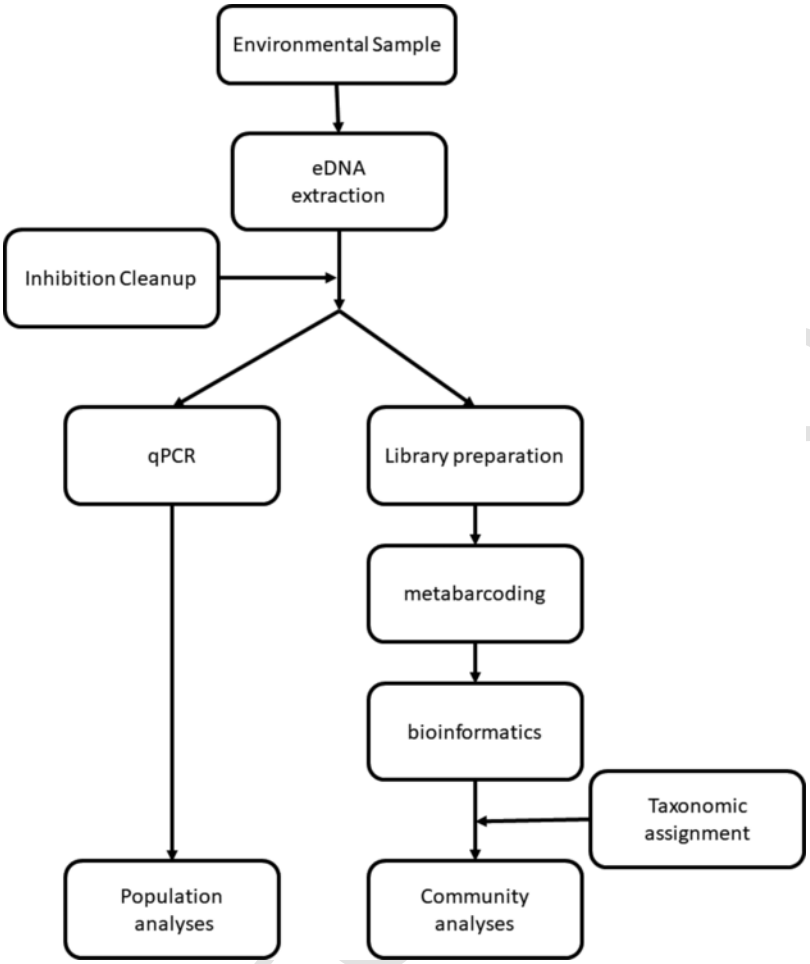


Fig. 2 Simplified workflow for single and multiple species detection using eDNA sampling. eDNA is extracted from environmental samples (e.g., water or soil). Depending on the quality of the extraction, it may be important to perform an inhibition cleanup prior to any PCR based method, although this is not always necessary. The general process for single species via qPCR follows standard qPCR procedures to generate population level data. The steps involved in multi-species eDNA assessment via metabarcoding includes preparing the genetic libraries from the extracted samples, performing the metabarcoding on a high throughput platform, using bioinformatics to quality check and process the sequence output and linking sequence information to taxonomy using a reference database.

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Glossary

Amplicon—PCR amplified DNA fragment contained between two primer regions.
Barcode—A specific region within a gene that is used to genetically identify individual species or other taxonomic levels.
Control samples—field or lab comparison that matches the eDNA sampling type (e.g., sterilized water when aquatic eDNA is collected) and serves to assess false positives in downstream analyzes due to sampling contamination. Laboratory controls are used to assess contamination during lab analysis due to contaminated equipment.
Environmental DNA (eDNA)—DNA isolated from an environmental sample (e.g., soil or water) that does not target a species or community of interest.
False negative—When a species/taxon is not identified from a sample even though it is present.
False positive—When a species/taxon is incorrectly assigned to a sequence due to contamination of the sample or incorrect assignment methodology.
Metabarcoding—High throughput sequencing of PCR-generated sequence libraries, which allows for multiple unique sequences to be read from a single sample.
Polymerase chain reaction (PCR)—A molecular technique used to replicate small (usually specific) segments of DNA.
Primer—A short single-stranded nucleic acid (~ 20–50 base pairs) utilized in the initiation of DNA synthesis during PCR (e.g., extension stage of PCR). Primer specificity refers to how stringent the primer is binding to target DNA sequences.

Quantitative PCR (qPCR)—A molecular technique that monitors the amount of amplification during a PCR via fluorescence.