Environmental DNA and its applications to Fisheries and Oceans Canada: National needs and priorities

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Annex A. Technical considerations for eDNA studies

Acronyms

AIS: Aquatic Invasive Species

bp: Base pair

CFIA: Canadian Food Inspection Agency

ddPCR: Digital droplet polymerase chain reaction

DFO: Department of Fisheries and Oceans

D-loop: Displacement loop

DNA: Deoxyribonucleic acid

dPCR: Digital polymerase chain reaction

eDNA: Environmental DNA

eRNA : Environmental RNA

EEZ: Exclusive Economic Zone

H-T: High-throughput

IYGPT: International young gadid pelagic trawl

L-T: Low-throughput

MI: Minimum information

MPA: Marine protected areas

mtDNA: Mitochondrial deoxyribonucleic acid

NGS: Next-generation sequencing

OTU: Operational taxonomic unit

PCR: Polymerase chain reaction

QA/QC: Quality assurance and quality control

qPCR: Quantitative polymerase chain reaction

RNA: Ribonucleic acid

ROV: Remote operated vehicle

RT-PCR: Real-time polymerase chain reaction, also known as qPCR

SAR: Species at Risk

SARA: Species at Risk Act (S.C. 2002, c. 29)

SCUBA: Self-contained underwater breathing apparatus

SMRT: DNA sequencing using single-molecule real-time methods (e.g., PacBIO)

Glossary

Abiotic: physical, chemical, and other non-living environmental factors

Abundance: the total number of individuals of a taxon or taxa in an area, community, or population

Allele: one of two or more alternative forms of a DNA sequence

Amplicon: a piece of DNA or RNA that is the product of replication events. It is typically considered as a result of using various amplification methods including polymerase chain reactions

Annotation: the process of identifying the locations of putative genes within contigs or genome assemblies and referencing the known functions of similarly coded genes in other species

Barcode: a short genetic marker in an organism's DNA to identify it as belonging to a particular (target) species or taxon

Base pair (bp): a pair of complementary bases in a double-stranded nucleic acid molecule, consisting of a purine in one strand linked by hydrogen bonds to a pyrimidine in the other. Cytosine always pairs with guanine, and adenine with thymine (in DNA) or uracil (in RNA)

Biodiversity: the variability among living organisms from all sources including terrestrial, marine, and other aquatic ecosystems; and the ecological complexes that they form including the diversity within and between species, and of ecosystems

Bioinformatics: the development and use of software to study genetic and protein sequence data

Biotic: relating to or resulting from living things, especially in their ecological relations

Chimera: a chimera can be an artifact of PCR amplification. It occurs when the extension of an amplicon is aborted, and the aborted product functions as a primer in

the next PCR cycle. The aborted product anneals to the wrong template and continues to extend, thereby synthesizing a single sequence sourced from two different templates

Clade: a group of organisms believed to have evolved from a common ancestor

Copy number: the number of copies of a DNA fragment

Digital droplet PCR (ddPCR): a biotechnological refinement of digital PCR (dPCR). A sample is fractionated into 20,000 droplets rather than in fixed arrays as in dPCR, and PCR amplification of the template molecules occurs in each individual droplet. This end-point amplification PCR is capable of absolute quantification independent from any reference material and is less prone to PCR inhibition than qPCR. The cost per reaction is higher for ddPCR than traditional quantitative PCR, however, the need to include (different) standard curves for the quantification using qPCR somewhat mitigates the difference in cost. ddPCR costs far less than dPCR technologies, thus it is thought that ddPCR (e.g., Bio-Rad Quantalife) may replace traditional PCR methods once costs are lowered

Digital PCR (dPCR): a biotechnological refinement of conventional PCR and qPCR methods that can be used to directly quantify DNA or RNA (e.g., Fluidigm systems). The key improvement of dPCR over traditional PCR is that the reaction is divided up into arrays of several hundred, smaller reactions (based on water-oil emulsion droplet technology) and thus provides greater precision than PCR and qPCR. It is particularly useful for low-abundance targets, targets in complex backgrounds, and for monitoring subtle changes in target levels that cannot be detected with real-time PCR

Endosymbiotic: a relationship between two symbiotic organisms, in which one organism lives inside the other

Environmental DNA (eDNA): genetic material that can be extracted from bulk environmental samples, such as water, air, biofilms, or sediment, and analyzed to determine the organisms present

Extraction: a process of purification of DNA or RNA from any given sample using a combination of physical and chemical methods

Hybridization: any mating of individuals of different genetic composition, typically belonging to different populations

Functional genetics (or genomics): uses genomic data to study gene and protein expression and function, focusing on gene transcription and translation, and protein-protein interactions, and often involving high-throughput methods. Functional genomics involves understanding the genetic control mechanisms, and how an organism’s genes respond to its environment, or environmental change

Gene: a unit of information corresponding to a discrete segment of DNA that codes for the sequence of an RNA molecule or for the amino acid sequence of a protein

DNA enrichment (also known as enrichment capture): any number of molecular techniques (e.g., PCR, hybridization in microarrays, various emerging forms of non-PCR solution-capture methods) that increase in the presence of target nucleic acid (DNA, RNA) sequence(s) in solution. Regardless of the capture method, DNA enrichment uses synthetic DNA probes designed from reference sequences that are complementary to regions in the genomes of the target taxa

Gene expression: the process of transcription and translation of the DNA code into a protein

Genome: the entire DNA of an organism

Genomics: a branch of biotechnology concerned with applying the techniques of genetics and molecular biology to the genetic mapping and DNA sequencing of sets of genes or the complete genomes of selected organisms, with organizing the results in databases, and with applications of the data

High-throughput (H-T) sequencing: see next-generation sequencing

Inbreeding depression: the reduction in fitness of inbred offspring relative to outbred

Introgression (also known as introgressive hybridization): the spread of alleles from one population into the gene pool of another through repeated backcrossing with members of one of the parent populations

in silico: conducted or produced by means of computational modeling or simulation

in situ: in its original place

in vitro: performed or taking place in a test tube, culture dish, or elsewhere outside a living organism

Laser transmission spectroscopy (LTS): a real-time quantitative detection technology that rapidly measures the size, shape, and number of nanoparticles in a solution by measuring the wave-length of light transmitted through a sample. LTS has the potential to become a quantitative and rapid DNA detection method suitable for many real-world applications

Locus (pl. loci): the physical location on the chromosome (e.g., of a gene or genetic marker)

Macrobial: of macro-organisms

Marker: a DNA sequence that can be used to identify individuals or species where a variation is observable

Massively parallel sequencing: any of several high-throughput approaches to DNA sequencing using the concept of massively parallel processing. Also referred to as next-generation sequencing (NGS) or second-generation sequencing

Mesocosm: experimental water enclosure designed to provide a limited body of water with close to natural conditions, in which environmental factors can be realistically manipulated

Metabarcoding: a rapid method of biodiversity assessment that combines two technologies: high-throughput DNA sequencing and sequence similarity-based identification. It uses one or more sets of PCR primers to mass-amplify DNA barcodes from mass collections of organisms or from environmental DNA

Metagenomics: the study of the metagenome, or the collective genome of microorganisms from an environmental sample, to provide information on the microbial diversity and ecology of a specific environment. Shotgun metagenomics refers to the approach of shearing DNA extracted from the environmental sample and sequencing the small fragments

Microarray: a printed or synthesized grid of DNA fragments of known sequence used to profile RNA, DNA copy numbers, DNA genotypes, antibodies, or proteins

Microfluidics: the engineering or use of devices that apply fluid flow to channels smaller than 1 millimetre in at least one dimension. Microfluidic devices can reduce reagent consumption, allow well controlled mixing and particle manipulation, integrate and automate multiple assays (known as lab-on-a-chip), and facilitate imaging and tracking

Single-molecule real-time (SMRT) sequencing: a third-generation parallelized sequencing technology that consistently produces some of the longest average read lengths available to date in the industry to date (average > 10,000 bp, some reads > 60,000 bp; but also see SMRT sequencing hand-held devices (e.g., MinION) that have high error rates currently)

Microsatellite: variable number of tandem base pair repeats where the repeat motif is generally two or more repeated bases. These markers are typically scored manually, although some automated programs have now been generated

Mitochondrial DNA: a circular molecule of DNA present in the mitochondria of eukaryotic cells. This DNA is at higher copy number than the nuclear genome and is therefore favoured for environmental DNA studies

Mitogenome: the whole mitochondrial genome

Next-generation sequencing (NGS): also known as high-throughput sequencing, is the catch-all term used to describe a number of different modern (non-Sanger-based) sequencing technologies including (e.g., Illumina (Solexa) sequencing). Millions or billions of DNA strands can be sequenced in parallel, yielding massively higher throughput and minimizing the need for the fragment-cloning methods that are often used in Sanger sequencing. Reads are typically shorter than Sanger sequencing (i.e., 100-300 bp relative to ~700 bp)

Operational taxonomic unit (OUT): An operational definition, where taxonomic definitions are difficult or unknown (as in many protist groups), used to classify groups of closely related individuals. Sequences can be clustered according to their similarity to one another, and operational taxonomic units are defined based on the similarity threshold (usually 97% similarity) set by the researcher

Outbreeding depression: when offspring resulting from crosses between genetically distant individuals (outcrossing) exhibit lower fitness in the environment of either of their parents

Pipeline: a series of formal steps in metagenomics bioinformatics tools designed to organise, analyse and classify the sequences (often to species) read in shotgun sequencing

Polymerase chain reaction (PCR): a DNA enrichment method for making millions of copies of short (0.1-4.0 kilobase) fragments of DNA

Population genetics: a subfield of genetics that deals with genetic differences within and between populations, and an integral component of evolutionary biology. Studies in this branch of biology examine such phenomena as local adaptation, speciation, population sizes, and connectivity

Primers: short DNA sequences manufactured to a match specified sequence of an organism, which are used in the polymerase chain reaction process

Proteome: the entire set of proteins that are produced or modified by an organism or system

Proteomics: the study of all proteins in a cell, or tissue, and their collective and individual functions

Quantitative PCR (qPCR; also called real-time PCR): the real-time quantification of amplified PCR fragments during the polymerase chain reaction. Depending on

approach, this method can use absolute quantification within a sample or relative quantification between samples

Read: a sequence of nucleotides resulting from a sequencing reaction of a single DNA fragment. A typical sequencing experiment involves fragmentation of the genome into millions of molecules to produce a library, which is then sequenced to produce a set of reads

RNA transcript: a complement copy of a gene that is composed of RNA. Many transcripts encode proteins with putative functions known based on sequence similarity to genes within model organisms. The collection of transcripts in a cell, tissue, or individual is referred to as a transcriptome

Sanger sequencing: a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication

Shotgun metagenomics: see Metagenomics

Taxon (pl. taxa): a grouping of organisms assigned to a particular category of classification (e.g., a species, genus, order)

Ultra-deep sequencing: refers to the general concept of aiming for a high coverage for each region of a sequence

ABSTRACT

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Environmental DNA (eDNA) detection is a revolutionary approach that aims to determine the presence of organisms, and other population parameters, by extracting the genetic material released from living and dead organisms in environmental samples, such as water. The rapid development of tools and technologies associated with eDNA has generated an unprecedented ability to detect species for biosecurity, biosurveillance, aquaculture, commercial fishery, and aquatic conservation management activities around the globe. In this report, we discuss the growing use of eDNA in Canada and its applications to DFO Programs related to at-risk and aquatic invasive species, fisheries management, sustainable aquaculture, aquatic animal health, and sustainable aquatic ecosystems. We highlight the challenges and technical considerations inherent to the use and interpretation of eDNA, and provide recommendations towards development of best practices and minimum reporting standards. Also, we review the fast-advancing field methods and DNA sequencing technologies used to study eDNA, where we see the most potential for growth within DFO. Altogether, this report describes the beginning of a national dialogue on research priorities and other science needs essential to providing the best possible advice on the use of eDNA for management decisions regarding the protection Canada’s aquatic resources and environments.

RÉSUMÉ

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La détection d’ADN environnemental (ADNe) est une approche révolutionnaire qui vise à déterminer la présence des organismes, et d’autres paramètres populationnels, en extrayant matériel génétique relâché précédemment par des organismes vivants et morts, dans les substrat environnementaux, tels que l’eau. Le développement rapide des outils et des technologies associés à l'ADNe a engendré une capacité sans précédent opur détecter des espèces aquatiques pour la biosécurité, la biosurveillance, l'aquaculture, la pêche commerciale, et en gestion de la conservation des écosystèmes aquatiques à l’échelle mondiale. Dans ce rapport, nous présentons les utilisations croissantes de l’utilisation de l’ADNe au Canada et de ses applications aux seins de divers programmes du MPO, tels que les espèces aquatiques envahissantes et en péril, la gestion des pêches, l’aquaculture durable, la santé des animaux aquatiques, et les écosystèmes aquatiques durables. Nous soulignons les défis et les considérations techniques inhérents à l'utilisation et à l'interprétation de l'ADNe et proposons des recommandations afin de développer les meilleures pratiques et les normes minimales lors de présentation de l’information écologique obtenu par l’ADNe. Nous révisons aussi les développements méthodologiques liés à l’échantillonnage de l’ADNe et les technologies de séquençage ayant un potentiel croissant au sein du MPO. Le présent rapport décrit le début d’une concertation nationale visant à élucider les priorités de recherche et les autres besoins essentiels qui permettront de fournir des avis scientifiques sur l’utilisation de l’ADNe en gestion des ressources et des écosystèmes aquatiques du Canada.

Executive Summary

The following discussion paper was prepared in response to inquiries from resource managers within Fisheries and Oceans Canada (DFO) on the current state of science vis-à-vis the emergent field of environmental DNA (eDNA) and its possible applications to conservation and management of aquatic resources and the associated ecosystems. The eDNA field is a revolutionary cross-disciplinary area of biological science that uses genetic material, shed or contained by organisms, extracted from environmental samples, such as water or sediment, to determine the organisms present and other population parameters. Increasingly, eDNA is being used globally for biosecurity, biosurveillance, aquaculture monitoring, fish health, commercial fishery, and aquatic conservation management activities. Within DFO, key programs that stand to benefit from eDNA technologies are Integrated Fisheries Management, Sustainable Aquaculture, Aquatic Animal Health, Species at Risk, Aquatic Invasive Species, Ocean Protection Plan, Climate Change Adaptation, and Oceans Management Programs. eDNA monitoring has the potential to help address the practical needs of multiple international biodiversity obligations (e.g., the Convention on Biological Diversity Aichi Targets). Therefore, it is timely to ascertain the current state of the technology, explore how it is currently used within DFO, and initiate a discussion on the development of guidelines.

Fisheries Management—In recent years, studies have demonstrated that eDNA analysis has the capacity to estimate fish diversity better than, or equal to, more conventional sampling methods. eDNA technology has great potential for monitoring the abundance of fish populations.

Sustainable Aquaculture—eDNA analyses can be applied to aquaculture research in two main areas, through 1) monitoring the consequences of organic and inorganic release of nutrients from aquaculture sites to assess the impacts on local biodiversity through changes in community structure, and 2) identifying the presence of farmed fish where they may interact with wild fish.

Aquatic Animal Health—eDNA has huge potential to contribute to the protection of farmed and wild fish through the 1) monitoring of diseases, water-borne viruses, and parasites, 2) monitoring for the increasing prevalence of genes resistant to therapeutants and antibiotics, 3) monitoring the microbiomes of fish and other organisms as a proxy for internal state of health, and as a 4) tool to improve disease ecology investigations.

Species at Risk—eDNA has the potential to improve monitoring and reporting through enhanced data on species occurrence, which can assist with the delineation of critical

habitat and allow for better population assessment estimates crucial to assessing recovery efforts. Additionally, the use of eDNA for providing forensic evidence of poaching and illegal trade of species may have benefits for the protection of at-risk finfish and shellfish.

Aquatic Invasive Species (AIS)—eDNA has served as an important tool for early detection of AIS (e.g., sea lamprey *Petromyzon marinus* in Great Lakes streams), so that management measures can be implemented swiftly. Early and more broad detection will help further research on aquatic invasive species across Canada, particularly in relation to climate change where species distributions are expected to change at all trophic levels.

Sustainable Aquatic Ecosystems—Indicator species are chosen for their sensitivity to environmental conditions, and can help characterize ecosystem health. eDNA has been used to help enhance monitoring of indicator species at offshore oil rig sites, salmon aquaculture farms, and in freshwater systems undergoing habitat change. eDNA studies also could be integrated systematically into Marine Protected Area management science to augment existing and developing monitoring methods to improve efficiencies and link existing and future eDNA projects nationally.

At present, eDNA technologies appear to be under-utilized in most of the DFO mandated responsibility areas, except for species at risk and aquatic invasive species. Now that the eDNA field is expanding, its capacity beyond targeted single-species studies, predominant in species at risk and aquatic invasive species studies, its applications to DFO's other mandated responsibilities are expected to increase.

Characteristic of an emerging scientific field, the approaches for sampling and interpretation of aquatic eDNA data vary across multiple independent research groups both internationally and within Canada. Field sampling, sample preparation and storage, laboratory preparations and extraction protocols, DNA sequencing technologies, reference databases, and computational analyses are important considerations for any eDNA study, and each area presents its own set of challenges. Advancements in field sampling methodology, including improved sample preservation methods, use of hand-held real-time sampling devices and remote and autonomous vehicles, as well as on-site satellite-based data transfer, may further increase the appeal of eDNA studies by lowering sampling costs, simplifying field logistics, and improving DNA yields. Growth of eDNA use within DFO, in the short term, may be limited by requirements for high performance computing processing power, technical bioinformatics skills and software, and shared annotated databases of aquatic organisms.

The development of national guidelines for sampling, data analysis, reporting, and results interpretion are key to moving eDNA science forward.

1. Introduction

1.1 Aims and Scope

Given the cross-cutting implications of environmental DNA (eDNA) analyses, this discussion paper has been developed to review the current state of eDNA and discuss a variety of potential uses for this technology in Fisheries and Oceans Canada (DFO) programs. Here we refer to eDNA as genetic material that can be extracted from bulk environmental samples such as water, air, biofilms, or sediment, and analyzed to determine the organisms present.

The scope of this paper explicitly encompasses eDNA applications to DFO mandated responsibilities such as fishery management, sustainable aquaculture, fish health, species at risk (SAR), aquatic invasive species (AIS), climate change adaptation, and protection of aquatic ecosystems. Since new eDNA technologies are developing rapidly, we also identify the exciting frontiers of eDNA applications, and highlight fast-advancing field sampling and DNA sequencing methods used to study eDNA where we see the most potential for growth within DFO. In a section on understanding our ability to collect, identify, and interpret eDNA, we review eDNA studies on aquatic species and environments and synthesize important concepts and critical challenges. As our focus is on the applications and innovations of eDNA research, a detailed discussion on methodological procedures and guidelines is beyond the scope of this paper. However, we have provided an Annex that elaborates on technical considerations associated with eDNA sample collection and preparation, sequencing methods, bioinformatics, and databases. With thoughtful discussions and a cohesive approach, we can address research priorities, information gaps, and technical challenges of this rapidly evolving field of eDNA analysis. In doing so, DFO can harness the enormous potential of this technology to monitor and assess aquatic organisms, populations, communities, and even entire ecosystems.

1.2 Background

Characteristic of an emerging scientific field, the approaches for sampling and interpretation of aquatic eDNA data vary across multiple independent research groups both internationally and within Canada (Annex A). Consequently, the time is right to initiate discussion on the development of national guidelines for optimally collecting field samples, preventing contamination, standardizing laboratory protocols and methodology, sharing data and bioinformatics scripts, and interpreting results. To this end, a national DFO eDNA workshop was held in March 2018 to examine the current state of eDNA research in the Department, as well as the possibilities and challenges associated with eDNA technologies. The workshop had participation from key management programs and clients from DFO, the Canadian Food Inspection Agency

(CFIA), and Transport Canada including the SAR Program, AIS, Conservation & Protection, Fisheries Protection Program, Aquatic Animal Health, Aquaculture Management, Ecosystem Management, and initatives under the Oceans Protection Plan. The primary objectives of the workshop were to:

- stimulate discussion among DFO scientists and resource managers on the opportunities eDNA offers to fisheries and aquatic resources;
- identify and prioritize information gaps and technical challenges of the rapidly evolving field of eDNA;
- identify eDNA research needs within DFO; and
- initiate discussion on developing best practices and guidelines for the validation and interpretation of eDNA data, to encourage systematic comparisons among studies.

Analysis of eDNA is a developing advancement in non-invasive genetics that may enable DFO to expand monitoring and reporting efforts on SAR (Mauvisseau 2017), AIS (Simmons et al. 2016), key food production industries such as fisheries and aquaculture (Bass et al. 2015), changes to ecosystems due to climate change (O'Dor et al. 2010; Jiao and Zheng 2011; Paerl and Paul 2012; Pawlowski et al. 2018), and other conservation initiatives. Building Canadian eDNA capacity now will also help DFO remain consistent with other international initiatives to monitor and manage aquatic environments [e.g., Convention on Biological Diversity, European Water Union Framework Directive (WFD Directive 2000/60/EC), *Clean Water Act* (CWA, 33 U.S.C. & 1251 et seq. 1972 from the US EPA), United Nations Convention on the Law of the Sea].

DNA in the environment can exist in two forms, either contained within the cells of an organism where it runs the metabolic functions or in free-form where it has been shed from an organism and drifts in the water column or settles on the substrate of the water body. Whole cells in environmental samples generally comprise microorganisms (e.g., microbes, single-celled algae, zooplankton) in the water column, surface biofilms, or in sediment while the free-form DNA molecules (e.g., water, benthic substrate) can originate from urine, faeces, mucus, saliva, gametes, and epidermal cells of living or decomposing aquatic organisms. Free-form eDNA can also be used to detect transient animals such as birds and mammals that deposit DNA into water when visiting or flying over water bodies (Rees et al. 2014; Pedersen et al. 2015). Once DNA is deposited in the environment, its persistence may vary from hours to weeks in temperate water to several months or years in soil, caves, permafrost, and sediment. From a single standardized environmental sample, eDNA from different species belonging to one or more taxonomic groups, or even whole communities, can be analyzed simultaneously. Studies that employ eDNA technologies can avoid direct contact with sensitive aquatic

species (Waits and Paetkau 2005), while potentially providing equivalent or improved species detection probabilities in less time, with less effort, and with lower costs for sampling compared to conventional survey methods (Smart et al. 2015; Sassoubre et al. 2016; Shaw et al. 2016; Tsuji et al. 2017; Boussarie et al. 2018; Pikitch 2018). Initially, most eDNA studies had focused on freshwater species because marine systems were more complex and vastly larger than freshwater systems, making it difficult to apply eDNA technology, but this is now changing. To effectively apply this relatively new technology in the field, it is critical that researchers and managers understand both the strengths and limitations of this monitoring tool. Only with careful validation and interpretation of results, can eDNA technology contribute significantly to DFO mandated responsibilities.

Interest in the applications of eDNA for biological conservation is growing rapidly, concurrent with fast-paced advances in innovative technologies (e.g., next-generation sequencing (NGS) and microfluidics quantitative PCR (qPCR), associated significant declines in monetary costs per sample). Construction of purpose-built dedicated eDNA facilities, with consistent quality control protocols is underway in several locations around the world (e.g., Spygen, France; Centre for Environmental Genomics Applications (CEGA), St. John's, Newfoundland and Labrador; EnvMetaGen, Portugal; Trace and Environmental DNA (TrEnD) Laboratory at Curtin University, Perth, Australia). Long-term eDNA monitoring has already been adopted by the United States government for early detection and monitoring of invasive Asian carp *Hypophthalmichthys* spp. in the Laurentian Great Lakes basin (Jerde et al. 2011). It is also being used to assess organic loading impacts on the ecosystem from salmon farming in Australia and New Zealand (Pochon et al. 2015a; White et al. 2017; Keeley et al. 2018). The scientific literature regarding this tool is rapidly expanding, and several high impact scientific peer-reviewed journals have published special issues devoted to amassing review papers on eDNA practices and pitfalls (e.g., Biological Conservation (Elsevier) journal in 2015; the March 2018 Biological Conservation Special Issue), and new journals are entirely dedicated to the field (e.g., Environmental DNA (Wiley) journal).

In the following text, we outline existing knowledge on eDNA and its applications, emerging opportunities in eDNA technologies and key results of the national 2018 DFO eDNA workshop, and suggest a cohesive approach to eDNA research within DFO.

2. Current eDNA applications and use within DFO

As a tool for the detection of aquatic organisms, eDNA technology has much to offer DFO, especially in cases where traditional sampling strategies do not meet the Department's rapidly increasing monitoring needs. According to the DFO Science

Monitoring Implementation Team 2006 report, fish habitat, AIS, food webs, SAR, integrated management initiatives, marine protected areas (MPAs), and any effects of cumulative anthropogenic impacts are not well monitored in Canada as a result of limited resources and vast remote areas that need to be covered. eDNA analyses on species relative abundance and distributions over broad temporal and spatial scales can help enhance the development of systematic monitoring programs of ecosystem health. The technology can be used to identify habitats critical to important fisheries and protected species, to improve the detection probability of rare and cryptic aquatic species, and to cover large and remote areas that are difficult to monitor consistently over time. In the following sections, we elaborate on current eDNA applications specific to DFO where it can be a beneficial complement to conventional surveys that inform decisions on the conservation and management of aquatic species and ecosystems. It should also be recognized that there are a number of cross-cutting issues that are relevant between the various categories below since the identification of species composition and abundance over time are metrics that several disciplines use in the delivery of their programs.

2.1 Fisheries Management

Fisheries stock management is a process that aims to assess, as precisely as possible, how many of which species of fish are in Canadian waters. Thus, high quality fish population estimates and clear understanding of stock structure are critical for the determination of total allowable catch for sustainable harvests. The potential of eDNA technology for monitoring marine fish populations was first studied by Thomsen et al. (2012a, 2012b), who isolated eDNA from seawater samples and detected commercially important species, species rarely detected by conventional methods, and rare vagrant species such as migrating seabirds. This was a groundbreaking proof-of-concept step in marine eDNA fisheries science, demonstrating that eDNA analysis could estimate fish diversity better than, or equal to, nine other more conventional sampling methods. Since that study, several other research groups have shown that eDNA analyses generally are comparable to conventional survey techniques, and are much more efficient (e.g., Civade et al. 2016; Thomsen and Willerslev 2015; Thomsen et al. 2016; Valentini et al. 2016; Yamamoto et al. 2017). Recent studies have shown that eDNA analysis can be an easier and more rapid way to estimate seasonal changes in biomass than conventional monitoring methods (Takahara et al. 2012; Lacoursiere-Roussel et al. 2016a, 2016b), though quantification using eDNA generally can provide only relative, not exact, abundance data.

Within DFO, researchers currently are developing eDNA as a tool to monitor fish distributions in the Northwest Territories (Robert Bajno, pers. comm.) and a pilot study is underway to assess the associations between eDNA data and quantitative metrics of abundance (catch per unit effort, biomass) in the Pacific Region (Kristi Miller, pers.

comm.). A more detailed discussion of eDNA and organismal abundance can be found in the "*Potential future applications of eDNA for DFO*" section of this paper and also in the review paper on "*The future of biotic indices in the ecogenomic era: Integrating (e)DNA metabarcoding in biological assessment of aquatic ecosystems*" (Pawlowski et al. 2018).

2.2 Sustainable Aquaculture

Two of the key concerns in aquaculture, particularly finfish aquaculture, are 1) farms may be point sources of input for waste nutrients (organic or inorganic) into the ecosystem and could be sites for increased biomagnification of contaminants (Johnsen et al. 1993; White et al. 2017), and 2) the possible negative effects of farmed fish on the demographics of wild fish populations. Therefore, aquaculture research areas where eDNA analyses could be applied pertain to impacts of aquaculture sites on local biodiversity and domestic fish interactions with wild fish.

Analysis of benthic community biodiversity (both prokaryotes and eukaryotes) can be a useful tool for characterizing the organic enrichment associated with salmon net pen farming (Keeley et al. 2012a, 2012b, 2018; Stoeck et al. 2010, 2018a, 2018b; Pawlowski et al. 2016) and environmental monitoring through NGS metabarcoding of protists (Pawlowski et al. 2014). It can also be a useful monitoring tool to document shifts in benthic communities or trophic structure, which may result in increased bioaccumulation and biomagnification of contaminants to higher trophic levels (White et al. 2017). In New Zealand, Pochon et al. (2015b) found that eDNA collected from marine biofilms could be used for early detection of fouling organisms. Canadian studies are beginning to appear in the peer-reviewed science literature (e.g., bacterial communities under salmonid aquaculture sites in Newfoundland; Verhoeven et al. 2016). Recently identified aims within DFO include the development of benthic eDNA tools for cost-effective ongoing biomonitoring of impacts of benthic organic enrichment from salmon aquaculture (Cathryn Abbott, Shawn Robinson, pers. comm.).

Monitoring farmed fish that have escaped and interact with wild fish populations is a priority for the aquaculture industry, governments at all levels, commercial and recreational fishers, Indigenous Peoples, and environmental advocacy groups. Wild-domestic hybrids in natural systems pose a risk of maladaptive hybridization and introgression in local wild salmonid populations. Consequently, there is a need for sensitive, relatively low cost, and near-real-time methods to detect and monitor the presence of escaped and/or stocked salmonids in wild rivers. Researchers within DFO are in the initial stages of designing tailored eDNA marker panels to enable accurate assessments of genetic impacts of domestic strains on native populations (Ian Bradbury, pers. comm.). Similarly, current work at DFO's West Vancouver Laboratories

is examining the potential to use eDNA to identify genetically modified fish from farm effluent, for use in compliance and enforcement (Robert Devlin, pers. comm.).

2.3 Aquatic Animal Health

To protect the health of farmed and wild fish, and to help maintain sustainable populations, DFO, in collaboration with CFIA, strives to reduce disease risks, manage disease and parasites, and treat outbreaks. Consequently, there is a need for reliable monitoring of pathogens and the development of alternative tools to augment disease ecology investigations. Researchers at the University of Manitoba recently have shown that eDNA technologies can be used to determine pathogen presence and abundance in freshwater systems and can provide many advantages over conventional survey methods, including lower cost and effort (Huver et al. 2015). Gomes et al. (2017) used eDNA and water quality data to predict protozoan parasites outbreaks in Australian freshwater fish farms. In Great Britain, Sana et al. (2018) have used eDNA to evaluate the risk of disease emergence of a generalist parasite, *Sphaerothecum destruens*, that is associated with the spread of an invasive freshwater fish. Researchers from the United Kingdom and Norway are also benchmarking methods to use eDNA to detect and monitor important parasitic species of salmonid aquaculture including salmon lice *Lepeophtheirus salmonis* and amoebic gill disease *Paramoeba perurans* (Peters et al. 2018). In experimental tanks at the DFO Pacific Biological Station in British Columbia, Polinski et al. (2017) demonstrated a potential for using eDNA to monitor an intracellular parasite Denman Island disease *Mikrocytos mackini* in wild or farmed oyster populations during periods of disease remission. Broad-based infectious agent monitoring of viruses, bacteria, and microparasites is also currently being undertaken in the Pacific Region to inform optimal fallowing periods and transmission risk to wild salmon (Kristi Miller, pers. comm.). As research in natural systems continues to expand, real-time eDNA pathogen test kits are becoming commercially available and being integrated into disease monitoring. Microfluidics qPCR is also being applied to monitor the activity of biomarker panels predictive of the presence of specific stressors or disease states (Kristi Miller, pers. comm.). One such panel was recently developed to identify salmon in an active viral disease state that was validated across multiple viral infectious diseases and only required a small non-destructive gill clip (Miller et al. 2017). In the future, eDNA approaches may help assess pathogens that degrade or disappear quickly or be used to rapidly survey large samples to assess parasites that show a highly aggregated distribution in host populations.

eDNA techniques can also be used to study the development of resistance to antibiotics by bacteria or by parasites to therapeutants during the treatment of disease in fish and other cultured animals. Antimicrobial resistant genes have been found in salmon farming operations in Chile (Miranda and Zemelman 2002; Buschmann et al. 2012; Shah et al. 2014), in finfish and shellfish culture operations in China (Dang et al. 2006a,

2006b; Li et al. 2008; Wang et al. 2017b, 2018) and in several tropical countries such as Taiwan (Huang and Hsueh 2008) and India (Van Boeckel et al. 2015; Elmahdi et al. 2016). eDNA-type research is also contributing to the understanding of the development of pesticide resistance in parasites such as sea lice (Yanez et al. 2014; Braden et al. 2015; Chavez-Mardones and Gallardo-Escarate 2015; Valenzuela-Munoz and Gallardo-Escarate 2016).

One of the latest discoveries in the study of organism health has been the relationship between the microbial flora in the gut of an animal (known as the microbiome) and its overall state of health. In human medical research, the development of eDNA techniques has opened up an entire field that is relating the microbiome to human diseases (Clemente et al. 2012; Sun and Chang 2014; Wang et al. 2017a). The same healthcare monitoring approach is now being applied to aquatic organisms such as fishes (Fong et al. 2016; Hashizume et al. 2017). As this technology develops, and the correlation between an organism's microbiome and state of health is established, eDNA will likely play a major role in monitoring an organism's well-being and be a useful tool for the DFO and CFIA National Aquatic Animal Health Program.

2.4 Species at Risk

eDNA technology can be used as a complementary tool to detect fishes, marine mammals, reptiles, and molluscs listed under the *Species at Risk Act* (SARA) in Canada. Specifically, eDNA has the potential to increase and improve the data available on the presence/absence or occurrence of SAR. Improved occupancy data may lead to better understanding of present and historical (i.e., through sediment profiling) patterns of distribution, better population assessment estimates, improved monitoring and reporting on recovery implementation efforts, remediation and restoration efforts, and better estimates of range limits, which is crucial to delineating critical habitat. Additionally, the use of eDNA for providing forensic evidence for poaching and illegal trade of SAR may have benefits for the protection of at-risk finfish and shellfish (e.g., Northern abalone *Haliotis kamtschatkana*). The development and testing of hand-held eDNA devices would allow enforcement officers to test real-time and on-site, enhance enforcement capabilities, and improve the recovery of at-risk populations.

eDNA has already been successfully applied to several at-risk fishes that occupy freshwater habitats in Canada, including Chinook salmon *Oncorhynchus tshawytscha* (Laramie et al. 2015), spotted gar *Lepisosteus oculatus* (Glass and Mandrak 2014; Boothroyd et al. 2016), redside dace *Clinostomus elongatus* (Serrao et al. 2015; Reid et al. 2018), Eastern sand darter *Ammocrypta pellucida*, Northern madtom *Noturus stigmosus*, and silver shiner *Notropis photogenis* (Balasingham et al. 2018), in addition to various mussel SAR in Ontario, New Brunswick and Nova Scotia (Northern riffleshell *Epioblasma torulosa rangiana*, snuffbox *Epioblasma triquetra*, wavy-rayed lampmussel

Lampsilis fasciola, round pigtoe *Pleurobema sintoxia*, kidneyshell *Ptychobranchus fasciolaris*, mapleleaf *Quadrula quadrula*, rayed bean *Villosa fabalis*, rainbow *Villosa iris*, brook floater *Alasmidonta varicose*). Fewer eDNA studies have focused on marine species and ecosystems. Marine mammal detection rates (e.g., harbour porpoise *Phocoena phocoena*, long-finned pilot whale *Globicephala melas*) in natural locales thus far have been shown to be less consistent than in controlled environments, and less successful than conventional acoustic detection methods (Foote et al. 2012).

2.5 Aquatic Invasive Species

AIS have contributed to the decline and disappearance of some of Canada's native aquatic species as well as the collapse of some local fisheries. eDNA has been identified as an important tool for early detection of AIS, so that preventative management measures can be implemented swiftly. For example, eDNA could be used to screen ship-ballast water (a common source of species introduction) for AIS (Darling and Frederick 2018), or to confirm the eradication of all invaders following an intensive eradication program (Pilliod et al. 2013). eDNA methods have been applied to the early detection and biosurveillance of AIS in the Great Lakes, which is often listed as one of the top threats to the recovery or survival of many imperilled freshwater fishes and molluscs in North America. In the United States, development of eDNA applications have focused on Asian carps (Jerde et al. 2011) and more recently on round goby *Neogobius melanostomus* (Nevers et al. 2018). In Japan, eDNA has been used for surveillance of the invasive bluegill sunfish *Lepomis macrochirus* with less disturbance to the environment compared to conventional methods (Takahara et al. 2013). In New Zealand, protocols are being developed for invasive dreissenid molluscs, including zebra mussels *Dreissena polymorpha* and quagga mussels *D. bugensis* (Pilliod et al. 2013). In China, eDNA-based methods have been applied to the early detection of the invasive golden mussel *Limnoperna fortune* (Xia et al. 2018). Some of the Canadian projects include: use of eDNA in AIS surveillance of the Great Lakes commercial bait trade (Nathan et al. 2014, 2015); detection and identification of sea lamprey *Petromyzon marinus* in Great Lakes streams (Gingera et al. 2016); detecting high-risk freshwater fishes in the live trade (Roy et al. 2015, 2017); eDNA as an early detection tool for zebra mussels in Lake Winnipeg (Gingera et al. 2017); and development and field testing of metabarcoding-based eDNA biosurveillance tool for AIS including finfish and dreissenid mussels in British Columbia (Cathyrn Abbott, pers. comm.).

2.6 Sustainable Aquatic Ecosystems

The characterization of ecosystem health through the measurement of biological indicators or "bioindicators" (e.g., species, biological source material) is another application of eDNA technology being used today (Barnes and Turner 2016). Bioindicators are chosen for their sensitivity to environmental conditions. If the chosen

bioindicator declines or increases in numbers, the respective change can be taken as a sign to look for detrimental influences such as water pollution, community structure shifts, and climate change. Besides being sensitive to change, bioindicators need to be easily observed and sampled (Stein et al. 2014). In freshwater stream environments, eDNA monitoring results were found to be comparable to conventional kick net sampling of benthic macroinvertebrates (Machler et al. 2014). Bista et al. (2015) showed that eDNA could be used to monitor lake ecosystem health by detecting chironomid midges. The use of eDNA to evaluate bioindicators eDNA to determine impacts of human activities on marine environments already has been demonstrated for both offshore oil drilling (e.g., first evaluation of foraminiferal metabarcoding for monitoring environmental impact from an offshore oil drilling site; Laroche et al. 2016, 2017) and salmon aquaculture (e.g., benthic monitoring of salmon farms in Norway; Pawlowski et al. 2016). With the advent of eDNA tools that are capable of measuring a much wider suite of species with shotgun sequencing, there is an opportunity to examine a wider range of potential indicator species to give us more precision in assessments based on cause-effect relationships.

There is a growing body of literature suggesting that eDNA has the potential to be an efficient method to sample marine biodiversity (Thomsen et al. 2012a, 2012b; Kelly et al. 2014; Port et al. 2016; Schmelzle and Kinziger 2016; Thomsen et al. 2016; Yamamoto et al. 2016; Gargan et al. 2017; Sigsgaard et al. 2017; Stoeckle et al. 2017; Evans et al. 2018). Of note, metabarcoding studies by Port et al. (2016) and Yamamoto et al. (2016) indicated that eDNA methods detected significantly more native or cryptic marine species not observed by conventional methods [> 20 species in the case of the Yamamoto et al. (2016) study]. eDNA biodiversity studies also could be integrated systematically into MPA management science to augment existing and developing monitoring methods, as well as link existing eDNA projects nationally to avoid redundancies and maximize collaborations. Current methods for evaluating ecosystem function within the marine environment, including MPAs, largely involve fish biodiversity and trophic structure assessment using ship-based whole organism sampling (e.g., IYGPT trawl surveys) and SCUBA-based visual assessment methods that are time and labour intensive, difficult to repeat, and ignore changes in marine microbial communities.

3. Understanding our ability to collect, identify, and interpret eDNA

As identified during the 2018 national DFO eDNA workshop, though new frontiers of eDNA applications to fisheries and aquatic science generate exciting possibilities for future management and research, several factors associated with our ability to collect, identify, and interpret eDNA (i.e., the source, yield, transport, and degradation of eDNA

must be understood as thoroughly as possible by researchers, managers, and reviewers). The following is a literature review on the ecology of eDNA.

3.1 Sources of eDNA

In all studies, it is important to acknowledge the uncertainty regarding the origins of the sloughed materials, rather than assuming origins (Figure 2a; Barnes and Turner 2016; Valentini et al. 2016). The shedding rate of DNA from aquatic organisms is often dependent on the characteristics of the organism itself, including its physical size, life stage, skin or scale properties, and stress and/or activity level (Sassoubre et al. 2016), as well as local abiotic (e.g., temperature) versus biotic (e.g., microbial activity) environmental conditions. Seasonal activity of organisms (e.g., peaks in spawning or die-off activity) has been shown to influence eDNA detection probabilities (de Souza et al. 2016; Tillotson et al. 2018). DNA from outside the study area or system sampled may lead to false positives (Shaw et al. 2016, 2017; Stoeckle et al. 2017; Yamamoto et al. 2017) via fecal matter deposited in or near water bodies, human effluent, translocation of individuals by scavengers, predators or humans, movement of boats or barges between systems, or movement of soil for construction purposes or restoration projects. Also, remnants of aquatic organisms (e.g., shells, bones) or the stirring of sediments may lead to positive detections of species that no longer occur in the study area.

3.2 Collection of eDNA

Systematic comparisons among eDNA collection methods are lacking, and trial and error has dominated the field thus far (Barnes and Turner 2016). Few eDNA studies to date consider, much less report, the physical form (e.g., intracellular, extracellular, particulate, or free/dissolved) of eDNA collected. In a study on common carp eDNA, Turner et al. (2014) found that the highest yield of eDNA was found as aggregations of cells on the larger pore filters compared to fine pored filters designed to capture threads of extracellular DNA. Further size fractionation studies, using serial filters, are needed to determine the size distribution of various particles in the aquatic environment and optimal filter size. This is important because different filter types could yield different eDNA concentrations that reflect particle size classes rather than population size differences. Inevitably, filters become clogged, and a trade-off exists between filter pore size and the amount of water that can be processed before a filter clogs. It is currently unknown as to whether there are trends across taxa and environments for optimized eDNA yield.

3.3 Transport, degradation, and persistence of eDNA

The interpretation of eDNA-based research and inferences can be affected by how DNA is transported through the environment. For example, Foppen et al. (2011) inoculated

two streams with synthetic extracellular DNA tracers and compared their physical movement with sodium chloride (NaCl) tracers. The NaCl tracer remained detectable ~2 km downstream, yet the eDNA did not. They found that DNA quantity progressively reduced as it moved downstream, suggesting adsorption, biological uptake, and other interactions between DNA and environment were common. Furthermore, similar studies have shown the downstream transport distance of eDNA can also be species-specific (Deiner et al. 2014). It is important that these types of preliminary, or pilot studies are done as part of the validation process for each new project.

From bacterial and plant research, it has been suggested that DNA molecules in water bodies begin to degrade immediately after dead cells are shed and that degradation increases exponentially with time (Nielsen et al. 2007; Thomsen et al. 2012a; Barnes et al. 2014; Maruyama et al. 2014; also see Annex A). Most eDNA persists in the water for only a few days or weeks, however, eDNA can persist for very long times given the right conditions (e.g., undisturbed substrate versus turbulent water in a tidal bay. Barnes et al. (2014) offer a comprehensive review on factors influencing the persistence of eDNA in aquatic environments. Several reviews on eDNA persistence indicate three broad categories of factors:

- DNA characteristics (conformation, length, and association with cellular/organellar membranes);
- abiotic environment (light, oxygen, pH, salinity, and the abundance and composition of substrates); and
- biotic environment (the composition and activity of the microbial community and extracellular enzymes).

The wide range of persistence times in the literature suggest that there is still much to learn about eDNA degradation (Barnes and Turner 2016). Uncovering this knowledge will likely require more research on eDNA itself, as well as the interactions between eDNA and its environment.

4. Technological opportunities associated with eDNA

The rapid development of eDNA has led to different approaches and challenges to sampling and interpreting eDNA data across research groups in DFO, Canada and internationally. Thus this section of the paper provides a brief contrast of the advantages and challenges of existing and emerging eDNA field sampling methods, DNA sequencing and high-throughput (H-T) DNA amplification technologies, and computer-based analysis of eDNA, of which we feel have the most potential for growth within DFO for eDNA research. Field sampling methods, including sample collection, preservation, and transportation are important considerations for any eDNA study, as

the chosen method has its own advantages and disadvantages, and can greatly affect the quantity and quality of eDNA used for sequencing. Likewise,we include a discussion on existing and emerging amplification and sequencing technologies, and computational proficiency and pitfalls for DNA data analyses. The definitions and descriptions of the various sequencing technologies can be found in Box 1.

4.1 Improved preservation methods

eDNA concentration in the aqueous environment is generally low and unevenly distributed. The reliability of eDNA analysis and the ability to detect species is therefore highly dependent upon capture efficacy and sample preservation methods. Comparisons of field methods are currently underway to maximize eDNA yield from water samples and improve detection probabilities (Goldberg et al. 2016; Hinlo et al. 2017). There are several common preservation methods: alcohol precipitation, filtration-based methods (e.g., use of cellulose nitrate, carbonate filters), chemical preservation (e.g., cationic surfactants, lysis buffers), or combinations of these methods. Originally, alcohol preservation was used to preserve eDNA (Ficetola et al. 2008), but eDNA retention was found to be low. In contrast, filtration-based methods retain more eDNA, and are now the most commonly used field method for eDNA preservation. However, the need for on-site filtration equipment, coolers to store filters, followed in some cases by rapid transportation of filter samples to research stations or laboratories can significantly impact the scope, length, and cost of eDNA projects. Recently, a fully integrated eDNA sampling system called ANDe™ (Thomas et al. 2018) was developed that consists of a backpack portable pump, long-pole extension and support biopod for sampling, remote pump controller, custom-made filter housings with single-use packets for each sampling site, and onboard sample storage.

Given the considerable amount of effort associated with filtration-based methods, several studies have focused on chemical additives that can be added to water samples or around filters to preserve DNA at ambient temperatures prior to DNA extraction (Renshaw et al. 2015; Yamanaka et al. 2016; Williams et al. 2017a, 2017b). Advances in chemical preservation methods may include reductions in their effect as a polymerase chain reaction (PCR) inhibitor, better understanding of their effectiveness among target species or different biomasses, and their application for metabarcoding studies. Many different labs internationally are trying to resolve the best protocols for handling DNA once it sampled (e.g., Wegleitner et al. 2015, Hinlo et al. 2017). DFO can build on this knowledge to produce best practices for handling of samples appropriate for the department. Continual advancements in field methodology may further increase the appeal of eDNA studies by lowering sampling costs, simplifying field logistics (e.g., lengthy decontamination protocols), and improving DNA yield across varying environmental conditions (see Annex A).

4.2 Hand-held real-time sampling devices

Hand-held devices offer rapid and on-site sequencing and assessment of DNA derived from aquatic ecosystems. Such devices have the advantage of automated real-time detection of sensitive species, aquatic microbiome and viruses, AIS, or lucrative fisheries in the field. In some devices, raw data are analysed using cloud computing by internet access (Bleidorn et al. 2016). This real-time reporting is beneficial when management decisions are needed quickly while crews are in the field (e.g., to confirm and respond to early detection of AIS, identification of illegal catches by enforcement officers). Such devices bypass the need to preserve and transport large volumes of sensitive samples from field to lab, and the DNA is used in its best form immediately before further degradation occurs. If eDNA technology continues to advance and innovate, additional benefits may be realized. Use of hand-held devices has great potential to revolutionize the detection of aquatic organisms and change the way we collect sequencing data; however current hand-held technologies cannot profile multiple targets at the time of writing.

4.3 Remote and autonomous sampling and analysis

Environmental samples from aquatic environments also can be easily sampled using autonomous vehicles and remote sensing technologies like submersible drones (Barnes and Turner 2016). Remote operated vehicles (ROVs) are generally available to DFO scientists and have been successfully used to collect benthic eDNA samples for bacteria in water depths of 30 to 50 m. Similarly, in the United States, aerial drones have been used to remotely collect water samples to survey macrobiota (Ore et al. 2015). Several purpose-built eDNA collection systems already are commercially available, although costs at this stage are prohibitive for most conservation studies. The most sophisticated remote sampling system autonomously conducts water filtration, DNA extraction, and genetic assays by qPCR underwater while deployed on an ocean mooring, then it transmits genetic data via satellite (Preston et al. 2011). The use of remote and autonomous sampling may also facilitate future large-scale geographic eDNA sampling involving citizen scientists (Biggs et al. 2015).

4.4 Amplification and sequencing technology and eDNA

Ultimately, different studies will use different DNA sequencing approaches depending on budget, sample number, community composition, and research questions (Creer et al. 2016). Initially, first-generation Sanger sequencing of PCR products were used for eDNA analyses (see Box 1 for explanations on and definitions of all sequencing technology terms mentioned in this section). Rapid advancements in NGS technologies (e.g., H-T genome-wide sequencing, long-range PCR, DNA enrichment) have had an enormous impact on genomic applications and improved cost-effectiveness per sample

(Kulski 2016). Looking forward, emerging technologies in microfluidics, laser transmission spectroscopy, and possibly single-molecule real-time (SMRT) sequencing may have potential to become powerful tools of eDNA science (see Box 1).

For eDNA studies that target one species or genus, it can be more cost-effective to use DNA amplification technologies like qPCR (see Box 1; Doi et al. 2015). On the other hand, there are benefits in using quantitative digital PCR (dPCR) over qPCR, because dPCR offers more accurate estimation of low concentrations of eDNA than qPCR and does not rely on standard curves (Nathan et al. 2014). Another new potential approach to sequencing eDNA is digitial droplet PCR (ddPCR). Evans et al. (2017) compared the cost and effort of fish detection through electrofishing to that of ddPCR, and found ddPCR to be a potentially cost-effective approach that could improve species detection per unit effort (but see Box 1). For multi-species eDNA assays, the use of H-T sequencing and PCR-based metabarcoding microfluidics (i.e., dPCR, ddPCR) (Box 1), or microarray methods to target large taxonomic groups such as teleost fish or phytoplankton can be more cost-effective than qPCR assays (Vuong et al. 2013).

Genetic methods that do not rely on PCR to characterize communities include shotgun sequencing (Simon and Daniel 2011), direct DNA enrichment, and SMRT sequencing (Zhou et al. 2013). Environmental shotgun sequencing is preferred for study of microbial communities (see Creer et al. 2016). There are promising developments in DNA enrichment methods that bind sequence probes to target DNA during DNA extraction and can help overcome the monetary cost and inaccuracy problems associated with PCR-based methods (Ballester et al. 2016; Dowle et al. 2016; García-García et al. 2016). The DFO Pacific Biological Station (pers. comm. Kristi Miller) laboratory effectively has used such a target enrichment approach to concentrate salmon viruses for full genome sequencing (Di Cicco et al. 2017). Finally, although current error rates are prohibitive, SMRT sequencing is being examined as a future metagenomics tool for genetically characterizing aquatic community structure (Bowman et al. 2015; Bleidorn et al. 2016; Creer et al. 2016; Deiner et al. 2017b).

Box 1. Expanded definitions of existing and emerging amplification and sequencing technologies

Low-throughput PCR-based analyses

Polymerase chain reaction (*PCR)* involves species-specific probes (i.e., primers) that bind to and amplify (i.e., many rounds of duplication) target DNA in an eDNA sample, so that it can be visualized after amplification (tradition PCR) or during amplification (real-time PCR (RT-PCR); Table 1).

Quantitative PCR (*qPCR*) can estimate quantity of a specific DNA sequence in a sample, which may then be used as a proxy for relative species abundance, by comparing the ‘unknown’ eDNA concentration to standard curve created by amplification of a known material (Table 1).

The qPCR method uses RT-PCR and has a high detection sensitivity, and enhanced quality control and assay validation compared to traditional PCR. It is beneficial for the detection of aquatic species in low abundance.

Long-range PCR via Illumina sequencing can generate DNA sequence fragments between 16 and 17 kilobases from eDNA. Recently, Deiner et al. (2017b) used long-range PCR to amplify entire mitochondrial genomes of invasive species from DNA collected from lake, pond, and stream water samples. Not all eDNA is highly degraded, and use of long-range PCR can alleviate many problems associated with identification of species from short-fragment PCR-based methods.

High-throughput (H-T) PCR-based metabarcoding

PCR-based metabarcoding is targeted simultaneous detection of multiple species in an environmental sample, where primer pairs target highly-conserved regions of one or more amplicons that bound highly variable (e.g., species-specific) regions.

PCR-based microfluidics and nanotechnology

Digital PCR (dPCR) is an emerging technology in microfluidics (the science of manipulating and controlling fluids in networks of tiny channels) likely to advance our ability to quantify eDNA (Table 1; Doi et al. 2015; Goldberg et al. 2016). First commercialized in 2006 by Fluidigm and subsequently Life Technologies, dPCR can amplify DNA held in hundreds to millions of massively parallel partitions on plates the size of a microscope slide (Baker 2012). Within each partition a PCR reaction either happens (sequence is detected) or not (sequence is not detected). The number of reactions on a plate quantifies the exact number of copies of target DNA in a sample, which helps to eliminate false negatives. dPCR may someday replace traditional qPCR.

Digital droplet PCR (ddPCR) first commercialized in 2011 by Bio-Rad, works by portioning samples, with no manual pipetting, of extracted eDNA into thousands of individual droplets that can be screened for the target species' DNA. ddPCR is similar in concept to dPCR, except that partition walls are made of oil and other chemicals rather than wells in a microscope slide. However, ddPCR has no true quantification capabilities. ddPCR costs far less than dPCR technologies, thus it is thought that ddPCR will replace traditional PCR methods once costs are lowered (Baker 2012).

Shotgun metagenomic sequencing

Shotgun metagenomic sequencing (SMS) refers to the random sequencing of all genomic material in an environmental sample, without the use of PCR primers, into short sequences which are subsequently assembled into longer sequences. Described in more detailed below.

Direct sequence capture and DNA enrichment

DNA enrichment uses an enormous number (e.g., 20 000) of short (e.g., 100-mer) synthetic DNA probes, designed from reference sequences, that are complementary to regions in the genomes of the target taxa. The complementary sequences capture target DNA regions (Maricic et al. 2010; Mertes et al. 2011). The captured DNA is a reduced representation of the

genome-wide material, and this in turn permits increased throughput and sequencing power. Thus far, DNA enrichment has been shown to produce up to one order of magnitude more sequences than the PCR-based metabarcoding of environmental samples (Dowle et al. 2016). DNA enrichment methods do not require PCR amplification, and thus avoid biases associated with universal primers (Dowle et al. 2016).

Laser transmission spectroscopy (LTS)

Laser transmission spectroscopy (LTS) is increasingly recognised as a powerful, cost-effective, and user-friendly new addition to the DNA detection arsenal (Li et al. 2011; Mahon et al. 2013; Barnes and Turner 2016; Goldberg et al. 2016). LTS is a real-time quantitative detection technology that rapidly measures the size, shape, and number of nanoparticles in a solution by measuring the wave-length of light transmitted through a sample. LTS has already been used to detect invasive aquatic species, and demonstrates highly sensitivity identification of target and non-target species DNA in multispecies samples (Mahon et al. 2013).

Single-molecule real-time (SMRT) sequencing

eDNA relies on the efficiency of genomic sequencing and analysis. A third generation of sequencing techniques recently became available that may make that possible through single-molecule sequencing with no PCR amplification step. Much of the current technology can "read" strands hundreds of base pairs (bp) long. With single molecule sequencing, however, the average output can be reads of 15,000 bp long (see Table 1). These methods produce extremely long sequences that are desired for metabarcoding and metagenomic eDNA studies, because they improve genome assembly, which allows for more reliable assignment of DNA sequences to reference species compared to second generation sequencing methods (Taberlet et al. 2012; Bleidorn 2016). Base calling error rates are the largest downfall, but error rates are improving each year. Third generation sequencing can be an invaluable tool when used in conjunction with paired-end read Illumina sequencing. Several third generation technologies are in various stages of development and beta-testing:

- Helicos—https://www.thebalance.com/helicos-biosciences-corporation-375516
- PacBIO—http://www.pacb.com/
- Nanopore—With hand-held devices (e.g., MinION), automated identification of species from eDNA may be achieved.

4.5 Computer-based analysis of eDNA sequence data

H-T sequencing technologies often generate massive volumes of complex data that is difficult to manage using standard desktop and laptop computers (Edgar et al. 2016). For many projects, high performance computers, computer clusters, and/or cloud computing systems are required, where problems are split into many small tasks and run in parallel (Gangloff et al. 2016). Mendoza et al. (2015) provide a useful overview for navigating the bioinformatics challenges associated with using massive datasets to evaluate low abundance sequences from eDNA samples. Furthermore, many of the programs that manage or manipulate H-T sequencing data require knowledge of the command line, as well as the ability to navigate, edit, annotate, and provide versions for

code to conduct reproducible eDNA analysis. Therefore, it is highly important for purpose-built eDNA facilities to include include bioinformatics when considering facility capacity.

For those that will be working with H-T sequencing data, it is important to have access to available toolkits for improved computational proficiency. For example, there are books designed to teach practical computing skills to biologists (Haddock and Dunn 2010), and training courses to improve eDNA analysis capabilities. Improvements to computer proficiency, including scripting methods (e.g., analysis in R, Bash, Perl, Python) as well as code documentation (e.g., git, GitHub), will result in more robust, reproducible outputs. Even when computing power is available, without careful analysis, bioinformatics analysis of H-T sequencing data from eDNA samples is susceptible to artefacts such as sample contamination, amplification errors, sequencing errors, computational artefacts, and inaccurate taxonomic assignment (Barnes and Turner 2016), although there are approaches to address most of these issues. Many software methods favoured by bioinformatics are open source, and therefore can benefit from continued community development. Taxonomic assignment, for example, is possible using programs such as MEGAN, whereby eDNA amplicons are assignment taxonomic identifiers using a lowest common ancestor (LCA) algorithm (Huson et al. 2007). Sample barcode switching, or misassignment of samples during demultiplexing may also be an issue, and the level of contamination that occurs through phenomenon can be addressed by having species not expected in the normal samples as a mock positive control (Deiner et al. 2017a). Problematically, there remain many amplicons that do not have voucher samples or representation in databases, which will result in unknown assignments. Unknown assignments can comprise a large component of eDNA metabarcoding results, thus there is a strong need to accumulate whole genome (and/or mitogenome) sequences, or at very least standard amplicon coverage for important fish and indicator species, SAR, and AIS. With a comprehensive database of whole genome sequences, researchers can better address the challenges concerning conservation of wild stocks and sustainability of aquaculture operations (Kumar and Kocour 2017).

5. Potential future applications of eDNA for DFO

5.1 Estimation of organism abundance

The ability to use eDNA concentrations to measure species abundance as a proxy for biomass estimation or catch per unit effort would greatly enhance the Department’s capacity to collect information on aquatic species. To estimate biomass, the abundance of whole organisms is quantified based on the number of recovered DNA sequences in environmental samples. Numerous studies have begun to explore quantification of eDNA as a means of estimating population size or biomass (Elbrecht et al. 2015;

Gomez-Rodriguez et al. 2015; Barnes and Turner 2016; Goldberg et al. 2016; Kelly 2016; Doi et al. 2017; Stoeckle et al. 2017; Weltz et al. 2017). One of the first eDNA-biomass studies (Takahara et al. 2012), showed that eDNA analysis was an easier and more rapid way to estimate seasonal changes in biomass of the Common Carp *Cyprinus carpio* than conventional monitoring methods. Environmental factors (e.g., temperature, pH, microbial communities), species-specific factors (e.g., spawning period), and other factors (e.g., species location in the water, live/dead ratio) may significantly affect biomass estimations over the course of a day or even within hours. Thus, recent research has focused on investigating how, and to what degree, these different factors influence eDNA-biomass estimations (Lacoursiere-Roussel et al. 2016b). Furthermore, shedding rates of eDNA can differ based on the life stage or reproductive status of the organism, which additionally confounds quantification. Due to the variable community composition that can be found in a location, the relative quantities of reads can depend on the sequencing of other taxa within the sample that takes up sequencing reactions, further reducing quantification; for this reason and others, qPCR is a more likely candidate for quantification of biomass than metabarcoding. Although eDNA quantification has advanced since earlier assessments on the tool (e.g., Takahara et al. 2013; Kelly et al. 2014), only careful study design can help overcome some inherent issues (see Evans et al. 2016). Because it is difficult to predict PCR primer performance despite best practices, the relationship between organismal abundance and PCR amplicon abundance is not predictable with high precision (Evans et al. 2016). With the advent of non-PCR and improved PCR technologies, as discussed below, new possibilities for evolutionary research will open up in the near future.

5.2 Description of whole communities

eDNA metabarcoding is emerging as a powerful tool to assess aquatic community structure, because the distributions of multiple interacting species can be assessed in a single survey (Yamamoto et al. 2017). It is quite possible that the biodiversity of an ecosystem has a fractal quality about it, where patterns in the microscale are reflected in the meso- and macro-scales. The implication of this hypothesis is that monitoring biodiversity might be accomplished at any scale and the techniques chosen based on efficacy and cost. An eDNA study from New Zealand on the validation of a multi-trophic meta-barcoding biotic index demonstrated strong correlations in the enrichment stages around salmon farms among prokaryotes and eukaryotes which included foraminiferans and larger infaunal macrofauna (Keeley et al. 2018). Marine fish community structure is difficult to investigate, often due to a lack of taxonomic expertise, damaged or fragile specimens, and requirement for extensive fieldwork. Some marine environments are simply difficult to observe (e.g., the deep sea). Since fishing practices and environmental factors can alter community structure, rapid and continual investigations

of marine communities are becoming increasingly essential (Yamamoto et al. 2017). eDNA has been used to successfully identify multiple species in a variety of marine and flowing freshwater ecosystems (e.g., Thomsen et al. 2012a, 2012b; Balasingham et al. 2017; Stoeckle et al. 2017). In a recent study, eDNA metabarcoding based on a 6-h collection of water samples detected 128 fish species, of which 63% (40 species) were also observed by underwater visual censuses conducted over a 14-year period (Yamamoto et al. 2017). However, the reliability of eDNA models and NGS to estimate whole community composition from eDNA is still in its beginning stages and requires ongoing field-testing and refinement (Rees et al. 2014). When whole organisms are present in a sample during metabarcoding and not only residual shed material, in some cases it is desirable to sequence more than barcodes and perform metagenomics. However, sequencing the genomes of mixed communities of bacteria, for example, compared to using metabarcoding to sequence short targeted sequences from the mitochondrial genome, requires a huge increase in sequencing power and bioinformatics resources. This consequently limits sample throughput in both quantity and time, reducing experimental flexibility in sampling design (Knight et al. 2012). The detailed description of communities will be particularly important in studies requiring time series data. Understanding the annual and inter-annual variability associated with the ecosystems DFO manages will be important to files such as Climate Change Adaptation and habitat impact and recovery.

5.3 Environmental RNA (eRNA) and functional genomics

Biological molecules other than DNA provide opportunities for conservation and research. There have been several studies sequencing RNA out of filtered water (eRNA), but also in sequencing microbial community transcriptomes from filtered water (e.g., metatranscriptomics). Since eRNA is less stable and degrades faster than eDNA, it has the singular advantage of detecting living organisms, rather than detecting a historical record of all organisms, living or dead, that may have occurred at a given location (Pochon et al. 2017). Thus, eRNA is increasingly being used to better estimate the time since deposition of biological material than eDNA. For metatranscriptomics, the microbial gene activity in water samples can be assessed for the community of microbes active in different conditions or associated with an event or phenotype of macro organisms within the water. eRNA has been used to assess impacts of offshore oil rigs on benthic communities in New Zealand (Laroche et al. 2017), and both eDNA and eRNA have been used to monitor the benthic communities under salmon farms in Scotland and Norway (Pawlowski et al. 2014, 2016) and New Zealand (Pochon et al. 2015a, 2017). Dowle et al. (2015) compared the efficiency of DNA and RNA using the 16S ribosomal RNA meta-barcodes for detecting organic enrichment from salmon farms. Also, quantification of proteins in the environment combined with a knowledge of proteomics is thought to be useful as an indicator of organismal activity or ecosystem

health, as the activation of certain genes could indicate responses to environmental stimuli (Barnes and Turner 2016).

Functional genomics addresses questions about the function of DNA at the levels of genes, RNA transcripts, and protein products. The practical conservation applications of functional analyses include identification of adaptive or fitness-related loci (Evans et al. 2017; Miller et al. 2011; Bradbury et al. 2013, 2010), monitoring genetic loci related to stress events (Prunet et al. 2008), using transcript markers predictive of disease/parasite responses and progression (Sutherland et al. 2014a; Miller et al. 2017) or physiological changes such as smoltification (Sutherland et al. 2014b), and describing the molecular basis of inbreeding and outbreeding depression (Paige 2010; Barnes and Turner 2016). Microbial metagenomics has laid the foundations for functional genomic analysis of eDNA (e.g., Inskeep et al. 2013; Staley et al. 2014; Mendoza et al. 2015), though these studies typically have intact organisms to filter and sequence at the level of the genome. This has not yet been possible in macro-organismal eDNA, due to the low concentration and degraded nature of the sample. In their review on eDNA, Barnes and Turner (2016) state that because molecules containing functional genomic information from macro-organisms are clearly present in environmental samples (see Orsi et al. 2013; Maki et al. 2017), "nothing prevents functional genomics applications for macrobial eDNA". Whole genomes continue to be published for many important macrobiota, bioinformatics tools continue to advance, and therefore if the sample acquisition of sufficient quantities and qualities of genomic material can be successful, this type of analysis may be a possibility in the future (Mendoza et al. 2015; Barnes and Turner 2016).

5.4 Population genetics and genomics

Population genetics is one important tool to help understand demographic parameters such as population size as well as connectivity (i.e., gene flow) over spatial and temporal scales. The study of population genetics—analysis of genetic differences within and between populations—using eDNA is another application with growing interest (Barnes and Turner 2016). However, despite interest levels, delineating populations, evolutionarily significant units, and individuals based on eDNA will require improvements in sample acquisition (potentially enrichment), identification of effective target markers for individual identification, and potentially sequencing power to achieve longer reads with low error rates for characterization of haplotypes (Stat et al. 2017). A major challenge to this aspect of eDNA is the fact that the nuclear genome is typically used in population genetics whereas the mitochondrial genome is used for eDNA metabarcoding (due to copy number and resultant availability in samples). For example, DNA fragments of different lengths and abundances persist in the environment differently, where eDNA fragments are typically short (< 300 bp). Furthermore, a pool of individuals in an eDNA sample, without knowledge of exactly the number of individuals

present, confounds allele frequency calculations needed to estimate population genetic parameters. Most mitochondrial DNA (mtDNA) eDNA studies involve slowly evolved loci, thus do not have high enough resolution for population genetic analyses. Microsatellite markers, commonly used for population genetic analysis, are not ideal for eDNA analyses because they usually are long fragments > 300 bp, and have low copy number relative to mtDNA. However, there are two eDNA studies published to date that successfully estimated population genetic parameters in humans using metabarcoding of mtDNA D-loop amplicons (Kapoor et al. 2014), and shotgun metagenomics sequencing (Afshinnekoo et al. 2015). Furthermore, Sigsgaard et al. (2016) were able to use eDNA and amplify the D-loop amplicons from mtDNA to identify whale shark *Rhincodon typus* individuals within the Arabian Gulf. As more markers are generated for this type of approach, and as long-read SMRT sequencing (see Box 1) improves in accuracy, more population genetic analyses may be possible using eDNA. This is one method of moving beyond the challenges of quantification from eDNA through use of individual level metrics. As the genomics field evolves, we expect to see more tangible innovations that may permit eDNA-population level studies.

6. Next Steps

DFO has a very broad and encompassing mandate. Canada, surrounded by the Arctic, Atlantic, and Pacific Oceans is home to a vast freshwater system, enjoys a strong reputation in fisheries, aquaculture, and aquatic environmental science and research. Therefore, new tools such as eDNA technologies should be evaluated as rigorously as conventional methods of research and monitoring used by DFO.

As the challenges facing Canada's marine and freshwater ecosystems increase and applications of eDNA are realised, collaboration among DFO researchers and the development of national guidelines are needed to adapt to new environmental realities and emerging technologies. It was agreed at the 2018 national DFO eDNA workshop that establishing a national DFO eDNA working group to address research priorities and other research needs (see Box 2) and the development of national guidelines would help to provide the Science Program with a common direction for the future and achieve a better balance between focussing on the long-term issues while maintaining flexibility to adapt and respond to priorities of the day. It would enable the Department to apply eDNA tools to emerging and present threats (e.g., AIS) and priority issues (e.g., aquatic animal health, SAR) that matter most to Canadians and possibly enhance our capacity to conduct long-term and forward-looking science on a broader array of issues.

Box 2. eDNA research priorities and other science needs discussed during the 2018 national DFO eDNA workshop

The 19 questions and statements below were presented and discussed by scientists and managers during the 2018 national eDNA workshop. They are assembled and grouped according to four themes that address priorities and science needs.

Improving fundamental scientific understanding of eDNA

1. How does eDNA behave, both chemically and physically, in different aquatic environments, particularly in the marine versus freshwater environment?
2. In what ways does eDNA in water and eDNA in sediment affect the efficacy of a chosen survey method (e.g., rate of exchange of eDNA between the water column and benthic sediments)?
3. What is the relationship between read (sequence) depth versus relative species abundance?
4. What is the relationship between eDNA concentration and species distribution in various environmental contexts (e.g., depth, temperature, seasonality)?
5. What types of parameters (physical, chemical, environmental) should be measured in order to decrease variability among field replicates?
6. Can inter-laboratory validation, including validation within the same research group, of eDNA measurements, analyses, or experiments, especially in new environments, be used to decrease uncertainty in results?
7. How cost-effective and efficient is eDNA for the detection of species (e.g., multi-species or area-based SAR monitoring, trawl-evasive species, marine species) and monitoring of ecosystems (e.g., for stock delineation, predator-prey relationships), especially those difficult to sample?
8. Can national guidelines with strong QA/QC be developed for sampling protocols and reporting for eDNA that maintain consistency with other DFO sectors and regions, with partners, proponents, other federal departments, and internationally?

Applications of eDNA tools to DFO mandated responsibilities

9. Can eDNA be used to support the investigation and/or prosecution for possession of prohibited species (e.g., using handheld devices, tracking genetically engineered sequences such as a growth hormone gene construct)?
10. What aspects of eDNA need to be developed further to allow for reliable management response to the early detection of AIS, disease and pathogens, harmful algal blooms, and other changes to important or sensitive habitats?
11. Can eDNA monitoring programs be developed or incorporated into existing monitoring programs at a national scale for MPAs, SAR distribution maps, initatives under the Ocean Protection Plan, etc.?
12. Can eDNA be used in biodiversity monitoring to establish multi-seasonal baseline surveys to develop biodiversity metrics (e.g., C3 Expedition)?

Technological needs

13. Can on-site tools for DNA extraction and sequencing be used for rapid management responses?
14. How can DNA capture during sampling and extraction be improved for specific needs through development of synergies across DFO and other agencies?
15. What non-PCR based methods (e.g., loop-mediated isothermal amplification assays, gene enrichment) can be used in place of traditional PCR methods for eDNA studies?
16. Can dedicated informatics (e.g., reference databases, keys with anatomical vouchers, standardized open bioinformatics pipelines) be developed for DFO?

Communication, collaboration, and coordination

17. How should knowledge built from expertise with eDNA be transferred to other DFO researchers/clients/managers, especially knowledge from freshwater studies to marine environments?
18. What research needs would be best undertaken through collaborative research with community, academic, federal, provincial, territorial, and international partners?
19. What materials and advice for QA/QC should be developed and shared with scientists and managers within and outside Canada?

6.1 Formation of a national DFO eDNA Technical Working Group

At the inaugural DFO national eDNA workshop on the current state of eDNA research in Canada held in Ottawa during March 6th to 8th, 2018, a clear need was identified for DFO scientists to create opportunities to collaborate and share expertise on technical aspects of eDNA. As a result, the formation of a National eDNA Technical Working Group within DFO was recommended and subsequently approved by the DFO Science Executive Committee (SEC).

The purpose of this national working group is to provide a forum for technical discussions on eDNA among DFO scientists and technical staff, working to:

- Share expertise on technical issues and advances related to sampling and analysis of eDNA in order to reduce uncertainty and variability associated with this new technology; and
- Develop best practices, guidelines, standards, and protocols for eDNA sampling and analysis in order to improve consistency and comparability among studies.

6.2 Development of guidelines for DFO eDNA studies

Given that variation in eDNA protocols can affect DNA yield and detection probabilities, our ability to compare results and inferences among different laboratories and studies

can be hindered (Goldberg et al. 2016; Piggott 2016; Hinlo et al. 2017). Therefore, before DFO eDNA research is expanded to assuage any research priority gaps, we

Table 1. Suggested Minimum Information Standards for reporting on DFO eDNA studies (modified from Goldberg et al. 2016).

Stage	Information
Design	• Inferential goal (presence/absence, quantity)
Ecological details on species	• Spawning time or period • Life history stage sought (e.g., veliger, sedentary mussel adult)
Water collection	• Contamination precautions including negative and positive controls • Collection volume, container material, replicates, depth • Site descriptions (flow rate, pH, temperature, salinity, area, etc.)
Sample preservation	• Method, temperature, duration • Filter type (if applicable), filtering location (e.g., in field)
Extraction process	• Contamination precautions (including dedicated laboratory), negative and positive controls • Methods including kit protocol
Probe-based PCR, qPCR, and dPCR	• Design and validation methods • Primer/probe sequences, amplicon length • Positive and negative controls • Inhibition detection and handling • Reaction concentrations, thermal profile • Technical replicates and their interpretation • Standard curve preparation and quality (qPCR only)
High-throughput sequencing	• Library type (shotgun or amplicon) and any enrichment strategy • Library preparation protocol or kit, PCR cycles • Platform, read length, read pairing, expected fragment size • Primers, sequencing adapters, sample index tags, exogenous spike-ins • Amplicon locus, target taxa, specificity, and bias
Bioinformatic analysis	• Read trimming and filtering of artefacts/chimeras • Reference database and/or *de novo* OTU generation • Positive and negative controls and their interpretation, if applicable • Technical replicates and their interpretation • Number of raw reads and final reads • Filtering steps and read audits at each step • Cutoffs for number of reads to retain OTU • Taxonomic assignment method and parameters (e.g., for taxonomic assignment report both close and exact-match results) • Statistical analysis and rarefaction • Open code (e.g., when using something like GitHub, this can make things very reproducible for others. • Data deposition (e.g., Short Read Archive; SRA)

suggest that DFO adopt a set of guidelines for eDNA studies. Exact protocols used in each study necessarily will vary with study sites and research questions, and protocols are expected to change as the technology evolves. As a starting place, we suggest that DFO scientists and managers from across Canada develop:

1. **Minimum Information Standards** for all federal eDNA studies (Table 1; see Goldberg et al. 2016);
2. **Common approaches for sample collection and analyses** to facilitate a common understanding among DFO Regions on interpretation and inference of eDNA data;
3. Sample and data storage capacity in each DFO Region, or possibly **a National data repository and sample archive**; and
4. **eDNA training courses** for regulatory managers, including a defined common language and decision making process.

6.3 Key recommendations for eDNA sampling, analysis, and reporting

The strengths and limitations of eDNA as a surveillance tool were discussed at the 2018 national DFO eDNA workshop, and several critical considerations for optimizing study design have been identified:

- choose validated and appropriate sample collection and analysis methods through use of pilot studies;
- prevent contamination in the field and the laboratory;
- validate genetic assays; and
- develop minimum reporting standards.

Table 2 contains a summary of recommendations that are of critical importance when conducting eDNA studies (from Goldberg et al. 2016). Finally, there is a need for development of consistent approaches and increased communication within DFO in order to continually improve upon the efficacy of eDNA analysis and interpretation of results as the field grows. Further, with the user bases across Canada, DFO is positioned to use this new approach to greatly improve many aspects of species detection and quantitation. Efforts will benefit the community by improving the inter-laboratory collaboration through sharing of methodologies (e.g., Protocols.io), coding methods (e.g., git, GitHub), and results through primary peer-reviewed articles. As genomics technology continues to rapidly improve and costs decline, the list of potential future conservation and research applications of eDNA is continually evolving.

Table 2. Recommendations for conducting eDNA studies (modified from Goldberg et al. 2016)

Recommendations for eDNA sampling, analysis, and reporting
Pilot study
• Implement field sampling protocol and evaluate detection rates with sampling and site data (e.g., filter material and pore size, sample volume, number of samples, spatial distribution of samples)
• Test extraction and analysis protocols
• Validate eDNA assays *in silico*, *in vitro*, and *in situ*
• Determine the variability in seasonal cycles and spatial variability in eDNA samples collected from the field to determine statistical power of the technique
Field
• Collect negative controls and deploy positive controls
• Employ strict decontamination protocols for all equipment and clothing that is reused
• Collect multiple samples at each site to address false negatives and estimate detection probabilities
Laboratory
• Process samples only in a dedicated clean laboratory (completely separated from PCR products) with restricted access, regular decontamination (bleach, UV), one-way workflow, use of filtered tips, and organize work flow of the day with eDNA done first
• Use probe-based qPCR if target is a well-characterized species; for many target or unknown species, use H-T sequencing or microfluidics qPCR (= digital PCR)
• For qPCR, use technical replicates (≥ 3), and internal positive control to test for inhibition
• Archive samples at -80°C
Reporting
• Report quantification values as copy #/volume sampled
• Acknowledge challenges to inferring: across space/time, presence vs. viable population and confounding sources of eDNA
• Maintain archived database with collection date and exact geographic location

7. Conclusions

The eDNA field is a revolutionary cross-disciplinary area of biological science and eDNA technologies are new disruptive technologies still at an early stage of development; therefore continued research and development is required to better interpret the information eDNA can provide. It is clear that eDNA is a promising technological field with the capacity to augment the Department's monitoring and reporting efforts on key fisheries, SAR, AIS, and other aquatic conservation and management initiatives. It is probably only limited by the imagination of the scientists for its application, given sufficient funding, available staff, and time. Ultimately, it will provide a pattern that needs to be interpreted. This implies a need for a body of work on the cause-effect relationship of these signals, however this also applies in equal measure to the current suite of metrics we are using. Due to the inherent variation among eDNA studies in different

locations and environments, we understand that it is neither possible, nor desirable, to strictly standardize protocols and methodologies across projects. However, it is timely to bring DFO scientists and managers together to discuss several important technical considerations for integrating this approach into DFO mandated responsibilities (fisheries management, sustainable aquaculture, aquatic animal health, SAR, AIS, and oceans protection and management). These technical considerations need to be better understood by DFO scientists and managers alike, and there is a need to make DNA-based protocols more transparent and understandable to non-geneticists in order for this technique to become a regular biomonitoring tool. The exchange of information and advice across the Regions should also be part of any strategy to develop the technology in order to avoid duplicated effort and to establish a more cohesive interpretation and inference of the data that is produced.

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Appendix A List of Department of Fisheries and Oceans Canada (DFO) eDNA projects and/or collaborations, and their associated publications to date.

Aquatic Invasive Species
Abbott, Cathryn (Pacific) Development and field testing of metabarcoding-based environmental DNA biosurveillance tool for aquatic invasive species including finfish and dreissenid mussels in Britich Columbia. Funded by AIS through SPERA 2016-2019.
Bajno, Robert (Central & Arctic) Assessing distribution and monitoring dispersal of zebra mussels in Manitoba using environmental DNA techniques. Funded by AIS, SAR, A-Base 2015-ongoing.
Bajno, Robert (Central & Arctic) Detecting colonizing aquatic organisms using environmental DNA (eDNA) techniques. Funded by GRDI 2014-2015. http://www.dfo-mpo.gc.ca/science/rp-pr/grdi-irdg/projects-projets/004-eng.html
Gingera et al. 2016. Detection and identification of lampreys in Great Lakes streams using environmental DNA. *Journal of Great Lakes Research* 42(3):649-659. (Central & Arctic)
Gingera et al. 2017. Environmental DNA as a detection tool for zebra mussels *Dreissena polymorpha* (Pallas, 1771) at the forefront of an invasion event in Lake Winnipeg, Manitoba, Canada. *Management of Biological Invasions* doi: 10.3391/mbi.2017.8.3.03. (Central & Arctic)
Nathan et al. 2015. The use of environmental DNA in invasive species surveillance of the Great Lakes commercial bait trade, *Conservation Genetics* doi: 10.1111/cobi.12381. (Central & Arctic)
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Reist, James and Robert Bajno (Central & Arctic) Environmental DNA as a detection tool for zebra mussels *Dreissena polymorpha* at the forefront of an invasion event in Lake Winnipeg. Funded by DFO A-Base 2014-2017.
Roy et al. 2017. Development of environmental DNA (eDNA) methods for detecting high-risk freshwater fishes in live trade in Canada. *Biological Invasions* doi: 10.1007/s10530-017-1532-z (Gulf Region)
Roy et al. 2015. Protection of Canadian biodiversity and trade through improved ability to monitor invasive freshwater fish. Genome 58:273-273 *–Used COI and RHO genes to compare qPCR and HTS to detect a suite of invasive species in mesocosm (a simulated live shipment).* (Gulf)
(European study) Davison et al. 2017. Application of environmental DNA analysis to inform invasive fish eradication operations. *Science Nature* doi: 10.1007/s00114-017-1453-9
Fisheries Management
(United States study) Baldigo et al. 2017. Efficacy of Environmental DNA to Detect and Quantify Brook Trout Populations in Headwater Streams of the Adirondack Mountains, New York. *Transactions of the American Fisheries Society* doi: 10.1080/00028487.2016.1243578
Bajno, Robert and Karen Dunmall (Central & Arctic ; NWT Cumulative Impacts) Developing environmental DNA as a tool to monitor fish distributions in the NWT. Funded by SAR, A-Base 2016-2019.
Lacoursiere-Roussel et al. 2016. Estimating fish abundance and biomass from eDNA concentrations: variability among capture methods and environmental conditions. *Molecular Ecology Resources* 16(6): 1401-1414. doi: 10.1111/1755-0998.12522.
Lacoursiere-Roussel et al. 2016. Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. *Journal of Applied Ecology* doi: 10.1111/1365-2664.12598

Appendix A (continued) List of Department of Fisheries and Oceans Canada (DFO) eDNA projects and/or collaborations, and their associated publications to date.

Sustainable Aquaculture

Abbott, Cathryn (Pacific) Development and validation of a biomonitoring tool to assess the impacts of salmon aquaculture on marine benthic communities using metabarcoding. Funded by PARR 2016-2018.

Abbott, Cathryn (Pacific) Development of eDNA based biosurveillance for AIS to inform management and policy decision-making associated with shellfish aquaculture movements. Funded by PARR 2016-2018.

Devlin, Robert (Pacific) and Heath, Dan (University of Windsor) Detection of genetically modified fish in farm effluent and surrounding areas through eDNA. Funded by GRDI and A-Base Canadian Regulatory System for Biotechnology.

Robinson, Shawn (Maritimes) The Feasibility of Using Bacterial Community Profiling With Next-Generation DNA Sequencing to Assess Temporal and Spatial Environmental Disturbances. Funded by PARR-2016.

Robinson, Shawn and Lorraine Hamilton (Maritimes) Sampling bacterial populations near salmon farms for antibiotic resistant genes. Funded by CEPA-DAS 2018.

Verhoeven et al. 2016. Bacterial community composition of flocculent matter under a salmonid aquaculture site in Newfoundland, Canada. *Aquaculture Environment Interactions* doi: 10.3354/aei00204 (Newfoundland & Labrador)

Sustainable Aquatic Ecosystems

Gagne, Nellie (Gulf) Metagenomics Based Ecosystem Biomonitoring (EcoBiomics). Funded by GRDI Shared initiative project.

Miller, Kristi (Pacific), Brian Hunt (UBC), Steven Hallam (UBC), and Curtis Suttle (UBC). Trans-Canada eDNA Biodiversity Mapping Project: provide groundwork for pan-Canadian database of coastal marine biodiversity. Funded by Canada C3.

Morris, Todd (Newfoundland & Labrador) Validation of eDNA technology using DFO survey data. Funded by PPF 2011-2014.

Valentini et al. 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology* doi:i: 10.1111/mec.13428.

Waits and Paetkau 2005. Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data. *Journal of Wildlife Management* doi: 10.2193/0022-541X

Species at Risk

Bajno, Robert, Karen Dunmall, and Mochnacz (Central & Arctic) Application of environmental DNA (eDNA) in detecting and monitoring important areas and species in northern ecosystems. Funded by SPERA, SAR, A-Base 2015-2018.

Balasingham 2018. Environmental DNA detection of rare and invasive fish species in two great lakes tributaries. *Molecular Ecology*, 27(1), 112-127. doi: 10.1111/mec.14395 (Central & Arctic)

Balasingham et al. 2017. Residual eDNA detection sensitivity assessed by quantitative real-time PCR in a river ecosystem. *Molecular Ecology Resources*, 17(3): 523-532. doi: 10.1111/1755-0998.12598.

Boothroyd et al. 2016. Environmental DNA (eDNA) detection and habitat occupancy of threatened spotted gar (*Lepisosteus oculatus*). *Aquatic Conservation: Freshwater Ecosystems* doi: 10.1002/aqc.2617 (Central & Arctic)

Cho et al. 2016. Development of species-specific primers with potential for amplifying eDNA from imperilled freshwater unionid mussels. *Genome* doi: 10.1139/gen-2015-0196

Appendix A (continued) List of Department of Fisheries and Oceans Canada (DFO) eDNA projects and/or collaborations, and their associated publications to date.

Species at Risk (continued)
Currier et al. 2018. Validation of environmental DNA (eDNA) as a detection tool for at- risk freshwater pearly mussel species (Bivalvia: Unionidae). *Aquatic Conservation: Marine and Freshwater Ecosystems, 28*(3), 545-558. doi: 10.1002/aqc.2869
Glass and Mandrak 2014. Distribution of Spotted Gar (*Lepisosteus oculatus*) adults and juveniles in the Rondeau Bay, Long Point Bay, and Hamilton Harbour watersheds. *Canadian Management Report of Fisheries and Aquatic Sciences*, 3048: iii + 21 p.
Laramie et al. 2015. Characterizing the distribution of an endangered salmonid using environmental DNA analysis. *Biological Conservation* doi: 10.1016/j.biocon.2014.11.025
Reid et al. 2017. An environmental DNA-based survey for Redside Dace (*Clinostomus elongatus*) in Greater Toronto Area watersheds. *Canadian Manuscript Report of Fisheries and Aquatic Sciences* 3120 (Central & Arctic)
Serrao et al. 2015. Using environmental DNA to detect endangered Redside Dace, *Clinostomus Elongatus*. *Genome*, 58(5):278-278 (Central & Arctic)
Zhan et al. 2013. High sensitivity of 454 pyrosequencing for detection of rare species in aquatic communities. *Methods in Ecology and Evolution* doi: 10.1111/2041-210X.12037 (Pacific)
Aquatic Animal Health
Polinski et al. 2017. Seawater detection and biological assessments regarding transmission of the oyster parasite *Mikrocytos mackini* using qPCR. *Diseases of Aquatic Organisms* doi: 10.3354/dao03167 (Pacific)
Other
Li et al. 2017. Comparison of DNA-, PMA-, and RNA-based 16S rRNA Illumina sequencing for detection of live bacteria in water. *Scientific Reports* doi: 10.1038/s41598-017-02516-3
Morris, Todd (Newfoundland & Labraor) Validation of eDNA technology using DFO survey data. Funded by PPF 2011-2014.
Veldhoen et al. 2016. Implementation of Novel Design Features for qPCR-Based eDNA Assessment. *PLoS one* doi: 10.1371/journal.pone.0164907.

Note: The participating DFO Regions are noted in parentheses, as well as project funding sources.

ANNEX A – Technical Considerations for eDNA Studies

Supporting information. Baillie, S.M., McGowan, C., May-McNally, S., Leggatt, R., Sutherland, B.J.G, and Robinson, S. 2019. Environmental DNA and its applications to Fisheries and Oceans Canada: National needs and priorities. Can. Tech. Rep. Fish. Aquat. Sci. 3329: xiv + 84 p.

ANNEX A – Technical Considerations for eDNA Studies

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Introduction

This Annex to the discussion paper on eDNA applications to DFO provides a detailed review of some of the technical considerations associated with environmental DNA (eDNA) field sampling, laboratory set-up, DNA enrichment and sequencing, bioinformatics, and DNA databases. The term eDNA, as used in this Annex, refers to the molecules of DNA (deoxyribonucleic acid) that are found in the environment; the DNA found in living microorganisms, as well as the free DNA or DNA contained in whole cells that has been shed by plants and animals (Maruyama et al. 2014). It also refers to a series of innovative technologies and techniques that have emerged from the field of genomics, and are beginning to transform the manner in which scientists measure biodiversity (Bohmann et al. 2014; Stoeckle et al. 2017). The technology allows scientists to collect and analyze a sample of eDNA from the environment, to identify the species of organisms present, and obtain an estimate of the biodiversity from the equences themselves. It has been tested successfully in terrestrial and aquatic sediments, ice and soil, and both freshwater and marine environments (see Thomsen and Willerslev 2015). When it comes to the management of aquatic resources, or the monitoring of aquatic ecosystems, eDNA can offer a comprehensive dataset of biodiversity from an environment that can be costly to explore. Whereas traditional methods used to study open water diversity (fishing and trapping) requires funding, skilled labour, and other various resources, data collected with eDNA will only require samples of water, taken from the location, or environment of interest (Sassoubre et al. 2016; Shaw et al. 2016; Tsuji et al. 2017). With proper validation and quality assurance control, the technology could contribute significantly to fisheries management, without incurring a significant cost.

Field sampling considerations

Cost effectiveness

Sampling simply involves taking water samples, and therefore eDNA can be a relatively quick and cost-effective approach to monitor aquatic species (Rees et al. 2014). In comparison of person-effort needed to detect one individual of an invasive fish species, it took 93 days by electrofishing compared to 0.174 day using eDNA (Jerde et al. 2011). Similarly, Sigsgaard et al. (2015) reported that the successful detection of a target fish species required ca. 300 hours of fishing compared to ca. 60 hours of effort with eDNA sampling. When costs were compared, fishing cost was estimated at 8100 USD whereas only 4250 USD was used to collect samples and conduct laboratory work for eDNA analysis. Stoeckle et al. (2017) reported that workflow of sample collection could be slow when many samples are collected and analyzed on a machine that requires accumulating multiple samples before running (e.g., Illumina MiSeq). Outside of the sampling time, they noted that the entire eDNA process could be accomplished at a reasonable pace and with present technology in one week, and in 24 hours if necessary. However, it is important to note that the cost-effectiveness of eDNA varies considerably with sampling effort and start-up costs. The cost per sample is suggested to decrease linearly as the number of sampling sites increased (Smart et al. 2016). Therefore, eDNA technologies can be

most beneficial in complement with conventional surveys, and also when conventional surveys are logistically difficult to complete, costly and/or large-scale, or may pose some harm to the field biologist, a sensitive species, or an ecosystem. A final additional important consideration is that typically fishing assessments target a specific species or group of species, whereas a collection of eDNA can be re-interrogated at different taxonomic levels as long as extraction methods are consistent among taxa; in this way, eDNA can be truly comprehensive at an ecosystem-level biodiversity evaluation.

Sample Collection

In general, sampling for eDNA involves collecting 2-3 samples of water from each site and concentrating the eDNA sample using laboratory methods before DNA extraction (e.g., see Ficetola et al. 2008; Laramie et al. 2015). Contamination is the greatest experimental challenge to eDNA studies and lengthy decontamination protocols are required for the preparation and sterilization of equipment before heading out into the field. Water samples are ideally filtered on-site and the filters are kept cold, frozen, or preserved with chemical additives until DNA extractions are conducted in the laboratory (methods may vary by protocol). Alternatively, chemical additives can be applied directly to water samples to preserve eDNA prior to transport. Once the eDNA in water samples are concentrated and extracted in the laboratory, a polymerase chain reaction (PCR), or quantitative PCR (qPCR, Real-Time PCR). Digital droplet PCR (ddPCR) with mitochondrial gene primers (or probes for other sequences) can be run with the DNA extract and appropriate controls to identify samples that are positive or negative for the presence of the target species' DNA.

There is considerable variation in the sampling methods available for collecting water samples for eDNA analysis. Common methods include filtration-based methods and alcohol and/or chemical preservation methods. Filtration-based methods require the use of in-line or vacuum-line pumps to pass water samples through one or more filters (e.g., cellulose nitrate, carbonate). The filters are then folded, placed into sterile tubes and preserved using ethanol, chemical additives, and/or in cold storage on ice or in a portable refrigerator. All-in-one filters can also be used, whereby the filter is contained within a single use plastic container, which is then dried by syringe expulsion, ends are sealed with Parafilm, and frozen. Alternatively, alcohols and/or other additives (e.g., sodium acetate) can be added directly to water samples to preserve eDNA at ambient or cold temperatures. Water samples may also be stored on ice without chemical additives and brought back to the laboratory to be centrifuged if they can be transported rapidly, but it is recommended that the water samples be kept refrigerated (Goldberg et al. 2016). Following centrifugation, the pellet of DNA can be preserved in ethanol and stored at -20-80°C for long-term storage. Of note, methods for profiling viruses will differ from the methods described here and are beyond the scope of this document.

Sampling equipment and personnel should always remain slightly downstream of the intended sampling location to prevent the potential contamination of field samples, which can compromise entire eDNA projects (Carim et al. 2015). Sampling in areas with significant backflow (e.g., eddies, splash pools, whirlpools) where DNA on clothing may enter water and flow upstream should also be avoided to prevent cross-contamination of field samples.

Sampling volume and replicates

Currently, there are no standards regarding the ideal volume of sample to capture complete community diversity (Shaw et al. 2017). The ideal volume to sample likely varies with sample and ecosystem type, expected abundance and diversity of organisms, total biomass, properties of the sample media, as well as targeted organisms (see Shaw et al. 2017). Flaviani et al. (2017) found there was little difference in microbiome richness in marine water samples from 10 to 1000 mL, and low variability in structure was observed when using greater than 50 mL. Studies have reported great biodiversity measures in biological replicates than in simply increasing volume of a single sample (e.g., Andruszkiewicz et al. 2017), and replicates of two, three, or more per site could be sufficient for accurate biodiversity measures.

Field Negative Controls

Negative controls should always be incorporated into any field protocol to detect potential contamination. Negative field controls include sterile freshwater or seawater that is preserved and processed in the same manner as the field samples to ensure that cross-contamination is not occurring between replicate sites. If possible, field negative controls, where the species is known not to be present, may help to validate non-detection. If any negative field control tests positive for contamination, the positive detection can act as a 'low expression threshold' whereby the number of reads must exceed the negative control all samples. Depending on the study and situation, the researcher may choose to consider the positive sample compromised and discard it.

Sampling Supplies

Given proper handling, disposable sampling supplies can greatly limit contamination of negative field controls and water samples. Preferably, single-use gloves and sterile collection containers or grab bottles should be used; however, if any bottles are to be re-used, thorough decontamination is necessary to maintain sample independence. Studies have shown that autoclaving and lower concentrations of bleach or quaternary ammonia may not sufficiently clean DNA from surfaces. At minimum, it is recommended that a 5-50% commercial bleach solution be used to decontaminate extraneous eDNA from equipment or supplies used more than once (Goldberg et al. 2016; Wilcox et al. 2016). The review by Goldberg et al. (2016) and the USGS sampling protocol for eDNA (Laramie et al. 2015) have suggested disinfecting equipment and surfaces that cannot be UV sterilized with ≥50% bleach solution, as it is the lowest concentration of bleach that can effectively remove extraneous eDNA and PCR products. The ≥50% bleach solution can be applied to contaminated equipment or surfaces for at least one minute then rinsed off thoroughly with 70% ethanol or distilled water stored in a sterile container.

Collection and Preservation

The reliability of eDNA analysis is highly dependent on sample preservation to maintain the initial state of eDNA. It is suggested that eDNA degradation occurs immediately and rapidly

following shedding, and that this rate can vary depending on the species and environmental conditions (Thomsen et al. 2012a; Thomsen et al. 2012b; Pilliod et al. 2014). Given the limited chemical stability of DNA in water (Lindahl 1993), the decay rate of eDNA in water bodies can be on the scale of hours or weeks (see Table 1 for recorded eDNA detection limits and/or degradation rates of aquatic taxa, but note that different laboratory and natural conditions can greatly influence the decay rate of eDNA).

Table 1. eDNA persistence in water in laboratory conditions and natural environments.

SPECIES	ENVIRONMENT	DETECTABILITY ESTIMATE (>5%)	SOURCE
Atlantic salmon (*Salmo salar*)	Lotic	0.5 days (11.5 hours)	Balasingham et al. 2016
Threespined stickleback (*Gasterosteus aculeatus*)	Marine	0.9 days	Thomsen et al. 2012a
Bluegill sunfish (*Lepomis macrochirus*)	Laboratory	1 day	Maruyama et al. 2014
Common carp (*Cyprinus carpio*)	Laboratory	1.9-6.6 days	Eichmiller et al. 2016
Common carp (*Cyprinus carpio*)	Laboratory	2-4.2 days	Barnes et al. 2014
Japanese sea nettle (*Chrysaora pacifica*)	Laboratory	4 days	Minamoto et al. 2017
Common carp (*Cyprinus carpio*)	Laboratory	4.2 days	Barnes et al. 2014
European flounder (*Platichthys flesus*)	Marine	6.7 days	Thomsen et al. 2012a
Mud crab (*Rhithropanopeus harrisii*)	Laboratory	7 days	Forsstrom and Vasemagi 2016
Sturgeon (*Acipenser baerii*)	Laboratory	17 days	Dejean et al. 2011
New Zealand mudsnails (*Potamopyrgus antipodarum*)	Laboratory	21-44 days	Goldberg et al. 2013

For studies aiming to detect or quantify rare sequences in water samples [i.e., species at risk (SAR) and early detection of aquatic invasive species (AIS)], it is crucial to consider the best preservation option available to limit false negatives arising from rapid DNA degradation. A variety of factors may influence the choice of less than ideal preservation method, including equipment requirements, resource limitations, limited personnel, and time constraints.

Common sample preservation methods involve storing water samples in a cooler, on ice, or in a freezer to reduce the degradation rate of eDNA until samples are filtered or fixed in ethanol (Takahara et al. 2012; Eichmiller et al. 2014). Alternatively, on-site filtration or addition of a preservative (e.g., 200-proof molecular grade ethanol, Longmire's lysis buffer or chemical additives) followed by the transportation of samples to a laboratory under cooling or freezing temperature can be used to maintain the initial state of eDNA (Renshaw et al. 2015; Yamanaka et al. 2016; Williams et al. 2017). Several studies suggest that on-site filtration provides the best estimate of the actual eDNA concentration in water bodies and improves the probability of detecting rare sequences (Yamanaka et al. 2016, 2017; Doi et al. 2017a; Williams et al. 2017). This is followed by on-site addition of ethanol, Longmire's lysis buffer or chemical additives, then cold storage alone.

Water temperature has a strong influence on the decay rate of eDNA. The majority of current sampling protocols recommend that water samples or filters be cooled to at least 4-5°C to slow the degradation rate of eDNA (Goldberg et al. 2016). A study examining the eDNA decay rates of Salamander (*Dicamptodon aterrimus*) and Common Carp (*Cyprinus carpio*) reported that the eDNA exhibits an exponential decay rate in lake water, with slower degradation rates at lower temperatures (Pilliod et al. 2014; Strickler et al. 2015; Eichmiller et al. 2016). A study on eDNA decay rate found that decay was slowest at 4-5°C and that at least 90% of the eDNA in samples stored at 4-5°C persisted past six days, compared to approximately 1 day for samples stored at temperatures of 15°C or higher (Eichmiller et al. 2016). While cooling can slow the decay rate of eDNA, it is important to consider that most eDNA degrades during the first 24-48 hours of sampling and will continue to decline at 4-5°C.

The need for on-site filtration equipment, coolers, or portable refrigerators, followed in some cases by rapid transportation of water or filter samples to research stations or laboratories, can significantly impact the scope and length of field sampling. Therefore, several studies have focused on testing different chemical additives that can be added to water samples to preserve DNA at ambient temperatures prior to DNA extraction (see Table 2 for examples of common additives (Yamanaka et al. 2016; Williams et al. 2017).

Ethanol is the most commonly used additive to eDNA prior to DNA extraction since it is widely available, inexpensive and can be used directly to facilitate the precipitation of DNA. In general, ethanol precipitation (often coupled with ammonium acetate or sodium acetate) and filtration-based methods are the most frequently used methods for collecting eDNA from water bodies. Ethanol precipitation was first used to collect eDNA from aquatic environments (see Ficetola et al. 2008) but recently there has been a shift to filtration based methods, primarily glass fiber filters (GF), filter capsules (e.g., Sterivex-GP polyethersulfone) or membrane filters, specifically cellulose nitrate (Minamoto et al. 2012; Yamamoto et al. 2016; Hinlo et al. 2017; Minamoto et al. 2017; Spens et al. 2017).

Table 2. Chemical additives used for eDNA preservation at ambient temperatures.

CHEMICAL AGENT	CATEGORIZATION	SPECIES	RESULTS	SOURCE

Ethanol	Alcohol	Rocky Mountain tailed frog (*Ascaphus montanus*); Idaho giant salamanders (*Dicamptodon aterrimus*); Common Carp (*Cyprinus carpio* L.)	Effective at preserving eDNA, but amount of recoverable DNA is lower than with isopropanol for the same reaction volume.	Goldberg et al. 2011; Pilliod et al. 2013a; Doi et al. 2017b
Isopropanol	Alcohol	Common Carp (*Cyprinus carpio* L.)	Isopropanol precipitation recovered 2x more eDNA than ethanol precipitation when reaction volumes were equal.	Doi et al. 2017b
Sodium acetate (3M)	Deliquescent Salt	American bullfrog (*Lithobates catesbeianus*); Siberian sturgeon (*Acipenser baerii*)	When combined with absolute ethanol, effective to preserve eDNA for >17 days with a detectability greater than 5%.	Dejean et al. 2011; Thomsen et al. 2012b
Benzalkonium chloride (BAC)	Quaternary ammonium - Cationic surfactant	Bluegill sunfish (*Lepomis macrochirus*)	92% of eDNA retained in 8-hr incubation test, compared to only 14% in water samples without BAC.	Yamanaka et al. 2016
Benzethonium chloride (BEC)	Quaternary ammonium - Cationic surfactant	Bluegill sunfish (*Lepomis macrochirus*)	Decelerated the loss of eDNA, but not as effective as BAC.	Yamanaka et al. 2016
Didecyldimethyl-ammonium chloride (DDAC)	Quaternary ammonium - Cationic surfactant	Bluegill sunfish (*Lepomis macrochirus*)	Decelerated the loss of eDNA, but not as effective as BAC.	Yamanaka et al. 2016
Longmire's solution[1]	Lysis buffer	Bluegill (*Lepomis macrochirus*) ; Wild pigs (*Sus scrofa*)	Preserved eDNA when samples left at room temperature for two weeks or frozen the samples at −80 °C out to 56 days.	Williams et al. 2017
Cetyl trimethyl-ammonium bromide (CTAB)[2]	Lysis buffer	Bluegill (*Lepomis macrochirus*)	Preserved eDNA at ambient temperatures for up to two weeks.	Renshaw et al. 2015

[1] 100 mM Tris, 100 Mm EDTA, 10 mM NaCl, 0.5 % SDS, 0.2 % sodium azide. **[2]** 1.4 M NaCl, 2% (w/v) cetyltrimethyl ammonium bromide, 100 mM Tris, 20 mM EDTA and 0.25 mM polyvinylpyrrolidone.

Transportation of samples

When water samples are not immediately preserved in the field, transportation can become a crucial factor that affects the quality of the eDNA analysis, specifically the ability to accurately detect or quantify rare species and/or compare eDNA data among sampling sites or species. Most protocols recommend that refrigerated water samples be preserved within 24 hours of collection (Pilliod et al. 2013a; Hinlo et al. 2017). Given the current understanding of the rapid degradation rate of eDNA (see Table 1 for more detail), field biologists should aim for water sample preservation within 12-16 hours. This is similar to the USGS eDNA sampling protocol, which recommended 16 hours.

The potential effects of abiotic and biotic factors (e.g., temperature, water quality, substrate type) should always be considered when designing and planning field surveys, especially when non-preserved samples require transportation. This consideration becomes even more important when sampling in remote areas and when multiple pieces of sampling equipment are required.

For remote sampling, it is recommended that samples be filtered on-site or preserved immediately using precipitation-methods or chemical additives to reduce eDNA degradation. If sampling sites can be accessed by road, some studies have designed protocols for filtering water samples in vehicles during transit or between sites (see Yamanaka et al. 2016). Once samples are collected and processed, they should preferably be refrigerated. Some studies have found that refrigeration results in higher yields than freezing for short-term storage (3-5 days, Takahara et al. 2015; Hinlo et al. 2017).

Laboratory set-up considerations

Quality assurance and quality control (QA/QC)

Attention to quality assurance and quality control (QA/QC) is an important consideration of eDNA methods. The eDNA method faces many of the same contamination issues as other genetic methods that rely on low-quantity and low-quality DNA. Several major reviews focus on strategies for lowering error rates (i.e., false positives, false negatives, Thomsen et al. 2012b; Rees et al. 2014; Goldberg et al. 2016). Error rates are especially important when the goal of the eDNA study involves the detection or quantification of rare aquatic species, such as species at risk or early detection of AIS. It is suggested that contamination is more likely to occur when the DNA is in a low concentration, such as during water sample collection, DNA extraction, and PCR set-up (Cooper and Poinar 2000).

Several reviews suggest that false positives (Type I Error, positive eDNA detection where target species is not present) are the most prevalent source of error with current eDNA methods (Bohmann et al. 2014; Rees et al. 2014; Thomsen and Willerslev 2015; Goldberg et al. 2016). False positives may arise from contamination in the field (e.g., sewage or wastewater, dead animals, ballast water discharge, faeces from birds and predators, unsterilized equipment) or in

the laboratory (e.g., low specificity of primers and probes to non-target eDNA, Rees et al. 2014). There is also a large disparity in the way samples are scored as positive with genetic tools that can lead to reproducibility issues. False positives can also be introduced by poor database annotation, where a species is improperly annotated with the incorrect species information. Solutions to reduce false positives include adhering to QA/QC standards to reduce contamination, repeated temporal sampling in the same areas (i.e., to limit influence of external sources of eDNA and/or dead and decaying animals), and *in silico* testing of DNA probes and primers to ensure specificity to the target species (Bohmann et al. 2014), as well as amplicons to ensure expected results from reference databases.

False negatives (Type II Error, negative eDNA detection when target species is present) may occur when the concentration of eDNA is too low or too unequally distributed to be detected. This is a common issue for species at risk found in low abundances or for the early detection of AIS. False negatives can also occur in cases when DNA amplification is inhibited, such as by humic substances released by decomposing foliage in autumn (Jane et al. 2015; Sigsgaard et al. 2015). Solutions to false negative in this case include sampling water in triplicates, filtering larger volumes of water, and rigorous testing of DNA probes and primers to ensure high specificity and successful amplification. Without positive controls or consideration of primers and databases, false negatives could also occur due to a species being absent from the reference database and therefore unable to be properly scored. Depending on the species and efficiency of eDNA detection, the rate of false negatives may be low, even for species present in low densities (Laramie et al. 2015). In these situations, future sampling could collect fewer water samples to reduce costs.

Laboratory personnel

Given the high sensitivity of eDNA methods and potential for contamination, rigorous field sampling and laboratory analysis strategies are essential for ensuring sample independence. As a reference, ancient DNA protocols and laboratory set-ups are a good model to follow regarding the necessary controls and standards for processing high-quantity, low-density DNA samples, while minimizing the risk of false positives.

Additionally, it is important that every new employee who begins eDNA work is trained and receives the appropriate guidance on both the theory and applied aspects of the Regional or National laboratory organization and QA/QC procedures. At minimum, each new employee should be able to demonstrate that they can capably perform the required laboratory work through analyses of positive and negative control samples before attempting any eDNA analyses without supervision.

Laboratory Infrastructure

From ancient DNA work, best practices include physically separate and appropriately designed work areas for pre-PCR and post-PCR stages to reduce carry-over contamination, which can lead to false-positive results (Cooper and Poinar 2000; Pedersen et al. 2015). The movement of equipment and personnel should be unidirectional (i.e., follow the DNA concentration gradient)

to reduce contamination from high-copy post-PCR products (Shaw et al. 2017). Shaw et al. (2017) suggest the use of overnight ultraviolet (UV) irradiation on surfaces (or at minimum 15 minutes), especially in post-PCR areas. However, UV radiation will not completely decontaminate and the use of bleach of the same surfaces is also recommended. Decontamination of equipment should also be completed before and after use. Biannually, the working surfaces of the laminar-flow hoods and the PCR workstations should be thoroughly decontaminated.

For DNA extractions, a negative control or extraction blank (where the sample is omitted or replaced with nuclease-free water) should be extracted at the same time to help identify laboratory contamination. Barrier pipette tips, polypropylene centrifuge tubes should be used for extractions and should be lot-certified, DNase-free, RNase-free, and pyrogen-free. Additionally, separate centrifuges should be used for sample preparation, pre- and post-PCR procedures. At high centrifugation speeds, tubes may leak in the centrifuge, leading to increased probability of contamination and loss of sample. Testing and selecting leak-proof centrifuge tubes is highly recommended (e.g., see Doi et al. 2017b).

At the PCR stage, DNA samples are often analyzed in triplicate to ensure detection of short and degraded DNA fragments. Additionally, disposable DNase-free, RNase-free, and pyrogen-free laboratory supplies should be used as applicable. To ensure great specificity and reproducibility, thin-walled tubes should be used to provide the best heat transfer during PCR and help ensure that the reaction volume reaches its specified temperature as rapidly as possible.

Filtration and centrifugation

Filtration and centrifugation are commonly used to concentrate water samples that may be collected in volumes too large for most extraction protocols. If water samples can be rapidly transported to a research station or facility, centrifugation can be used to concentrate water samples into a DNA pellet which can then be washed and cleaned. However, filtration seems to be the preferred method because of its transportability to the field, although notably some protocols use both filtration and centrifugation methods together (Shaw et al. 2017). Centrifugation is suitable for water samples with large amounts of particulate matter. While centrifugation speeds can be adjusted depending on the target organism, it sometimes may not be possible to reach the speeds necessary to pellet some eDNA.

DNA preparation

DNA extraction protocols can vary depending on species or degree of organic matter present. Most studies use manufactured DNA extraction kits to streamline and standardize the DNA extraction process while limiting cross-contamination of samples (Davison et al. 2016; Goldberg et al. 2016). Extraction kits, such as the silica-based QIAGEN DNeasy Blood and Tissue Kit, including the DNeasy PowerWater Kit designed specifically for extraction of DNA from filtered water, or even the DNeasy PowerWater Sterivex Kit, designed for extraction from Sterivex filters. These methods provide a rapid and safe method to extract DNA from water samples and

can help avoid processing bottlenecks (Goldberg et al. 2011). The kits generally yield high-quality DNA even from difficult environmental samples.

Alternatively, liquid-liquid or “in-house” DNA extraction methods (precipitation and phase-separation methods; phenol-chloroform and CTAB-chloroform) are often less expensive and can improve DNA yield compared to silica-based extraction kits. However, risk of contamination is increased with liquid-liquid based methods given the reuse of solutions and it is recommended that solutions be routinely tested for contamination. Several studies have also reported that DNA quality is generally lower with liquid-liquid based methods compared to silica-based kits (see Shaw et al. 2017 review). Few studies have examined the effect of PCR inhibition, such as by humic substances, on DNA amplification with different extraction methods; however, several strategies have been proposed to identify and overcome this problem (Pedersen et al. 2015).

Physical sample management and data management should be considered prior to the start of an eDNA survey and maintained appropriately. Following DNA extraction, samples should be archived at -20-80°C. It is also crucial to maintain an archived database that includes the exact collection date and conditions, abiotic/biotic variables measured, and precise GIS coordinates of sampling sites.

DNA amplification and sequencing considerations

As Shaw et al. (2017) outline in their detailed review of methodologies associated with eDNA, eDNA-based surveys typically comprise five main steps: sample collection, DNA extraction, target DNA amplification, sequencing, and bioinformatics analysis. This section contains a review of the literature on the most common DNA amplification and sequencing methodologies used in eDNA studies today, as well as explanations on developing genomics technologies that are being tested for use in eDNA studies.

PCR methods for target eDNA amplification and detection

Before any eDNA analysis is performed, primers and/or probe design should be tested to ensure positive detection of the target species’ DNA. Although both nuclear and mitochondrial gene sequences have been used for primers, the majority of eDNA studies have focused on mitochondrial primers due to the substantially greater copy number of mtDNA than nuclear DNA per cell. The mitochondrial cytochrome *b* gene [range of 90-120 base pair (bp)] has been the popular choice for primers.

Since eDNA rapidly degrades in water, a small fragment size gene is important to use over a larger fragment as it will be more likely to persist long enough to allow for species detection (Rees et al. 2014). This is especially critical for water samples originating from natural water bodies where eDNA is often present at low concentrations and is degraded (Rees et al. 2014). For qPCR probe chemistry, short and species-specific sequences (ideally 50-150 bp) should be

selected for a primer and probe set, improving the amplification and detection of the target species' DNA (Pilliod et al. 2013b).

Both PCR and qPCR methods have been used to reliably detect the presence of non-native fishes in water samples (Janosik and Johnston 2015). Conventional PCR methods can be a straightforward, rapid, and cost-effective approach (Nathan et al. 2014, 2015). While studies have successfully used PCR to detect the presence or absence of aquatic animals (Deiner and Altermatt 2014; Davison et al. 2016), PCR is only semi-quantitative at best (if the intensity of amplified bands on a gel are compared with DNA ladder standards), and has low sensitivity and poor precision compared to qPCR (Goldberg et al. 2016).

Given several issues with conventional PCR methods for eDNA studies, probe-base quantitative qPCR is currently the preferred method for single-species detection and/or biomass quantification. The qPCR method has increased sensitivity compared to traditional PCR methods and includes inherent measures of quality control and assay validation, which is beneficial for the detection of species at risk or biomonitoring of AIS. The practicality of qPCR comes from the use of known-concentration standard curves to quantify the starting eDNA concentration. Studies have used qPCR to successfully detect the eDNA of species found at low natural densities, including red swamp crayfish (Treguier et al. 2014) and great crested newt (Biggs et al. 2015). Alternatively, in a mesocosm experiment, a study found that there was no difference between PCR and qPCR for detecting species presence (Nathan et al. 2014). Given the disparity in results, future studies should continue to compare the effectiveness of PCR and qPCR in field surveys and under different environmental conditions when feasible. However, the potential for automated analysis of qPCR combined with a slight increase in cost and an ability to retrieve quantitative results suggests qPCR is optimal for these purposes. Furthermore, qPCR is superior to PCR in determining whether multiple amplicons are being amplified simultaneously based on melt curve analysis.

PCR inhibition can commonly occur with eDNA samples, and the use of chemical additives may exacerbate the inhibition process further. PCR inhibition can result in failed or delayed amplification of target DNA and may lead to an incorrect assumption that a target species has not been detected during the eDNA survey (Goldberg et al. 2016). Goldberg et al. (2016) provide a good overview of the PCR inhibition process and several options for alleviating inhibition.

PCR-based metabarcoding

Metabarcoding is targeted detection of DNA, where primer pairs target a specific location of the genome (typically mitogenome for eDNA) and this amplicon can be used to distinguish between multiple species to test for biodiversity measures in an environment. It can be used for eDNA detection, but is also used to detect DNA from whole organisms in a sample (e.g., bacterial and microorganism diversity studies in sediments, zooplankton diversity studies in marine samples). Shaw et al. (2017) provide a good overview of metabarcoding for aquatic biodiversity studies, including basic concepts and factors to consider in design, implementation, and analysis. Metabarcoding enables multiple species detection of individual species across broad taxonomic

groups (e.g., vertebrates, fungi, bacteria) by targeting less-specific markers. The targeted area (barcode) is usually a short sequence containing two evolutionary conserved areas as primer targets that flank a highly variable species-specific region. Thus, unique sequences of multiple species are targeted and amplified using a single or a few primer pairs, and amplified sequences are then sequenced via Next Generation Sequencing (e.g., Illumina and Ion Torrent platforms) and compared against reference databases using bioinformatics tools.

The primary use of eDNA metabarcoding is to examine biodiversity of a group of taxa in an environment or sample, and has been demonstrated to efficiently estimate biodiversity of fish communities in many different marine, estuary, and freshwater (lakes, rivers) ecosystems (e.g., see Thomsen and Willerslev 2015; Civade et al. 2016; Shaw et al. 2016; Thomsen et al. 2016; Valentini et al. 2016; Andruszkiewicz et al. 2017; Karahan et al. 2017; Maggia et al. 2017; Stoeckle et al. 2017; Yamamoto et al. 2017). Other demonstrated uses include characterizing zooplankton and/or larval fish species to identify non-indigenous species or aquatic invasive species in ecosystems or water ballasts (Brown et al. 2016; Hatzenbuhler et al. 2017), determining the pelagic prokaryotic and eukaryotic diversity in a marine water sample (Flaviani et al. 2017), and benthic monitoring of sediment organisms to determine sediment organic enrichment associated with fish farms (Pawlowski et al. 2016).

Metabarcoding is a technically demanding process, and more time consuming and complicated than single species eDNA detection (see Darling and Frederick 2017). Metabarcoding can theoretically or in some cases provide comparable or improved community biodiversity information relative to traditional surveys and with remarkably less effort and invasiveness. Potential advantages of metabarcoding over traditional surveying for biodiversity include those associated with single species eDNA detection such as less field effort (e.g., 6 h worth of sampling for a eDNA metabarcoding survey provided greater biodiversity results than 14 years of visual surveys in a coastal area; Yamamoto et al. 2017), less destructive field sampling, ability to detect all life stages including those not detected in some field surveys (e.g., larvae in some traditional sampling techniques), lack of reliance on taxonomic expertise and labour-intensive morphologically-based taxonomy, and improved ability to detect rare and cryptic species. Automated analysis once bioinformatics pipelines have been developed can also be applied to various different taxonomic groups, which in the traditional survey method would require a completely different skill set in some cases, for example for taxonomy identification, or for catch method. However, there are disadvantages with using metabarcoding for biodiversity studies such as strict quality control and protocol development, and complex analysis creating challenges in characterizing both false positives and false negatives. The complex data obtainable through metabarcoding provides strong power to biodiversity studies, but also adds to the challenges with the technique (Pawlowski et al. 2018). Optimally the traditional methods can be paired with metabarcoding to some extent to ground truth results, especially during the development of new assays. For example, eDNA can be rapidly used to assess many sites inexpensively, then the sites that are positive for a species can be specifically surveyed to validate the observations at a smaller, more targeted effort. This more extensive reach can allow for a much broader assessment than originally possible (e.g., Lacoursière-Roussel et al. 2016a,b)

Barcode choice and primer design for PCR Methods

Choice of targeted barcode(s) depends on numerous factors including whether targeting free eDNA or DNA from whole cells or organisms, type and range of targeted taxa, etc. Targeted genes for barcoding when examining free eDNA or detecting rare species are often from the mictochondria, ribosome, or chloroplasts (plants) due to high resolution at the species level, but also due to the high copy number per cell making them more likely to be detected than single-copy nuclear DNA (see Thomsen and Willerslev 2015). Ideally, primers should be specific to conserved regions in all targeted species, and the variable region among targeted or expected species should be different enough (i.e., greater than 1 bp difference in sequence) to distinguish among organisms to the desired taxa level – usually to the species level. However, a single primer pair often does not distinguish all desired taxa. Multiple primer pairs targeting a single barcode can be used if the conserved region differs among targeted taxa, and multiple primer pairs targeting different barcode sites may be used if the variable region is too similar in some taxa to distinguish to the desired level of taxa.

For most metabarcoding applications, there are no specific set of primer pairs that will work for all circumstances. Careful primer design and choices are critical and use of multiple genetic markers can increase probability of species detection or differentiation and act as cross-verification of taxa detection per sample (see Shaw et al. 2016; Valentini et al. 2016; Hatzenbuhler et al. 2017). Current barcodes used or in development include 12S mitochondrial vertebrate primers (see Table 3), which generally have good level of reference sequences, but may not be able to distinguish closely related fish species. Presence of other non-target vertebrates may saturate the amplification process thereby preventing detection of rare fish species (Shaw et al. 2016; Yamamoto et al. 2017). Miya et al. (2015) recently developed fish-specific primers for a hypervariable region of 12S rRNA that were able to detect and distinguish 168/180 species in an Okinawan aquarium, from rays to higher teleosts, and have been successfully used in coastal surveys (Andruszkiewicz et al. 2017; Yamamoto et al. 2017).

Table 3: Some primers used in aquatic eDNA metabarcoding studies

Name	Primer sequence (5' – 3')	Product size	Taxa	Reference(s)
MiFish-U-F (12S) **MiFish-U-R**	GTCGGTAAAACTCGTGCCAGC CATAGTGGGGTATCTAATCCCA GTTTG	163-185	Fish	Miya et al. 2015
MiFish-E-F (12S) **MiFish-E-R (12S)**	GTTGGTAAATCTCGTGCCAGC CATAGTGGGGTATCTAATCCTA GTTTG	163-185	Fish	Miya et al. 2015

16S fish- F **16S fish- R**	GGTCGCCCCAACCRAAG CGAGAAGACCCTWTGGAGCTTI AG	~ 100 bp	Fish	Shaw et al. 2016
teleo_F (12S) **teleo_R**	ACACCGCCCGTCACTCT CTTCCGGTACACTTACCATG	<100	Fish	Valentini et al. 2016
Ac16s	CCTTTTGCATCATGATTTAGC CAGGTGGCTGCTTTTAGGC	330	Fish, Amphibians	Evans et al. 2016, 2017
Fish F1 (COI) **Fish F2**	TCAACCAACCACAAAGACATTG GCAC TAGACTTCTGGGTGGCCAAAGA ATCA	652	Fish	Hubert et al. 2008
12S F (V5F) **12S R (V5F)**	TTAGATACCCCACTATGC TAGAACAGGCTCCTCTAG	73-110 bp	Vertebrates	Riaz et al. 2011; Shaw et al. 2016
16S-HF **16S-HR1** **16S-HR2**	ATAACACGAGAAGACCCT CCCACGGTCGCCCCAAC CCCGCGGTCGCCCCAAC	80-125 bp	Vertebrates	Horreo et al. 2013
L2513 (16S) **H2714**	GCCTGTTTACCAAAAACATCAC CTCCATAGGGTCTTCTCGTCTT	202	Vertebrates	Kitano et al. 2007; Evans et al. 2016, 2017
Uni18S **Uni18SR**	AGGGCAAKYCTGGTGCCAGC GRCGGTATCTRATCGYCTT	400-600	plankton	Zhan et al. 2013
s14F1 (18S) **s15**	AAGGGCACCACAAGAACGC CCACCTATCACAYAATCATG		Foraminifera	Pawlowski et al. 2016
1391F (18S) **EukB**	GTACACACCGCCCGTC TGATCCTTCTGCAGGTTCACCT AC		eukaryote	Stoeck et al. 2010

Development of fish-specific primers such as these may alleviate lack of specificity issue associated with general vertebrate markers. Mitochondrial cytochrome oxidase subunit I (COI) is also commonly used as a genetic barcode in diversity studies, but does not have suitably conserved regions for short-amplicon-based metabarcoding free eDNA studies (see Thomsen and Willerslev 2015; Shaw et al. 2016). Less utilized but more fish-specific primer sets (e.g., for 16S mitochondrial sequences) may not have reference sequences for all detected fish (Shaw et al. 2016). Other common eDNA metabarcoding primers target 16S rRNA (bacteria), 18S rRNA (eukaryotes), internal transcribed spacer (ITS, fungi and algae), and chloroplast rbcL and trnL (plants; see Shaw et al. 2017). Shaw et al. (2016) demonstrated use of both non-specific (12S rRNA) and specific (16S rRNA) primer sets for fish were able detect all species present in traditional surveys as well as additional species, but a use of a single primer set accounted for only 40-70% of traditional surveys. ECOPRIMERS software is a useful tool for identifying DNA markers and associated PCR primers (Riaz et al. 2011).

Inherent in metabarcoding methodology is a complexity to the work flow including multiple steps that must be planned carefully. For example, sample-specific tags can be attached at PCR amplification, so that all samples can be run simultaneously and sequences from different samples can be distinguished from on another (see Shaw et al. 2017). If a large quantity of non-

target DNA is expected to be present (e.g., human), blocking primers can be used to prevent non-target DNA saturating the PCR amplification process (see Valentini et al. 2016). Also, degenerate PCR tags can be used to confirm that a tag repeatedly viewed is or is not from the same PCR product.

In terms of fish biodiversity measures, eDNA metabarcoding is generally comparable to traditional survey techniques but is much more efficient (e.g., Civade et al. 2016; Thomsen et al. 2016; Valentini et al. 2016; Yamamoto et al. 2017). Samples at different depths, along a freshwater gradient, along the continental slope, and in different seasons have all demonstrated differences in local community biodiversity as measured by eDNA metabarcoding (Civade et al. 2016; Thomsen et al. 2016; Andruszkiewicz et al. 2017; Karahan et al. 2017; Stoeckle et al. 2017). Shaw et al. (2016) found water eDNA was a better determinant of fish species number than sediment eDNA in a river system. Metabarcoding can be particularly useful in biodiversity studies of plankton and/or larvae that are difficult to sample and distinguish by traditional methods (Brown et al. 2016; Hatzenbuhler et al. 2017; Maggia et al. 2017).

The potential minimum eDNA required for detection has not been well addressed, and is likely influenced by numerous factors including species, habitat, community composition, environmental conditions, lifestage, etc. In a controlled experiment Hatzenbuhler et al. (2017) examined the detection limits of larval fish species, and found “rare” targeted species could be detected as low as 0.02% of total biomass. However, limits to detection varied interspecifically, some species were susceptible to amplification biases, and in some combinations they were unable to detect non-target species. The authors suggested caution be applied when interpreting presence, absence, and relative abundance in fish assemblages until metabarcoding methods are optimized for accuracy and precision, and sensitivity and accuracy may vary with species composition. Issues with detection of rare species include sequencing errors resulting in overestimation of rare species richness, and artefact removal resulting in false negative detection (see Darling and Frederick 2017). It should be noted that false positives can arise from transportation of eDNA by secondary vectors such as predator faeces, boats, birds, water currents, and wastewater outflow (Shaw et al. 2016; Stoeckle et al. 2017; Yamamoto et al. 2017).

eDNA metabarcoding is not yet able to rigorously address population-level questions, but is a strong tool for early detection of depletion of exploited and cryptic species (see Karahan et al. 2017). It can also detect cryptic or rare species or species only present as larvae better than traditional surveys (see Yamamoto et al. 2017). Although eDNA metabarcoding sequence counts thus far remains only semi-quantitative at best in some circumstances such as within species comparisons (e.g., Shaw et al. 2016), currently quantitative abundance measures for multiple species are not recommended (see Shaw et al. 2017). However, Thomsen et al. (2016) found a good correlation with trawl catch size and eDNA sequence reads taken at depth in Greenland, suggesting in some circumstances eDNA can be used as both a qualitative and quantitative proxy for marine fish assemblages. Further uses will likely be demonstrated as knowledge of and skills in eDNA metabarcoding become more commonplace.

Shotgun metagenomic sequencing (SMS)

Metagenomics is another biomonitoring tool using detection and analysis of DNA, and is generally used for viral or bacterial-specific diversity studies. Metagenomics refers to the genomic analysis of microorganisms by direct extraction, cloning, and sequencing of all DNA in a sample (Handelsman 2004), typically from an environmental or other sample containing a community of microorganisms. While amplicon-based approach (i.e., metabarcoding) can be included in metagenomics, here we focus on "shotgun-based" approach to metagenomics to whole genome sequencing (WGS), where all genomic material in a sample is sequenced, assembled, and homology searched against a reference database to identify the gene and/or species (see Mineta and Gojobori 2016). Shotgun metagenomic sequencing is an efficient way to understand the microbial community diversity at a sampling point in ecosystems such as in the marine environment, and can also be used to identify characteristic sequences and novel genes associated with an environment (Mineta and Gojobori 2016). Also metagenomics, or rather metatranscriptomics, is used to characterize the functional potential of microbial communities by assessing gene expression patterns in complex communities (Creer et al. 2016). Shotgun metagenomic sequencing has been used for eDNA studies of microbial communities, phytoplankton, and microscopic larval forms (Valentini et al. 2016), as well as sequencing of eukaryote parasite communities (e.g., nematodes and trypanosomes in wild bees; Schoonvaere et al. 2017). Some researchers predict that recent technological developments in metagenomic sequencing of macro-organisms may resolve many of the issues prevalent in eukaryotic marker gene studies (Barnes and Turner 2016; Mendoza et al. 2016). When used in conjunction with targeted genome sequencing, SMS may facilitate detection of unexpected endangered or introduced species (Barnes and Turner 2016; Creer et al. 2016).

Sczyrba et al. (2017) recently reviewed available tools and methods for metagenomics analysis, and introduced a community-driven initiative for the Critical Assessment of Metagenomic Interpretation (CAMI) for ongoing assessment of metagenomics-related programs. The authors identified challenges with current data interpretation tools, such as difficulty in assembling closely related genomes, and that this can result in gaps in taxonomic reconstructions when closely-related organisms are present, and poor abundance estimates when plasmids and viruses were included in data.

In a review of viral metagenomics analysis in aquaculture, Munang'andu (2016) summarized potential and/or demonstrated uses including use of baseline viral data of environment intended for aquaculture to enable the design of effective disease control strategies, identification of most effective disinfectants for recirculating aquatic systems, and identifying viral composition of ballast water to design management strategies. Viral metagenomics can be used to understand viral diversity, and has been used to identify and describe novel aquatic pathogenic viruses (see Alavandi and Poornima 2012). Oh et al. (2011) used WGS DNA sequencing to examine the phytoplankton microbial community in a temperate freshwater lake, and Flaviani et al. (2017) used it in conjuncture with metabarcoding to determine the microbiome diversity in the marine environment. One advantage of metagemonics is that viral pathogens or other microorganisms can be identified without prior knowledge of genomics sequences (see Munang'andu 2016).

Marine metagenomic studies have led to the discovery of new plankton, bacteria and viruses (see Mineta and Gojobori 2016).

Current challenges associated with metagenomics include the vast quantity of data generated requires long computational time, high computer processing power, technical bioinformatics skills and software, lack of consensus on data analysis and interpretation, and ideally publicly available annotated databases of aquatic viruses which can be limiting (see Munang'andu 2016; Sczyrba et al. 2017). Analysis of a single sample through metagenomics results in a large volume of complex data, and if analyzing data from multiple sample points over several time periods, the data accumulation quickly becomes tremendous (Mineta and Gojobori 2016). Due to the large volume of complex data produced from metagenomics surveys, it is necessary to have databases that can extract useful knowledge from the data (Mineta and Gojobori 2016; Table 5).

Software and database considerations

The ability for any kind of DNA barcoding to distinguish organisms is determined by the completeness and accuracy of the reference database used to assign taxonomy (Keskin et al. 2016; Shaw et al. 2016; Darling and Frederick 2017; Hatzenbuhler et al. 2017). A species can only be identifiable if its target sequence has been determined and is distinguishable from similar species (Brown et al. 2016; Shaw et al. 2016). Several studies assemble their own database by sequencing targeted genes of expected species to add to existing reference databases to minimize lack of detection from incomplete reference databases (e.g., Valentini et al. 2016; Karahan et al. 2017; Maggia et al. 2017; Stoeckle et al. 2017). These issues regarding identification of unexpected, poorly sequenced organisms are expected to decrease with time as more reference sequences are made available and existing ones are screened for accuracy.

The most commonly used online genetic database is GenBank (see Table 4), but it is not extensively curated and consequently may have inaccurate sequences (Shaw et al. 2017). Other databases that may have fewer sequences but more curation with physical voucher specimens include Greengenes, RDP, SILVA, EZBioCloud, and BOLD (see Mineta and Gojobori 2016; Shaw et al. 2017). The International Barcode of Life is assembling vertebrate-specific libraries (e.g., base on COI) and libraries related to marine and freshwater biosurveillance (FISH-BOL). Reference databases can be skewed geographically and taxonomically, although a worldwide effort is rapidly addressing this issue (see Thomsen and Willerslev 2015).

Challenges associated with metabarcoding include the large quantity of data generated by high-throughput (H-T) sequencing, requirements for intensive or long computational times, technical bioinformatics programming skills, experience with software, effective data and code management, lack of consensus approaches for data analysis, interpretation, and reporting, and ideally publicly available annotated databases. Some more detail will be given here on the general workflows of eDNA analysis, although it should be noted that there are a very large

number of original workflows, each of which may have its own strengths and weaknesses. The methods described below are just one such approach.

Initially, data from a sequencer will be inspected for quality using a program such as FastQC (Andrews 2010) with an aggregator such as MultiQC (Ewels et al. 2016). MultiQC is very useful when the samples come already demultiplexed, meaning that each sample is within its own fastq file (and thus there will be many files). The aggregator MultiQC is an optimal method for viewing numerous fastq file quality values simultaneously. These methods will provide the total numbers of reads per library, which will be valuable as one moves through the pipeline, by auditing where reads are dropping out of the analysis. Once the initial sequencing data are inspected, the data can be moved into a pipeline. Below we focus on the OBITools pipeline generally (Boyer et al. 2016), but it should be noted that many others exist. For much more detailed explanation on OBITools and other programs, please see the respective citations for the software packages.

If samples have not yet been demultiplexed, it will be necessary to first extract the sample by the tag that was attached to each sample earlier in the preparation of libraries. At this point, methods will also remove the PCR primer site from the amplicon as well. In OBITools this process is conducted using the program *ngsfilter*. If samples are already demultiplexed, the primer site should be removed for example using a standard sequence removal program such as Cutadapt (Martin 2011). Importantly, if data comes from paired-end reads, prior to demultiplexing, sequences from the forward and reverse read will be compared to each other, identified as overlapping or not (i.e., not overlapping if the amplicon size is greater than the size of the two reads put together), and then merged into a single amplicon with consolidated quality scores. This should be conducted prior to any removal of low quality data or demultiplexing. Depending on the input data, the above steps may slightly vary, but in general, this will result in demultiplexed data that has been merged if paired-end, with the primer site removed.

Using OBITools, once samples are demultiplexed, they should have a sample identifier in the header of the reads. If they do not, one can add this information using built-in tools of the OBITools suite (i.e., *obiannotate*). Once the amplicons have been labeled with a sample identifier in the sequence header, they can be combined into one large file. Subsequently, a single amplicon is retained for all amplicons that share a certain amount of sequence identity (possibly a perfect match), as based on settings used by the program. The number of times this amplicon was seen in the various samples is then retained in the header of this amplicon, so that one can see the number of reads per sample per amplicon and can deal with a smaller file. After this, some filtering can be conducted, for example based on sequence identity (e.g., remove amplicons that vary only by a single base from another more frequent amplicon as they may be sequencing or PCR errors; Boyer et al. 2016), or by size (e.g., retain amplicons only within the size range expected for the selected taxon), or by the minimum number of read counts used as a threshold cutoff. After this has been conducted, one is left with a cleaned list of unique amplicons with the number of reads per amplicon per individual in a single file.

Once one has the unique amplicons, the next step is typically to use an automated sequence alignment program such as Basic Local Alignment Search Tool (BLAST) to collect a

number of annotations (e.g., the ten top annotations) from the database of choice (e.g., NCBI nucleotide database) for each unique amplicon. Depending on settings used, there may be some or many unannotated sequences. It would be important for the researcher to evaluate the settings to determine the best to use. Nonetheless, these parameter adjustments can be used later to do additional taxonomic filtering. Notably, BLAST and similar programs can be run in parallel (e.g., GNU Parallel; Tange 2011). The results of the BLAST can be input into a taxonomic clustering program, for example the MEGAN Community Edition, where one can explore and annotate unique amplicons with taxonomic identifiers based on settings within MEGAN. Finally, one can use custom scripts to connect the amplicons that have been annotated with the read counts (e.g., using OBITools *obitab* program to export read counts per sample per unique amplicon). At the end of this analysis, one is left with a table of species annotations and read counts per amplicon per sample.

These data are then further filtered if required (e.g., by low expression), and are the input for presenting in tables, use for statistical analysis, or for plotting. The statistical analysis of this type of data is continually being developed, and this topic is beyond the scope of this review. It is important to note that the number of reads per species will depend on various factors, but importantly on the sequencing depth of the library, the complexity and abundances of other species in that library (i.e., taking up sequencing reactions from the target species), as well as technical aspects of efficiency of amplification of that species' amplicon, which may be impacted by aspects of the sample itself (e.g., turbidity, inhibitors), as discussed above. In any case, this type of data is an important starting point for an analysis, and in many cases, as described above, leads to results that are verified by results obtained by other physical estimating methods. A final note is the value of looking into unknown and taxonomically unassigned amplicons. These can provide insight on where the database is lacking, and as discussed above, can point to areas that can be improved in the database by careful collection of voucher specimens to improve the use of the amplicon type for that species-database combination.

As discussed above, the approach described here is only one such analysis method, and many others also exist, some of which may be superior to the approach described here. In the scope of this review, we cannot exhaustively list such methodologies, but rather provide a general framework that puts some context to the bioinformatics approach. Where possible, it is best to carefully document within prepared manuscripts the details regarding quality control steps taken, parameters used by programs, the stages where reads are removed, the scripts used, and the versions of programs applied. These types of details improve the reproducibility of the analysis and can allow others to verify results for themselves. With this in mind, it is important to use open source programs that can be used freely so that any reader can reproduce the analysis, given that the reader has sufficient bioinformatics skills required to do so.

Importantly, during these analyses it is critical to facilitate communication between researchers/technicians doing the field collections, DNA extractions, library preparations, sequencing, and bioinformatics to make sure that any issues are fully understood and can be improved for future analyses. Further, these types of discussions can allow for the detection of

errors that may be misinterpreted when it comes to data reporting. Presentation of results to team members involved in earlier stages of the analysis is essential to avoid errors and to improve future projects.

Issues associated with metagenomics include the vast quantity of data generated requires long computational time, high computer processing power, technical bioinformatics skills and software, lack of consensus on data analysis and interpretation, and ideally publicly available annotated databases of aquatic viruses which can be limiting (see Munang'andu 2016; Sczyrba et al. 2017). Analysis of a single sample through metagenomics results in a large volume of complex data, and if analyzing data from multiple sample points over several time periods the data accumulation quickly becomes tremendous (Mineta and Gojobori 2016). Due to the large volume of complex data produced from metagenomics surveys, it is necessary to have databases that can extract useful knowledge from the data (Mineta and Gojobori 2016; Table 5). Mineta and Gojobori (2016) list other major databases contain marine and/or freshwater metagenome data, as well as pipelines for collection and analysis of metagenomics data. Lindgreen et al. (2016) provide an evaluation in terms of accuracy and speed of publicly available metagenomics analysis tools, and provided analysis of existing common tools to assist researchers to choose the best tool for their needs.

Table 4. List of software and databases used in metagenomics for species identification.

Metabarcoding			
Genbank **Greengenes**		https://www.ncbi.nlm.nih.gov/genbank/	annotated collection of all publically available DNA sequences
		http://greengenes.secondgenome.com	16S rRNA specific
	RDP	http://rdp.cme.msu.edu/	http://rdp.cme.msu.edu/, bacterial and archaeal 16S and fungal 28SrRNA
	SILVA	https://www.arb-silva.de/	rRNA from bacteria, archaea, eukarya
EZBioCloud		http://www.ezbiocloud.net/taxonomy	fungi-specific DNA
	UNITE	https://unite.ut.ee/	
Barcode of Life Data System	BOLD	http://www.boldsystems.org/	platform of integrated databases
International Barcode of Life	IBOL	http://ibol.org/phase1/	COI gene barcode references libraries through the BOLD platform
FISH-BOL	FISH-BOL	http://www.fishbol.org/index.php	

Metagenomics			
IMG/M		http://img.jgi.doe.gov/	microbe specific collection and analysis
MG-RAST		http://metagenomics.anl.gov/	pipeline for analysis
Megan6		http://ab.inf.uni-tuebingen.de/software/megan6/welcome/	interactive analysis of microbiome metagenomics data
CLARK		http://clark.cs.ucr.edu/	Ounit et al. (2015)
Kraken		http://ccb.jhu.edu/software/kraken/	Wood and Salzberg (2014)
iMicrobe		http://imicrobe.us/	microbe specific
VIROME		http://virome.dbi.udel.edu/	virus specific
EBI metagenomics		https://www.ebi.ac.uk/metagenomics/	pipeline for collection and analysis
Marine Metagenomics Portal	MMP	https://mmp.sfb.uit.no/	provides data resources and analysis services specific to marine domain, including reference databases of complete genomes (MarRef) and all prokaryotic genome data (MarDB), and marine genes and proteins (MarCat), analysis pipeline for marine metagenomics data (Meta-pipe), and a blast search for gene or protein coding (MMP BLAST)

Conclusion

The eDNA field of study and associated tools and technologies have the potential to improve our understanding of aquatic species and ecosystems, which can help identify habitats critical to important fisheries and protected species. If eDNA science is applied in situations where it can be advantageous, it can strongly complement conventional survey methods, allowing for more effective and efficient management decisions surrounding the conservation of species and aquatic ecosystems. Here, we have compiled several critical considerations from the peer-reviewed literature on aspects of the study of eDNA from field sample to database

management. Although technological developments associated with the field of eDNA are occurring rapidly, and their use will change over time, we recognize the imminent need for development of consistent approaches and increased communication within DFO. Collaboration across DFO, other Canadian government bodies, and internationally is needed to develop the relevant databases, models, and software for eDNA that can support and improve quantitative studies related to broader sampling and assessment (Yoccoz 2012; Roussel et al. 2015). In this collaborative and connected manner, DFO can continually improve upon the efficacy of eDNA analysis and interpretation of results as the field grows. As genomics technology continues to rapidly improve and costs decline, the list of potential future conservation and research applications of eDNA is genuinely inspiring.

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