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No. 12, Thiruvannamalai Nagar, Saravanampatti - Kalapatti Road, Saravanampatti,
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Ph: +91 9385339863 | www.threatenedtaxa.org
Email: sanjay@threatenedtaxa.org

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Cover: Dorsal view of Mantis Shrimp *Cloridina ichneumon* (Fabricius, 1798) & *Gonodactylus demanii* (Henderson, 1893). © Fisheries Research Station, Junagadh Agricultural University, Sikka.



Environmental DNA as a tool for biodiversity monitoring in aquatic ecosystems – a review

Manisha Ray¹ & Govindhaswamy Umapathy²

^{1,2} CSIR - Centre For Cellular And Molecular Biology, Uppal Rd, Hyderabad, Telangana 500007, India.

¹manisharay@ccmb.res.in, ²guma@ccmb.res.in (corresponding author)

Abstract: The monitoring of changes in aquatic ecosystems due to anthropogenic activities is of utmost importance to ensure the health of aquatic biodiversity. Eutrophication in water bodies due to anthropogenic disturbances serves as one of the major sources of nutrient efflux and consequently changes the biological productivity and community structure of these ecosystems. Habitat destruction and overexploitation of natural resources are other sources that impact the equilibrium of aquatic systems. Environmental DNA (eDNA) is a tool that can help to assess and monitor aquatic biodiversity. There has been a considerable outpour of research in this area in the recent past, particularly concerning conservation and biodiversity management. This review focuses on the application of eDNA for the detection and relative quantification of threatened, endangered, invasive and elusive species. We give a special emphasis on how this technique developed in the past few years to become a tool for understanding the impact of spatial-temporal changes on ecosystems. Incorporating eDNA based biomonitoring with advances in sequencing technologies and computational abilities had an immense role in the development of different avenues of application of this tool.

Keywords: eDNA, non-invasive, biomonitoring, endangered, eutrophication, anthropogenic

Tamil: நீர்குழியலமைப்புகளில் மாணுட செயற்பாடுகளால் ஏற்படும் மாற்றங்களைக் கண்காணித்தல், நீர்வாழ் பல்லுயிர் இனங்களின் நல்வாழ்வை உறுதி செய்தல் மிகமுக்கியம். மாணுட இடையூறுகளால் நீர்நிலைகள் தூர்ந்துபோதல், நீர்குழல்களில் ஊட்டச்சத்து வெளியேற்றத்திற்கு முக்கிய காரணியாக செயல்படுவதுடன், அதன் விளைவாக உயிரியல் உற்பத்தித்திறன் மற்றும் பல்லுயிர் சமூகக்கட்டமைப்பை மாற்றுகிறது. வாழ்விட அழிப்பு மற்றும் இயற்கை வளங்களின் அளவு கடந்த சுரண்டல் ஆகியன நீர்மண்டலங்களின் சமநிலையை எதிர்மறையாக தாக்கும் மூலங்கள் ஆகும். சூழல் மரபணு என்பது, நீர்வாழ் பல்லுயிர்களை மதிப்பிடவும், கண்காணிக்கவும் உதவும் ஒரு முறை. இந்த ஆய்வு களத்தில், குறிப்பாக பல்லுயிர் பாதுகாப்பு மற்றும் மேலாண்மை குறித்த பகுதிகளில், அண்மை காலங்களில் கணிசமான அளவில் ஆய்வுகள் நடந்து வருகின்றன. இந்த மதிப்பாய்வுரை, அழிவின் விளிம்பில் இருக்கும் அச்சுறுத்தப்பட்ட மற்றும் அருகிவரும் உயிரினங்கள், கண்டறிய கடினமான மற்றும் ஆக்கிரமிப்பு உயிரினங்கள் ஆகியவற்றை, கண்டறிதல் மற்றும் ஒப்பிட்டு அளவிடுதலில் சூழல் மரபணுவின் பயன்பாடுகள் குறித்து கவனம் செலுத்துகிறது. சுற்றுச்சூழல் மண்டலங்களில் பரப்பு-காலம் சார்ந்த மாற்றங்களின் தாக்கங்களைப் புரிந்து கொள்ள ஒரு வழிமுறையாக மாற, கடந்த சில ஆண்டுகளில் இந்த நுட்பம் எவ்வாறு மேம்பட்டுள்ளது என்பதற்கு நாங்கள் ஒரு சிறப்பு முக்கியத்துவம் கொடுத்திருக்கிறோம். மரபணு வரன்முறையிடல் நுட்பங்கள் மற்றும் கணக்கீட்டுத்திறன்களில் ஏற்பட்டுள்ள முன்னேற்றங்களை சூழல் மரபணு சார்ந்த பல்லுயிர் கண்காணித்தலுடன் ஒருங்கிணைத்தது, இவ்வழிமுறையின் பன்முகப் பயன்பாடுகளை மேம்படுத்துவதில் மாபெரும் பங்காற்றியுள்ளது.

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Author details: MANISHA RAY has post-graduation in Molecular Microbiology and is currently a PhD student at CSIR-CCMB, Hyderabad. Her research interest lies in studying microbial ecology and the novel functions of microbes that help ecosystems to thrive and support other life forms. Her doctoral work aims at using eDNA as a tool to decipher the species and genomic diversity of Phylum Cyanobacteria in relation to ecosystem services and changing physico-chemical factors of Chilika Lake, Odisha. GOVINDHASWAMY UMAPATHY is a Conservation Biologist. He works on understanding species extinction in human dominated landscapes and develops various biotechnological tools in biodiversity conservation at Laboratory for the Conservation of Endangered Species, CSIR-CCMB, Hyderabad.

Author contributions: MR and GU conceptualized the content of the paper. MR collected all relevant references and wrote the manuscript. Both MR and GU revalidated the content and proofread the manuscript.

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INTRODUCTION

Earth is an abode of numerous living organisms which exist in varying environmental conditions and all are ultimately interconnected. Major unknowns in estimating global biodiversity are: how many species inhabit Earth, and what is their rate of extinction. Only a fraction of total biodiversity is known, and a substantial number of species that have not yet been accounted for and are vanishing without our knowledge. Since all species are dependent on each other in some way or another, the removal of one drastically affects other species. Unravelling each point in this network of life is important to study how an ecosystem at large functions and also to understand the life history of a species and how new communities get established.

Aquatic ecosystems comprising freshwater, brackish, and marine water in nature are the sources of a lot of species diversity ranging from microbes to mammals. The impact of human activities on these life forms is multifactorial. An increase in the emission of carbon from anthropogenic actions is leading to an increase in water temperature, acidification and oxygen deprivation of aquatic systems (Jiao et al. 2015). The changes in the abiotic parameters of the ecosystem is accompanied with impacting the cycling and efflux of nutrients. These changes in turn regulates the geographic distribution of the life forms in that habitat (Nazari-Sharabian et al. 2018). According to the special report of IPCC (The Intergovernmental Panel on Climate Change) on changing ocean and cryosphere 2019, by the year 2100, the ocean will witness an increase in temperature by 2 to 4 times and oxygen levels will decline further resulting in increase in the volume of oxygen-deficient zones (OMZ). These changes will impact ecosystem services with a projected decrease in fish catch potential and global marine biomass, which will further impact revenue generation, food security and threaten livelihood. Analysing the world's biodiversity becomes a critical aspect of learning about the distribution of these "biodiversity hotspots" and applying conservation practices to protect these areas.

The traditional practices of estimating biodiversity are biased towards the sampling of particular species (Gunzburger 2007) or can also pose a risk to sensitive organisms. In recent times, molecular techniques are gaining importance in the estimation of biodiversity and its conservation in the world. One such molecular tool is the study of environmental DNA (eDNA), which has tremendous potential to develop our understanding of biodiversity science and provide implications for

conservation practices with census data of species present at a comprehensive scale in real-time.

What is environmental DNA?

The term 'environmental DNA' (eDNA) was introduced in the field of microbiology for the detection of microbial communities in sediments by Ogram et al. (1987). eDNA has been classified based on particulate size: aggregates of eDNA greater than 0.2 μm were termed as particulate DNA (P-DNA) while eDNA less than 0.2 μm is termed as dissolved DNA (D-DNA) by (Paul et al. 1987). DNA extracted non-invasively from environmental sources like soil, air, or water is termed environmental DNA (eDNA). It has a polydisperse nature, i.e., the origin of eDNA can have several sources like sloughed cells, faecal matter, spores, slimy coating (in amphibians), or dead carcasses. Based on the source of origin of eDNA, it undergoes selective decay and thus complicates the evaluation of decay rates (Wilcox et al. 2015). eDNA has been used in the aquatic system to either detect the presence or absence of a species or for quantitative estimation of a particular species. Its application varies between lotic and lentic ecosystems as their nature varies. The lotic ecosystem is flowing and can transport eDNA directionally downstream from the correct location of the target organism, whereas the lentic ecosystem is stagnant. eDNA is released into the environment and subsequently undergoes progressive decay due to many biotic and abiotic factors.

FACTORS GOVERNING THE CONCENTRATION OF EDNA IN THE AQUATIC ENVIRONMENT:

Based on the literature review, it has been perceived that there can be numerous factors that can govern the concentration of eDNA at a particular time and space, but can be primarily divided into three categories:

- 1) eDNA released by the organism
- 2) Persistence of eDNA in different environmental conditions
- 3) Capture protocols for eDNA and sensitivity of detection assay

eDNA release by the organism

The concentration of eDNA released by an organism and the degradation rate of DNA in a particular environment are the two attributes on which the concentration of eDNA varies on a given spatial-temporal scale. The release of eDNA is a complex interaction between environmental conditions, the natural history of an organism, its metabolic rate, and the developmental stage. With an increase in the temperature of the water,

Table 1. Few key studies on the applications of eDNA as a tool.

	Study details	References
A) Detection of species:		
1)	Detection of alien invasive species <i>Procambarus clarkii</i> (crayfish) in water from the natural pond and artificial aquarium	(Geerts et al. 2018)
2)	Detection of a threatened species <i>Glyptemys insculpta</i> (wood turtle) using qPCR by designing species-specific primers and Taq man probe	(Lacoursière-Roussel et al. 2016c)
3)	Detection of endangered Shasta crayfish (<i>Pacifastacus fortis</i>) and invasive crayfish (<i>Pacifastacus leniusculus</i>) in river water	(Coward et al. 2018)
4)	Comparing the sensitivity of detection of alien invasive species- American bullfrog (<i>Lithobates catesbeianus</i>)	(Dejean et al. 2012)
5)	Detection of invasive species, African jewelfish (<i>Hemichromis letourneuxi</i>) and determine the lower limit of detection and effect of fish density and time on detection in an artificial aquarium	(Díaz-Ferguson et al. 2014)
6)	Detection of invasive species, New Zealand mud snails (<i>Potamopyrgus antipodarum</i>) and to find the time till which eDNA remains detectable in the aquatic system	(Pilliod et al. 2013a)
7)	Detection of invasive submerged aquatic plant, <i>Egeria densa</i> in pond water	(Fujiwara et al. 2016)
8)	Differentiating between endemic species, Japanese giant salamander (<i>Andrias japonicum</i>) and exotic species, Chinese giant salamander (<i>Andrias davidianus</i>) using eDNA	(Fukumoto et al. 2015)
9)	eDNA detection rate has a positive relationship with flow volume in waterways and has a more pronounced effect on eDNA detection probability than other co-variables like temperature, dissolved oxygen concentration, pH	(Song et al. 2017)
10)	Detection of transient pelagic marine fish, Chilean devil ray (<i>Mobula tarapacana</i>)	(Gargan et al. 2017)
B) Estimation of biomass/abundance:		
1)	Effect of water temperature and eDNA capture method on altering the relationship between eDNA concentration and fish biomass of economically important salmonid, Brook Charr (<i>Salvelinus fontinalis</i>)	(Lacoursière-Roussel et al. 2016b)
2)	Killer whale (<i>Orcinus orca</i>) eDNA quantification using ddPCR from seawater	(Baker et al. 2018)
3)	Estimation of transport distance of eDNA of brown trout (<i>Salmo trutta</i> , L.) using a dual-labelled probe for relative quantification	(Deutschmann et al. 2019)
4)	Comparison of detection probability, density, biomass and occupancy with traditional methods of sampling of Rocky Mountain tailed frog (<i>Ascaphus montanus</i>) and Idaho giant salamander (<i>Dicamptodon aterrimus</i>)	(Pilliod et al. 2013b)
5)	Salmon DNA was measured from water samples during the spawning season using species-specific quantitative PCR probes and factors affecting the correlation between eDNA concentration and biomass of these fishes were also studied.	(Tillotson et al. 2018)
C) Studying the communities in the ecosystem		
1)	The direct impact of an anthropogenic activity like an oil spill on the coastal marine ecosystem was observed. The succession of communities after the event was monitored which included bacteria, metazoans and protists. Certain communities were found to be resistant to the effect of this incidence whereas few others were conferred with the sensitivity to this.	(Xie et al. 2018)
2)	The community-level response in cyanobacteria, diatoms and microbial eukaryotes were correlated to physicochemical parameters of Lake Constance like rising phosphorus and air temperature. Major environmental perturbations like eutrophication during the 20 th century were found to align with the reversion of resilience demonstrated by the communities.	(Elberri et al. 2020)
3)	The change in community structure of bacterial, protistan, and metazoan communities in response to pollution status of the river using eDNA metabarcoding. The varying level of nutrients in the ecosystem was shown to be the main driving factor in the relative abundance of OTUs and community structure.	(Li et al. 2018)
4)	The spatial distribution of bacterial communities was studied using metabarcoding. The change in the richness of these communities and the abundance was shown to be a measure of the degree of anthropogenic contamination and can be an area to focus on for biomonitoring of coastal ecosystems.	(Garlapati et al. 2021)
5)	The study focuses on identifying the association between the fish assemblages in the ecosystem and invasive species and how these get affected by environmental co-variables and human-induced disturbance.	(Pukk et al. 2021)

the mobility of fish has been reported to increase (Petty et al. 2012) hence the metabolic rate also increases (Xu et al. 2010) until a physiological limit of tolerance is attained. The timing of sample collection plays a vital role because it can help in capturing the presence of the migratory species based on its natural history or seasonal variability in levels of resident species (Lesley et al. 2016). It has been found that with different developmental stages, eDNA released also varied. eDNA

release rate per fish body weight is slightly more in the juvenile group when compared to that of an adult group due to factors related to ontogeny. But, the rate of eDNA release per individual is more from adult fish than juveniles because of the larger body size of adult fish (Maruyama et al. 2014). Hence, it is difficult to infer if the source of eDNA is from a higher number of juveniles or a lesser number of adults.

Persistence of eDNA in different environmental conditions

DNA has limited chemical stability (Lindahl 1993) and once it is shed into the environment, it can either persist in free form or get adsorbed to organic or inorganic matter or else get sedimented or degraded (Dejean et al. 2011). The persistence of eDNA depends on factors which are divided into three categories - abiotic (temperature, salinity, pH, oxygen, & light), biotic (extracellular enzyme & microbial community), and DNA characteristics (length, conformation, & membrane-bound) reviewed by Barnes et al. (2014).

Capture protocols for eDNA and sensitivity of the assay

Most efficient capture protocols are a combination of a selection of the most appropriate filter materials which allows filtering the maximum amount of water using powerful automatic motors along with optimized isolation protocols and preservation techniques to maximize the yield of eDNA. The pore size of the filter is also an important feature that decides which source of DNA shall be enriched- gametes, sloughed cells free DNA, etc, and also the target group of organisms. If microorganisms are the target, then very low pore size filters will capture most of them. Renshaw et al. 2015 found that there was no significant difference in copy number in the case of 0.8 µm cellulose nitrate (CN) filter or 0.8 µm polyether sulphone (PES) filters. In contrast to this, (Hinlo et al. 2017) and (Liang & Keeley 2013) found a CN filter to have a significant difference in DNA yield. This difference could be due to a different combination of isolation and preservation protocol.

Precipitation and filtration are the two methods that have been used to extract eDNA from water samples. Precipitation is generally used for smaller volumes by using salt and ethanol to precipitate extracellular DNA by using centrifugal forces (Maniatis et al. 1982). Filtration is more size-dependent and is based on the property of filter material to keep eDNA. Filtration had shown more yield of eDNA in combination with isolation protocols for DNA (Deiner et al. 2015). DNA isolation: three protocols generally have been used to extract DNA from filters, namely the phenol chloroform Isoamyl alcohol method (PCI), Qiagen's DNeasy® blood and tissue kit, and MoBio's PowerWater® DNA isolation kit. PCI method has been shown to yield more targeted DNA compared to Qiagen's DNeasy® blood and tissue kit using a 0.45 µm CN filter. While MoBio's PowerWater® DNA isolation kit has shown more yield than the PCI method using a 1.5 µm glass membrane filter (GMF) (Renshaw et al. 2015). However, filtration along with Qiagen's DNeasy

® blood and tissue kit has shown a higher diversity of eukaryotes being detected compared to that of limited species being detected in the case of the PCI method with filtration (Deiner et al. 2015). We believe that skipping the use of lysis buffer during isolation of eDNA from filter membranes will help in reduction of the microbial eDNA part as it will limit the lysis of microbial cell. This method will help in studying the non-microbial or eukaryotic taxa. The flow rate through filters had also been seen as a crucial step, as eDNA might start the process of degradation if the filtration time is too much. Hence, filters with higher flow rates have been preferred (Hinlo et al. 2017).

Preservation of DNA and storage is also a very crucial step in the case of detection of very low abundant species or quantification of the abundance of any species, as even a slight degradation in copy numbers might give faulty results. Freezing of filters at a very low temperature cannot always be workable in field conditions hence 95% ethanol (Minamoto et al. 2015), Longmire buffer (Renshaw et al. 2015; Williams et al. 2016), and CTAB (Renshaw et al. 2015) has been shown as alternatives. It was found that both the Longmire buffer and CTAB preserved filtered eDNA for over two weeks at 20°C but at 45°C Longmire, buffer outperformed CTAB buffer (Renshaw et al. 2015). Enhanced CTAB buffer has shown to have better inhibitor removal activity while Longmire buffer has the property to preserve eDNA for a longer time (Hunter et al. 2019). It is recommended to choose the best preservation buffer according to one's requirement by conducting a pilot experiment.

PCR inhibitors can be responsible for incorrect estimation of abundance or failure in the detection of very low copy number species. These inhibitors can either be co-extracted along with the extraction of eDNA or during isolation protocols. These inhibitors, like phenol and proteinase K, are removed by adding BSA to the PCR master mix (Deiner et al. 2015). These inhibitors might also be removed using inhibitor removal columns available in some commercial kits (McKee et al. 2015).

The specificity of primer and sensitivity of PCR is crucial. Nested PCR has been shown to improve detection compared with conventional PCR (Jackson et al. 2017). Detection rates of eDNA are greater with digital droplet PCR (ddPCR) than real-time PCR (qPCR) at lower concentrations (Doi et al. 2015). Quantitative estimation of biomass was shown to be more accurate by using ddPCR than qPCR. ddPCR was suitable for measurement of the natural sample as inhibitory substances have little effect on DNA quantification, as endpoint PCR amplification in each droplet can be

detected independent of amplification efficiency in ddPCR (Doi et al. 2015). There have been reports that base pair mismatches in the primer have more impact than that of the probe and the location of the mismatch also plays an important role. Base pair mismatch near the 3' end has shown a larger impact on specificity than in the 5' end or any other region (Wilcox et al. 2013).

APPLICATIONS OF eDNA AS A TOOL IN CONSERVATION AND BIODIVERSITY MONITORING

From deciphering single species to documenting entire communities, our understanding of eDNA study has progressed over the years. There is a multitude of applications of eDNA ranging from detection of invasive species, elusive species or any other ecologically important or threatened species to unravelling community dynamics and their response to changing spatial-temporal changes. This has paved new avenues in ecosystem management. In the case of microbes, less than two per cent of the total are culturable (Wade 2002). This necessitates the implementation of culture-independent methods for understanding their genomic and functional aspects. The eDNA technique has found a host of new applications over several years in the field of ecosystem monitoring and management.

1) Detection of species

Its advent revolved around the uncovering of single species like the detection of invasive species, Crayfish *Procambarus clarkia* (Geerts et al. 2018), endangered or vulnerable species, Wood Turtle *Glyptemys insculpta* (Lacoursière-Roussel et al. 2016c), or some elusive species, Oriental Weather Loach *Misgurnus anguillicaudatus* (Hinlo et al. 2017). A brief methodology for the detection of species from environmental aquatic samples using the eDNA method has been depicted in Image 1. eDNA technology along with occupancy modelling has been utilised for monitoring the presence of endangered species of Northern Tidewater Goby species *Eucyclogobius newberryi* and Southern Tidewater Goby species *Eucyclogobius kristinae* across the entire coast of 1,350 km (Sutter & Kinziger 2019). They found that eDNA technology showed double the rate of detection compared to the seining method, which resulted in improved site occupancy estimates as Northern Tidewater Goby was detected at two sites where their presence was never known before. A positive correlation was observed between eDNA concentration and catch per unit effort (CPUE). The implication of such objectives paves the path towards improved conservation goals. A list of key studies, along with the primers used in the detection and monitoring

of different species, is summarised in Table 2.

2) Population genetics studies

Population genetics has been a significant aspect in the study of ecology as it gives information about evolutionary history. But, research in this sector with the use of eDNA has just begun and is in its initial stage. Sampling in the case of population genetics has been a major challenge, especially in threatened organisms. eDNA approach helps to mitigate such challenges and helps in the study of organisms that are difficult to sample. Researchers have used eDNA that was extracted from sea water to examine the haplotype frequencies and genetic diversity at population level in Whale Shark *Rhincodon typus* (Sigsgaard et al. 2017). They used high throughput sequencing of two mitochondrial control region sequences and compared it with tissue samples from 61 individuals at the same locality from when samples for eDNA were collected. It was found that relative frequencies in both were similar. The more current study of elusive Harbour Porpoise *Phocoena phocoena* used high throughput sequencing for studying haplotype diversity and found eight unique mitochondrial DNA sequences from seawater sampling (Parsons et al. 2018). In another study, species and ecotypes of Killer Whales (*Orcinus orca*) were identified following encounters using digital droplet PCR and subsequently were sequenced. It was identified that the killer whale encounter was from a southern resident community (Baker et al. 2018). In a more recent study by Stepien et al. (2019), Silver Carp *Hypophthalmichthys molitrix* which is an invasive species in the U.S was studied for its introduction and spread using eDNA and mitochondrial markers targeting cytochrome b and c oxidase and nuclear DNA microsatellite markers.

3) Estimation of relative abundance

The scope of eDNA is more than just detecting the presence/absence of an organism. Estimation of copy number or biomass has been the major focus and extrapolation of avenues in which an eDNA study can be helpful. The information about an organism's relative abundance in the spatial-temporal scale helps to document the seasonal variations due to its response to the environment or due to other external forces like inter or intra-species competition. Estimation of abundance can have economic value in aquaculture if yield in a particular season can be known beforehand by studying the history of a few years about its seasonal variations. Even though numerous factors play a role in the persistence of eDNA in the environment along with its polydisperse nature, as discussed in the earlier section, if all protocols related to filtrations, isolation,

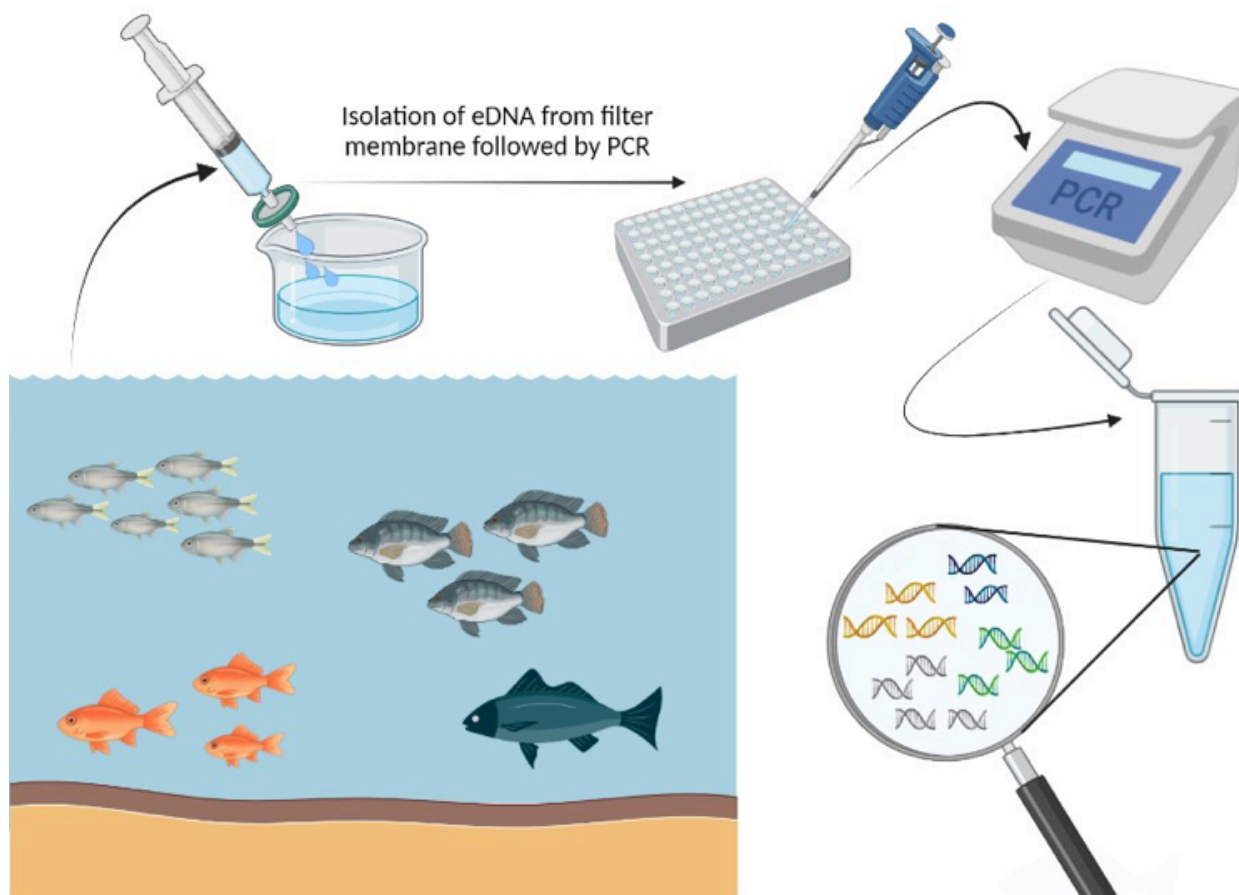


Image 1. Illustrative representation of methodology of the detection of the different species residing in an aquatic ecosystem. The collected environmental sample (water) can be syringe-filtered using membrane filters of desired pore size and material type, followed by amplification using PCR to detect the target species. In the case of metabarcoding based approaches, the entire biodiversity of the ecosystem can be deciphered. A repetitive sampling at the same site can give an idea about the quantitative change in biodiversity due to seasonal variations, anthropogenic disturbances, or some other abiotic attribute. As the abundance of a particular species varies, the same will be reflected in the copy number of eDNA which can give an understanding of how population dynamics are controlled by multiple factors. (Illustration created with BioRender.com)

and preservation are followed the same way for all samples across all seasons, then it can give an insight of its relative abundance. eDNA concentration of Lake Trout *Salvelinus namaycush* was estimated in 12 natural lakes and its abundance was compared to that of standardized gill net catches (catch per unit effort -CPUE and Biomass per unit effort- BPUE (Lacoursière-Roussel et al. 2016a) . Another study showed that the eDNA released from the target organism is a measure of its biomass for which laboratory and field-based experiments were conducted on Common Carp. This highlighted that the concentration of eDNA positively correlated to its biomass and can serve to understand its distribution in natural systems (Takahara et al. 2013).

An endangered amphidromous fish, Ryukyu ayu *Plecoglossus altivelis ryukyuensis*, was monitored to estimate abundance using qPCR with specific primers to

amplify the mtDNA ND4 region. The visual snorkelling surveys by individually fish counting positively correlated to eDNA copies/ml (Akamatsu et al. 2020). In another recent study by (Capo et al. 2019), digital droplet PCR was used to detect as well as quantify Brown Trout *Salmo trutta* and Arctic Char *Salvelinus alpinus* populations. While they compared between fish population estimated by conventional Catch per unit effort (CPUE) from gill netting method and eDNA concentration from digital droplet PCR, no significant correlation could be deduced, yet this paves a promising path for future research in this aspect by focussing on challenges and limitations which need to be overcome. This study also focuses on probable problems of stand-alone methods and how a congregation of various approaches, together with optimised protocols, can yield the desired result. In another method of individual

Table 2. Key studies for detection of important species in aquatic ecosystem.

	Aim of Study	Primer sequence used in the study				Reference
		Forward primer	Reverse primer	Probe	Amplicon length (in bp)	
1.	Detection of invasive rusty crayfish (<i>Orconectes rusticus</i>) in inland lakes using specific qPCR primers targeting the cytochrome c oxidase subunit 1 (COI) sequences	5'-CAGGGGCTCAGTAGATTAGGTAT-3'	5'-CATTGATCTATAGTATTCCCGTAG-3'		128	(Dougherty et al. 2016)
2.	Detection of invasive common Atlantic slipper limpet (<i>Crepidula fornicata</i>) from environmental seawater sample using species-specific primers targeting COI gene	Forward primers 5'-GATGATCACTATACAATGTA-3'	Reverse primers 5'-TAAACCGTTCAACCGG-3'		Amplicon length (in bp) 239	(Miralles et al. 2019)
3.	Detection of invasive signal crayfish (<i>Pacifastacus leniusculus</i>) in river and lake water samples using Taqman probe and species-specific primers targeting the COI gene	Forward primer 5'-ATAGTTGAAAGAGGAGTGGGTACT-3'	Reverse primer (5'-TAA ATCAACAGAACCCCTGCA-3')	Probe FAM-5'-CCTCCTCTAGCAGCGGCTATTGCTCATGC-3'-BHQ1	Amplicon length (in bp) 87	(Harper et al. 2018)
4.	Studying the distribution of silver carp (<i>Hypophthalmichthys molitrix</i>) and developing of novel methodology for on-site detection of the species	Forward primer 5'-GCAATTAATTCATCACCACAATTA-3'	Reverse primer 5'-TCCAGCAGCTAAACTGGTAAG-3'	Probe 5'-[FAM]-AAACACCTCTCTTTTGGAGCTGTG-[TAMRA]-3'		(Dol et al. 2021)
5.	Detection and quantification of European weather loach (<i>Misgurnus fossilis</i>) using digital droplet PCR targeting the COI gene. This species is cryptic and is facing population decline in recent times.	Forward primer 5'-CCCCGACATAGCATTTCCG-3'	Reverse primer 5'-AACTGTTACGCTGTCCAG-3'	Probe 5'-[6-FAM]CTCGTTCCTCTCTCTGCTGG(ZEN)/IBFQ]-3'	Amplicon length (in bp) 119	(Brys et al. 2021)
6.	Detection of endangered freshwater or Spectacle case Mussel (<i>Margaritifera monodonta</i>) using species-specific qPCR primers.	Forward primer 5'-AGTGGGTGATACCCWGTATCT-3'	Reverse primer 5'-TACCCCTAGCACCATTGTAT-3'	Probe 5'-SHEX/TCTAGCCCT/ZEN/AAGACTATGACAACTTTTCC/3IABKQ-3'		(Lor et al. 2020)
7.	Monitoring of river systems for detection of invasive Eastern mosquitofish (<i>Gambusia holbrooki</i>) and the consequent decline of two endemic species of killifish (<i>Valencia letourneuxi</i> and <i>Valencia robertae</i>) using species-specific qPCR targeting the COI region.	Species <i>Valencia letourneuxi</i> <i>Valencia robertae</i> <i>Gambusia holbrooki</i>	Forward primer (5'-3') TGGGGGTTTGGCAACTGAC ATGGCCTTCCCGGAAATGAA GTGCCCCAGACATAGCCTTT	Reverse primer (5'-3') GGAGGAGAGAAACGAGGGGG GCTAAGTTTCCGGCCAGAGG TACAGAAAGTCCGGCATGTG	Amplicon length (in bp) 113 137 167	(Mauvisseu et al. 2020)
8.	Detection of endangered Hay's Spring Amphipod (<i>Stygobromus hayi</i>) and a co-occurring species of <i>S. tenuis</i> in groundwater using species-specific qPCR targeting the COI region.	Species <i>Stygobromus hayi</i> <i>S. tenuis</i>	Forward primer (5'-3') GCATCTGTCGACTTAGCTATT CTGAACAGTATATCCCACT	Reverse primer (5'-3') CGGCACCTGGTCTATAGTTATT CATTCCAGGTCTCCGTATATT A	Probe (5'-3') 6-FAM-TCACCTTATTGACGAGGCTCTCTC-TAMRA -6-FAM-TGCAGTAGCCCATAGTGGAGCATCT-TAMRA	(Niemi et al. 2018)

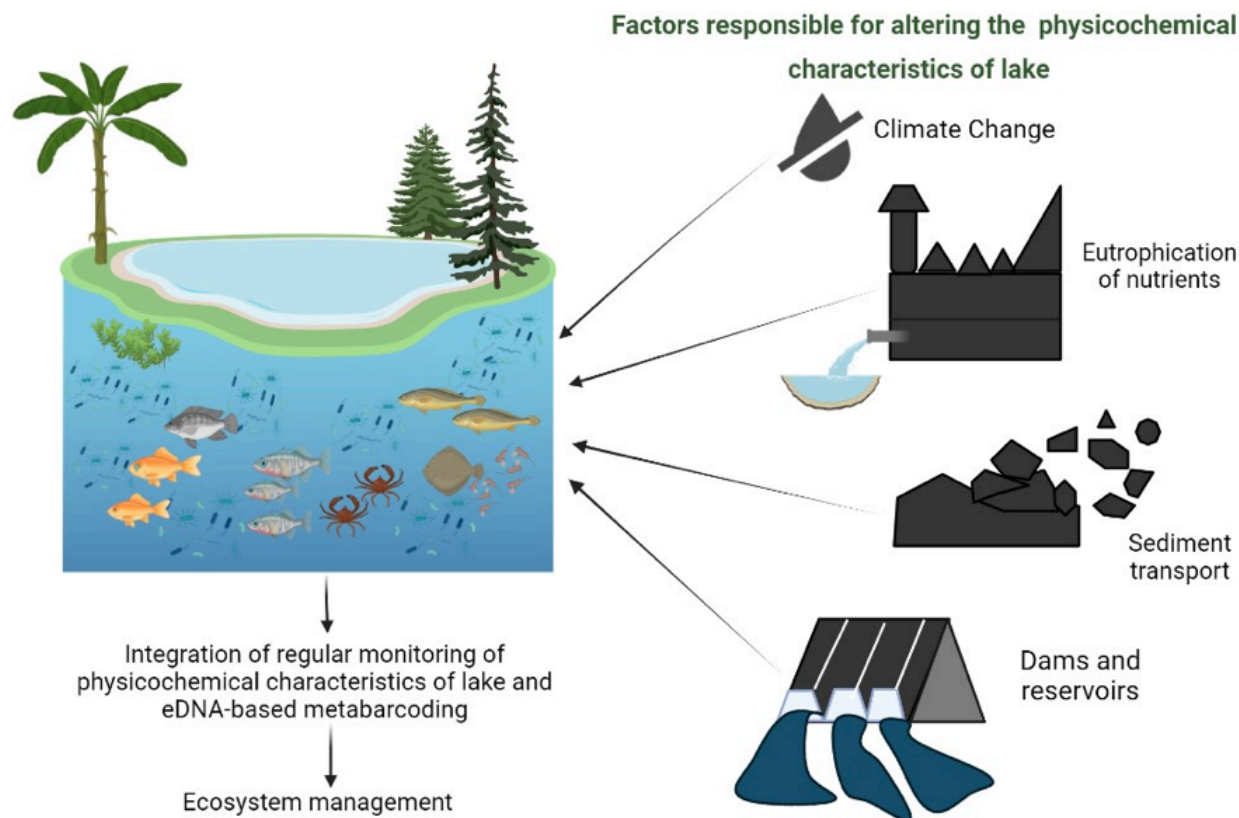


Image 2. The change in physicochemical parameters of the waterbody can be due to either seasonal variations or due to anthropogenic disturbances like the building of dams and reservoirs, sediment transport due to erosion of topsoil or eutrophication of nutrients from industrial and agricultural outflow which lead to subsequent climate change. These changes in physicochemical factors can be monitored along with the changes in species found in the ecosystem using eDNA metabarcoding. Continuous monitoring over all the seasons at a spatial scale might offer a link among these changes in biotic and abiotic components of an ecosystem and hence help in future restoration processes. (Illustration created with BioRender.com)

estimation in a population, a novel NGS based strategy was used which counted haplotypes in the mitochondrial D-loop sequence of eel. This method was named HaCeD-Seq and it was claimed to be better and more accurate in quantification than conventional qPCR. However, its accuracy decreased when the number of individuals increased because of lesser unique haplotypes and more overlap of sequence among individuals (Yoshitake et al. 2019). A much deeper understanding of factors affecting the abundance of eDNA copies in natural environments can help to boost this technology and can be of extreme importance, especially in fisheries management and has direct implications on increasing its economic value.

Though there are substantial volumes of research in this field of eDNA our understanding is still limited. There have been enormous volumes of reports concerning the release and persistence of eDNA in various environments, but there has been no noticeable research on the effect of stressed environments like human activities or predation pressure on the release

rate of eDNA and how it brings changes in our overall understanding of species abundance.

4) Studying the communities in the ecosystem:

Holistic study of ecosystem and metabarcoding gives more inferential insights and hence an upheaval in the use of eDNA has led to transitioning from DNA barcoding to metabarcoding, hence from studying single species to communities and their interactions. This in turn has enabled extracting more information and data using less time and manpower under field conditions.

Understanding ecosystem health in aquatic bodies

In the last few years, a new paradigm has got an increasing focus that aids in the understanding of the health of ecosystems using metabarcoding. This can be accomplished by establishing the link between changing abiotic factors and the ecology of the ecosystem to that of changing biotic interactions among communities inferred from metabarcoding data. Eutrophication is a process of enrichment of nutrients like nitrogen and

phosphorus (Conley et al. 2009) in water bodies. Although natural eutrophication occurs at a very slow pace due to the ageing of water bodies (Carpenter 1981), in the past century cultural eutrophication due to anthropogenic actions has led to rapid nutrient efflux into the water bodies (Smith 1998). Eutrophication is one of the major indicators of anthropogenic means of changing the physicochemical parameters of aquatic bodies along with the construction of dams, channelisation and sediment transport as depicted in Image. 2 (Bianchi & Morrison 2018). This can change the biological productivity and community structure composition of the water bodies (Sawyer 1966). There are manifold effects of eutrophication, algal blooms being the most noticeable of them. The change in Nitrogen (N): Phosphorus (P) ratio or dissolved organic carbon (DOC): dissolved organic nitrogen (DON) has been found as a variable in case of such blooms (Anderson et al. 2002). The major component of these blooms is Cyanobacteria and they produce cyanotoxins which act as neurotoxins and hepatotoxins for fishes, mammals and also humans (Oberemm et al. 1999; Carmichael 2001). Literature search shows that though there are some studies in this area using metabarcoding to find the community structure of bacteria and planktons (Wan et al. 2017; Banerji et al. 2018) in aquatic systems, we find very few studies relating how anthropogenic disturbances might affect the ecosystem services.

One such study by (Craine et al. 2017), showed the relation among the changing environmental variables like dissolved nutrient concentration with four taxonomic groups namely bacteria, phytoplankton, invertebrates, and vertebrates. Further, they found that increasing eutrophication of nutrients and river size were the crucial variables that changed the abundances of these broad taxa. Clark et al. (2020) demonstrated the impact of enrichment with fertilizers on the benthic communities in two estuaries that differed in its environmental attributes. The effect was studied using eDNA metabarcoding on bacterial (16sRNA), eukaryotic (18sRNA) and diatom only (rbcl) communities after seven months of nutrient enrichment. They found that there were clear changes in the case of bacterial and eukaryotic taxa but more obscure in the case of diatoms. Also, they found that these changes could be observed within 150 g N m⁻² of fertiliser treatment, suggesting that early signs of ecosystem degradation could be studied and the restoration process could be initiated using such shifts in the structure of communities as cues. Such methods were used initially for species detection and quantification, now it has been used for

ecosystem assessment and monitoring for its health. The focus on studying community structure as a measure of predicting ecosystem health has advantages as it brings about a holistic view of the same and helps acknowledge the fact how species interdependency is linked to abiotic factors as well. One such study in this regard was by Yang & Zhang (2020), where they used zooplankton community to assess the quality of the ecosystem. They showed across three seasons, i.e., dry, normal and wet, the species detected remained the same but their relative abundances changed at the temporal scale. The study also emphasised that though eDNA based abundance studies are the semi-quantitative presence of species along with changing relative abundances of indicator zooplankton species at spatial-temporal scale. The water quality index correlated with 60 different zooplankton indices which were both qualitative and quantitative. But such correlations need not always be direct/correct due to other confounding factors like interaction with other species communities, which in turn influence the zooplankton community. Such studies aren't limited to only aquatic systems but also have seen recent applications in analysing sediment pollution from coastal regions at a spatiotemporal scale. In a study by Lee et al. (2020), the changes in microbial diversity at phylum level showed variation concerning 13 environmental variables of sediment pollution and toxicity. Although certain phyla remained dominant others showed shifts in community structure.

Whole-genome or metagenome-assembled-genomes (MAGs) based studies

Most of the above-mentioned studies are based on the amplification of the universal marker regions of DNA or amplicon-based 16sRNA sequencing and can have bias during PCR (Jovel et al. 2016). They are based on the single-gene approach of identification of its taxa. One way to solve this is to bring in a multi-gene or whole genome-based taxonomic approach. This also helps in functional prediction of genes like those involved in the biogeochemical cycling by microorganisms and can be of great significance in studying ecosystem services. Another method used in recent times to study taxonomy based on phylogenetic reconstruction is by assembling the metagenomes also called metagenome-assembled genomes (MAGs) and is of immense importance in culture-independent microbial molecular studies. In a study by Tran et al. (2021), the role of specific taxa of microbes in biogeochemical processes in the lake, they had assembled 24 samples individually by de novo method and generated 24 MAGs which were then binned, then

finally used for the construction of concatenated gene phylogeny using single-copy ribosomal proteins. They found that MAGs showed an abundant genomic capacity for nitrogen and sulphur cycling. In another similar work by Reji & Francis (2020), MAGs were constructed for a lineage of Thaumarchaeota, a phylum of Archaea from the marine ecosystem. This lineage seemed to be devoid of genomic repertoire only for chemoautotrophy as it did not have ammonia-oxidising machinery and other pathways related to the same as in other archaeal lineages. This highlights the metabolic diversity among the microbial communities for nutrient acquisition and processing, which is generally not possible in the case of culture-dependent molecular studies as most of the bacteria are non-culturable.

We find studies in eDNA are becoming broader in perspective rather than only species detection, but this holds much more potential in coming years in terms of answering some basic ecological questions about the effect of anthropogenic disturbances that lead to changes in abiotic factors of an ecosystem which changes community structure composition at spatial and temporal scales and threatens ecosystem services and ecosystem health.

Also, there has been very little emphasis on understanding the functional role of eDNA studies i.e., how it can be used to compare eDNA and eRNA and decipher the active constituent of the genome which might have an important role in ecological functioning like genes responsible for biogeochemical cycling of various nutrients in nature. Since RNA has lesser stability than DNA, it is a better and more reliable measure for studying the presence of an organism or its abundance and hence has been used in forensic science to estimate the time since deposition of biological material (Bremmer et al. 2012).

TECHNICAL CHALLENGES OF eDNA-BASED METHODS

Although eDNA technology has provided a plethora of its applications and helped to understand nature in a holistic view, it still suffers from a few challenges which require more refinement and troubleshooting.

PCR Bias

The foremost problem arises in the estimation of relative abundance using a metabarcoding approach where PCR bias serves as a major issue. Those taxa having organisms that are not affected by seasonal variations and are more abundant in number having high dispersal ability tend to be over-represented during sampling than sedentary and seasonal ones.

Even the copy number of target loci may vary among taxa, individuals, or tissue types. There can be several possibilities that can cause bias in PCR amplification during metabarcoding. PCR is a stochastic process hence can become a source of bias like the number of PCR cycles, mismatch in primer binding site, annealing temperature, secondary structures in template DNA, multiple templates in the sample, more selectivity of primers for some specific taxa and copy number of target loci (Pinto & Raskin 2012; Elbrecht & Leese 2015; Fonseca 2018). Nichols et al. (2018), showed that polymerase can show bias toward GC sequence and can alter the relative abundance of molecules dramatically during metabarcoding and that this bias can be removed experimentally using a molecular identifier (MID) where starting material is disambiguated bioinformatically following PCR.

Unknown source of eDNA

There have been reports of transport of undigested material of higher organisms or their dead carcasses, which gives a false implication of their presence at that particular site (Song et al. 2017).

Problems with single-species detection and bias in eDNA extraction protocols

Single species detection in the marine environment is challenging due to increased dilution, higher salinity, and more intermixing of constituents (Cristescu & Hebert 2018). Higher salt concentration can also inhibit PCR and give false implications about the absence of the target organism. Continuous sample collection either monthly or seasonal depending on the research question might serve as a way to overcome false detections. Enrichment of extracellular DNA can help in reducing the signal from non-target microbial cells as they are more abundant in natural ecosystems.

Chances of false positives and false negatives

False positives errors (Type-I) arise when there is no actual presence of the target organism, but still, it is detected at that site which can be due to contamination issues or problems in PCR optimization or sequencing (Schmidt et al. 2013). The specificity of primers also plays a vital role in minimizing picking up related species having very little sequence variance than the target species. False-negative errors (Type-II) arise when a target organism fails to get detected even though it is present there. This can be attributed to reasons like inefficient sample preservation, faulty sampling practices, or less sensitivity of detection assay in the

case of low-abundant organisms.

Measuring the absolute abundance of the species is practically not possible

Factors governing the quantification of eDNA are dependent on countless factors. Many juvenile organisms or a lesser number of adult organisms, might release an equal amount of eDNA. Hence, biomass estimation can be made but estimating abundance can be difficult with PCR-based methods (Elbrecht & Leese 2015). Change in eDNA concentration due to seasonal variation has been reported by many, which can lead to difficulty in estimation of true abundance (Barnes et al. 2014). Maintaining many replicates for PCR and DNA isolation can increase the probability of capturing many taxa by the metabarcoding approach (Leray & Knowlton 2017).

eDNA shedding and decay rates in a particular environment govern the quantification of particular species. In a study by Sassoubre et al. (2016), eDNA decay and shedding rates in seawater mesocosm were assessed for three economically and ecologically important marine fishes- *Engraulis mordax* (Northern Anchovy), *Sardinops sagax* (Pacific Sardine), and *Scomber japonicas* (Pacific Chub Mackerel) by Taqman® qPCR assay. In another similar study, Round Goby *Neogobiusme lanostomus*, an elusive species, was assessed for the shedding and decay rate of eDNA. eDNA shedding was measured after fixed time intervals, and the effect of temperature on shedding rate was also studied. First order decay constants were calculated and the decay rate was found to be slightly lower in cold water than in warm water. A most significant part of the study was that a positive correlation between eDNA concentration and the number of round gobies collected using two capture methods could be established (Nevers et al. 2018). Knowledge about these factors together with factors affecting abundance can act as a lead in abundance estimation studies. The effect of various environmental factors affecting the persistence of eDNA and indirectly the abundance has been shown by (Barnes et al. 2014).

POTENTIAL SOLUTIONS TO THE CHALLENGES:

We have developed a few reflections that might be helpful for future eDNA research:

PCR- free methods

As mentioned in the previous section, PCR introduces several kinds of biases. Hence developing a new methodology to overcome this step during the

metabarcoding approaches can be of immense value in future. Following the same optimized capture and isolation protocols for all collected samples along with maintaining appropriate controls, increasing the number of replicates at each site of sample collection, seasonal collection of samples at the same points throughout the year and developing of PCR-free approach can help to give a picture of near-absolute abundance of organisms. Manu & Umapathy (2021), designed a novel metagenomic workflow which used PCR-free library preparation during Next-generation sequencing (NGS) and performed an ultra-deep sequencing and pseudo taxonomic assignment to get the biodiversity of an ecosystem across the entire tree of life.

Source of eDNA can be both from live and dead organisms: In aquatic systems, transport of eDNA has been observed for tens of kilometres (Andruszkiewicz et al. 2019), hence mere detection of eDNA at a particular time neither confirms the exact location nor the source since eDNA can persist in systems for approximately 48 hours (Collins et al. 2018). A probable way of accounting for this issue is by an increase in both the number of biological and technical replicates as well as sampling continuously for a minimum of three days at the same locations which might add more confidence to the data acquired.

Sampling criteria, filtration of samples and isolation of eDNA protocols

It should be based on the research question. The standardisations of all the protocols should consider the main hypothesis of the research. For example, if the purpose of the research question is only addressed towards deciphering prokaryotic diversity, then all the protocols should be tweaked to get enriched eDNA from that community and also to get maximum diversity of that taxa. This might help to get a better and more focused results. The enrichment of extracellular DNA should be targeted if the question needs studying the entire biodiversity of the system.

Reducing false positives and false negatives

It has been reported that increasing the number of replicates during PCR can minimize the chances of false negatives. The inclusion of positive control during PCR can help check the optimization of PCR conditions. To limit the detection of false absence, the number of replicates should be a minimum of six for a detection probability of 0.5, and for even lower detection probability, a minimum of eight replicates are needed (Ficetola et al. 2015). When both detection probability

and the number of replicates has been too low, it was found that this underestimated occupancy and overestimated the detection rate (Ficetola et al. 2015).

Only relative abundance can be quantified

Since eDNA yield depends on the developmental stage and size of an individual (Petty et al. 2012), mesocosm or aquarium-based studies can be standardised for a particular developmental stage or size of an individual of a species to get an estimate of the actual number of individuals, but mimicking natural environmental conditions of an ecosystem is very difficult and prone to errors. Also, since every ecosystem has its own abiotic and biotic features, the results might not be reproducible.

CONCLUSION

The use of eDNA and its multitude of applications has become a fast-developing area. This outpour comes in the light of the increasing need to monitor changes in our environment and how living organisms are affected by them. This helps to have better conservation focus on regions or species of special importance. In this era of unprecedented climate change and the concerns possessed by it, eDNA can help assist in the monitoring of biodiversity alongside other conventional methods to yield better results. Any new technology calls for new challenges and room for improvement, so is with eDNA where chances of contamination and bias for the detection of abundant species are higher. But with more stringent methodology and computational advancements, the risks are getting minimised. It has the potential to answer many deeper questions of research in this area.

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Dr. John Noyes, Natural History Museum, London, UK
 Dr. Albert G. Orr, Griffith University, Nathan, Australia
 Dr. Sameer Padhye, Katholieke Universiteit Leuven, Belgium
 Dr. Nancy van der Poorten, Toronto, Canada
 Dr. Kareen Schnabel, NIWA, Wellington, New Zealand
 Dr. R.M. Sharma, (Retd.) Scientist, Zoological Survey of India, Pune, India
 Dr. Manju Siliwal, WILD, Coimbatore, Tamil Nadu, India
 Dr. G.P. Sinha, Botanical Survey of India, Allahabad, India
 Dr. K.A. Subramanian, Zoological Survey of India, New Alipore, Kolkata, India
 Dr. P.M. Sureshan, Zoological Survey of India, Kozhikode, Kerala, India
 Dr. R. Varatharajan, Manipur University, Imphal, Manipur, India
 Dr. Eduard Vives, Museu de Ciències Naturals de Barcelona, Terrassa, Spain
 Dr. James Young, Hong Kong Lepidopterists' Society, Hong Kong
 Dr. R. Sundararaj, Institute of Wood Science & Technology, Bengaluru, India
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 No. 12, Thiruvannamalai Nagar, Saravanampatti - Kalapatti Road,
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