MACHEREY-NAGEL

Technical Note on eDNA isolation from aquatic systems

Things to consinder for the isolation of eDNA from aquatic ecosystems - tips, tricks, and references

This technical note aims to support all those seeking to isolate eDNA from aquatic ecosystems, e.g. by use of NucleoSpin eDNA Water kit or other methods. It gives tips and tricks to consider and an access to a selected number of references from a fast growing wealth of publications covering technical as well as application aspects of eDNA.

What is environmental DNA (eDNA)?

Any DNA which is released from organisms into the environment (air, water, soil) can be considered as environmental DNA (eDNA). For aquatic systems, eDNA can be derived from e.g. cells released into the water (e.g. epithelial cells, spawn) or decaying organisms or tissues. eDNA might be pieces of tissue, cells, organelles (e.g. nuclei, mitochondria), protein bound DNA (e.g. DNA histone complexes) or it might be secondarily adsorbed by mineral substances (e.g. clay, sand) or organic matter (e.g. cell walls, lignins, humic substances). The major amount of eDNA seems to be bound to particles and only a rather minor fraction of eDNA seems to float free without being associated to any other structure within the water. Typically, eDNA is found in particles in the range over 0.2–200 µm. eDNA in aquatic systems is subject to degradation due to physical, chemical and biological processes with observed half lifes of a few to several days, depending on water quality, temperature, UV and other factors.

Sampling strategy

Depending on the aim of the investigation planned to do by using eDNA, the following points should be considered:

- Number of samples
- Volume of water per sample or subsample
- Pooling of subsamples
- Longitudinal, cross-sectional, or vertical sectioning of the waterbody
- Season of sampling

Literature	
Grey *et al.* 2018	Effects of sampling effort on biodiversity patterns estimated from environmental DNA metabarcoding surveys
Cantera *et al.* 2019	Optimizing environmental DNA sampling effort for fish inventories in tropical streams and rivers
Wilcox *et al.* 2016	Understanding environmental DNA detection probabilities: A case study using a stream-dwelling char Salvelinus fontinalis
Dickie *et al.* 2018	Towards robust and repeatable sampling methods in eDNA-based studies
Beentjes *et al.* 2019	The effects of spatial and temporal replicate sampling on eDNA metabarcoding
Civade *et al.* 2016	Spatial Representativeness of Environmental DNA Metabarcoding Signal for Fish Biodiversity Assessment in a Natural Freshwater System
Sato *et al.* 2017	Usefulness and limitations of sample pooling for environmental DNA metabarcoding of freshwater fish communities

Season of sampling

For areas with distinct seasons consider the following points. For e.g. Europe take into account:

- Winter: some user of eDNA prefer winter sampling, however activity of the targeted organismes has to be considered. Risk of filter clogging is typically lower than for spring and summer samples.
- Spring and summer: Filtration is likely to be more difficult than for winter samples due to the higher filter clogging risks caused by typically increased numbers of microorganisms (e.g. bacteria, fungi, algae, protozoen).
- Fall: Due to increased amounts of rotting vegetation, water samples might contain highly increased amounts of inhibitory substances (e.g. humins, tannins) which might be co-purified. For eDNA eluates which might be contaminated with such inhibitor (often recognizable by a brownish color) a clean up can be advisable (e.g. by NucleoSpin Inhibitor Removal Kit, REF 740408.50).

Literature	
De Souza *et al.* 2016	Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms
Dunn *et al.* 2017	Behavior and season affect crayfish detection and density inference using environmental DNA
Rees *et al.* 2017	The detection of great crested newts year round via environmental DNA analysis
Wacker *et al.* 2019	Downstream transport and seasonal variation in freshwater pearl mussel (Margaritifera margaritifera) eDNA concentration
Wittwer *et al.* 2018	Comparison of two water sampling approaches for eDNA-based crayfish plague detection

Sampling volume – water volume per sample point

With respect to temporal, physical, and chemical factors influencing eDNA detectability in ecosystems, either filtration of several hundreds of milliliter up to a several liter of water sample or a direct precipitation method from e.g. up to 50 mL can be considered.

Literature	
Mächler *et al.* 2016	Fishing in the Water: Effect of Sampled Water Volume on Environmental DNA-Based Detection of Macroinvertebrates
Petra *et al.* 2019	An optimised eDNA protocol for detecting fish in lentic and lotic freshwaters using a small water volume
Hunter 2019	Improving eDNA yield and inhibitor reduction through increased water volumes and multi-filter isolation techniques

As described in Moushomi *et al.* 2019 (Nature Scientific Reports) isolation of eDNA directly from a water sample without filtration might be a good choice for some applications. However, this method is not commonly used.

Literature	
Moushomi 2019	Environmental DNA size sorting and degradation experiment indicates the state of *Daphnia magna* mitochondrial and nuclear eDNA is subcellular

Storage – transport – conservation

Several storage and conservation methods have been described.

- Cooling / freezing of water samples: Is possible for a limited time and described in several publications, however is rather inconvenient, if water sample have to be transported.

Immediate filtration and conservation of eDNA on the filter by

- drying the filter and storage under desiccante
- storage in ethanol
- freezing of the filter

Is recently a commonly used method. We recommend the treatment of the round filter with several milliliter of ethanol immediately after the water filtration process in order to remove residual water and subsequent storage of the ethanol-wet filter in e.g. a 5 mL tube.

Literature	
Ladell *et al.* 2018	Ethanol and sodium acetate as a preservation method to delay degradation of environmental DNA.
Minamoto *et al.* 2016 (ethanol treatment for preserving eDNA)	Techniques for the practical collection of environmental DNA: filter selection, preservation, and extraction.
Majaneva *et al.* 2018 (four different filter preservation methods)	Environmental DNA filtration techniques affect recoverd biodiversity.
Geerts *et al.* 2018 (filter storage at -20 °C)	A search for standardized protocols to detect alien invasive crayfish based on environmental DNA (eDNA): A lab and field evaluation
Pilliod *et al.* 2013 (filter storage in 95 % ethanol)	Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples
Hinlo *et al.* 2017 (filter storage at -20 °C or in ethanol at room temperature)	Methods to maximise recovery of environmental DNA from water samples
Carim *et al.* 2016 (filter storage dry with dessicant)	A protocol for collecting environmental DNA samples from streams

Choice of purification method

Filtration

Recently the most common and widespread method for eDNA isolation. It enables the purification of eDNA from large volumes of water. Small, free, nonbound DNA might be lost, because most filter materials do not have the inherent abilty to bind free DNA. Filtration typically allows isolation of particle bound DNA.

Literature	
Tsuji *et al.* 2019	The detection of aquatic macroorganisms using environmental DNA analysis – a review of methods for collection, extraction, and detection

Direct precipitation

Even though the direct precipitation is probably the first method described for eDNA isolation (Ficetola *et al.* 2008), publications describing direct precipitation (e.g. Doi *et al.* 2017, Dougherty 2016) are outnumbered by publications describing a filtration method. This is likeley to be due to the limitations of sample volume for precipitation methods.Precipitation of eDNA from 30 mL water samples by isopropanol requires 50 mL centrifugation buckets. Direct precipitation will be advantageous for isolation of smal, free, nonbound DNA.

Literature	
Doi *et al.* 2017	Isopropanol precipitation method for collecting fish environmental DNA.
Ficetola *et al.* 2008	Species detection using environmental DNA from water samples.
Dougherty *et al.* 2016	Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances.
Renshaw *et al.* 2014	The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol DNA extraction

Direct sedimentation of eDNA by centrifugation

- Used for eDNA isolation due to impracticability of centrifugation of large water volumes.

Choice of filter material and pore size

Numerous filter materials (CN, MEC, RC, glass fiber, PCM, PES) and pore sizes have been successfully used. Initially, membrane filter dominated the literature (approx. 2017–2018) but subsequently glass fiber filter were catching up (Tsuji *et al.* 2019) because of there superior filtration properties, while still catching high amounts of eDNA. Besides pore size, material quality and thickness influence filtration speed, clogging risk, and retention of eDNA and inhibitors. Membrane materials tend to clogg faster than paper materials, due to there complete lack of any depth filter effect (e.g. membrane compared to glass fiber filter).

Literature	
Li *et al.* 2018	The effect of filtration method on the efficiency of environmental DNA capture and quantification via metabarcoding.
Minamoto *et al.* 2016	Techniques for the practical collection of environmental DNA: filter selection, preservation, and extraction.
Tsuji *et al.* 2019	Techniques for the practical collection of environmental DNA: filter selection, preservation, and extraction.

For membrane filter, brittle materials like nitrocellulose should be avoided to prevent filter cracking and risk of water bypassing during filtration. Further, membrane filters tend to clog much faster compared to glass fiber filters.

Glass fiber filter usually require somewhat larger volume of buffer for eDNA release from the filter due to the larger water absorption capacity compared to membrane filter, which has to be considered during eDNA release.

Glass fiber filter can be ethylene oxide treated. Ethylene oxide treatment is recently the state of the art for minimizing DNA contamination risks (Ref. Shaw *et al.* 2008).

Literature	
Shaw *et al.* 2008	Comparison of the effects of sterilization techniques on subsequent DNA profiling.

Ethylen oxide treated glass fiber filter are commercially available (Glass Fiber Filter 45 mm diameter, EO treated, MN REF 740564).

Quantification of eDNA by spectrophotometry

Obtained eDNA yield values might be affected by copurified substances (e.g. humic substances, polyphenolics) leading to possible over-quantification and a low purity indicator (ratio A_{260}/A_{230}). Some type of contaminations are readily visualized by their color (e.g brownish humic substances) whereas others are uncoloured (e.g. some polyphenolics).

Quantification of eDNA by fluorescent methods

Due to strong UV-absorbent proterties and fluorescent properties of some contaminants (e.g. humic substances, polyphenols) quantification via fluorescent methods (e.g. PicoGreen fluorescent dye or similar) might be affected leading to erroneous results. Overquantification and underquantification (e.g. by intrinsic fluorescence or by UV absorption of the substance) might occur. For influence of humic acid on SYBR Green I and other DNA-fluorecend dye complexes see:

Literature	
Zipper H. 2004	Entwicklung analytisch-molekularbiologischer Verfahren zur Konstruktion einer Plasmid-Genbank aus Boden-DNA in Escherichia coli und deren Durchmusterung nach neuen Enzymen für die technische Anwendung. Dissertation Universität Stuttgart, *https://elib.uni-stuttgart.de/handle/11682/1674.*

Analysis of eDNA by gel electrophoresis

Analysis of eDNA by gel electrophoreis reveals, that – sample dependend – eDNA might show a size distribution from several dozen bp up to several kb. Size distribution strongly depends on water quality, time of sampling, delay of processing after sampling, purification method.

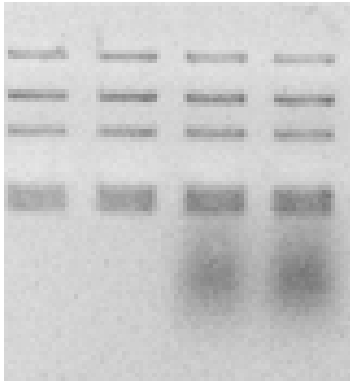

Fig. 1: TAE-agarose gel electrophoresis of DNA ladder (lane 1 and 2) and a DNA ladder spiked with humic acid (lane 3 and 4). Humic acid shows clear fluorescent signal in the low molecular range.

Due to UV-absorbent and fluorescent properties of some possible contaminants (e.g. humic substances), gel pictures might be misinterpreted. In an ethidium bromide stained TAE-agarose gel, e.g. humic acid appears as DNA in the size range of few dozen to several hundreds of base pairs (see figures below).

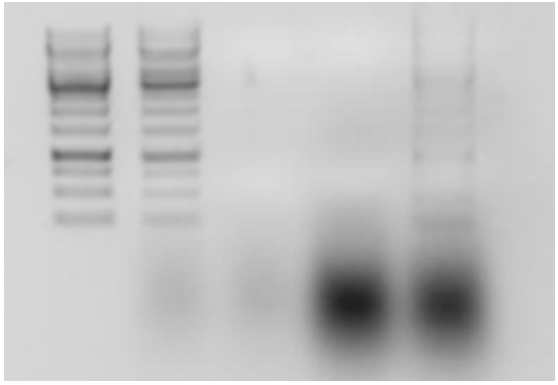

Fig. 2: TAE gel electrophoresis of 1 kb DNA ladder (lane 1); DNA ladder spiked with small amount of humic acid (lane 2); low concentrated humic acid (lane 3); humic acid in higher concentration (lane 4); 1 kb DNA ladder with higher concentrated humic acid (lane 5).

Moreover, humic substances can shield DNA fluorescence in an ethidium bromide stained gel, as shown in the following figure

Further, humic acid can cause electromobility shifts of DNA during gel electrophoretical analysis of DNA, as depicted in the following publication:

Literature	
Zipper H. 2004	Entwicklung analytisch-molekularbiologischer Verfahren zur Konstruktion einer Plasmid-Genbank aus Boden-DNA in Escherichia coli und deren Durchmusterung nach neuen Enzymen für die technische Anwendung. Dissertation Universität Stuttgart, *https://elib.uni-stuttgart.de/handle/11682/1674*.
Zipper H. 2004	Thus, humic substances contaminated DNA preparations should be analyzed with care. However, humic substance contaminated DNA preparations can be easily recognized by their brownish colour and can be cleaned up with e.g. NucleoSpin Inhibitor Removal Kit Ref 740408.50.

On the other hand, colourless, UV-absorbing and non-fluorecent contaminants (e.g. polyphenolics) might shield UV-irradiation causing underestimation of DNA amount in the gel.

Analysis by Bioanalyzer and Fragment Analyzer

Analysis might be affected by UV-absorbent and fluorescent properties of some possible contaminants.

PCR – qPCR

Inhibitor PCR controls are highly recommended to show that the sample eluate is a usable template for PCR analysis with negligible or acceptable PCR inhibition.

Literature	
Sidstedt *et al.* 2015	Humic substances cause fluorescence inhibition in real-time polymerase chain reaction. Analytical Biochemistry, Vol 487, pp. 30–37.

Negative PCR controls are highly recommended to show that PCR is not contaminated by e.g. previously generated amplicons.