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Methodology Article

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Environmental DNA Metabarcoding for Monitoring Fish Diversity in Turbid Estuaries

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Abstract

Estuaries are among the hardest places in which to survey fish. Visibility is poor, nets are selective and damaging, and the assemblage changes with every tide. Metabarcoding of environmental DNA, in which fish are detected from genetic material shed into the water, avoids several of these difficulties, but the standard protocols were developed in clear lakes and coastal seas. Suspended sediment clogs filters after small volumes, humic substances carried over from extraction inhibit amplification, and tidal mixing complicates the link between a detection and the place where a fish lives. This article proposes an eight-step procedure for estuarine fish monitoring that addresses these problems in turn: definition of sampling strata by salinity and tide, an audit of the reference library, a filtration pilot, field sampling with controls, extraction with an explicit inhibition test, replicated amplification of a short 12S marker, conservative bioinformatic filtering, and analysis by occupancy models that separate presence from detectability. Each step is described with its rationale in the published literature. The procedure is a proposal. It has not been applied or validated here, and the article sets out what a validation against conventional netting would need to show.

Keywords: *Environmental DNA, Metabarcoding, Estuarine fish, Turbidity, PCR inhibition, Occupancy modelling*

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1. Introduction

Fish assemblages in estuaries are monitored for many reasons: to judge ecological status under water quality legislation, to follow nursery function for commercial species, and to detect arrivals of non-native fish. The conventional tools are seine, fyke and trawl nets. In muddy, tidal water each of these catches a different fraction of the fauna, none can be used over soft mud, mangrove roots or oyster reef without difficulty, and all cause mortality among juveniles that the survey is meant to protect.

Environmental DNA (eDNA) metabarcoding offers a different route. Water is filtered, the DNA retained on the filter is extracted, a short gene region shared by the target group is amplified with general primers and sequenced in bulk, and the sequences are matched to a reference library (Deiner et al., 2017).

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The approach has given species lists for fishes that match or exceed those from established methods in lakes and rivers (Valentini et al., 2016), and in an urban estuary it tracked the seasonal turnover of the fish fauna (Stoeckle et al., 2017).

Turbid estuaries nevertheless strain the method at several points, and practitioners who follow a clear-water protocol unchanged are likely to obtain small sample volumes, failed amplifications and results that are hard to interpret. This article proposes a procedure designed for these conditions. It is assembled from published methods and from the general guidance on eDNA practice (Goldberg et al., 2016). It is not a report of its application, and no field results are presented.

2. Why Turbid Estuaries Are Difficult

Three properties of estuarine water matter. The first is the load of suspended particles. Much of the eDNA of fish and other animals occurs in or on particles larger than about one micrometre (Turner et al., 2014), so it is captured by filtration, but the silt, clay and phytoplankton that are captured with it block the filter. In turbid water a fine membrane may pass only a small fraction of the volume routinely filtered in clear water, and the amount of target DNA collected falls accordingly (Williams et al., 2017).

The second is chemistry. Estuarine sediments and the rivers that feed them are rich in humic and fulvic acids, which are co-extracted with DNA and inhibit the polymerase used in amplification. An inhibited reaction yields nothing, and unless the inhibition is recognised the result is read as an absence. Dilution of the extract and passage through a purification column both relieve inhibition, the former at the price of diluting the target (McKee et al., 2015).

The third is movement and persistence. Tidal currents carry DNA away from its source, the salinity and temperature gradients that structure an estuary also affect how fast DNA decays (Barnes and Turner, 2016), and decay rates differ between inshore and offshore waters (Collins et al., 2018). Sediment adds a further complication. Fish DNA is more concentrated and longer lived in bottom sediment than in the water above it (Turner et al., 2015), so resuspension by tides, wind or boat traffic may return old DNA to the water column. Table 1 summarises these problems and the countermeasure proposed for each.

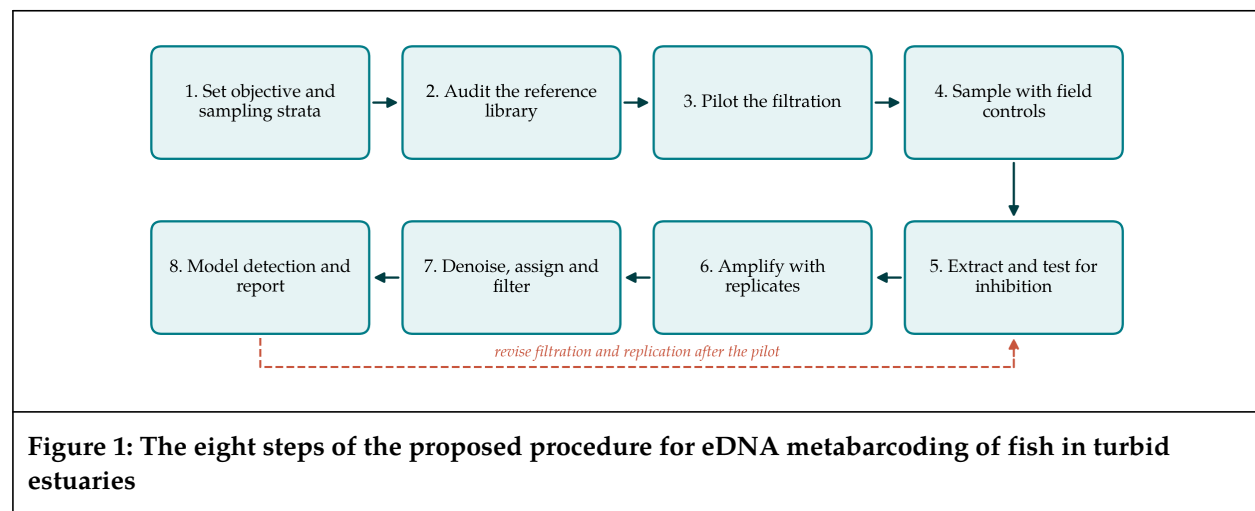
Table 1: Problems posed by turbid estuarine water for eDNA metabarcoding and the countermeasures built into the proposed procedure			
Problem	Consequence if ignored	Countermeasure	Step
Filter clogging	Small volumes and missed rare species	Larger pore size or pre-filter; several filters pooled per sample	3
Humic inhibitors	False absences from failed reactions	Inhibitor-removal extraction, internal control, dilution series	5
Tidal transport	Detections far from the source	Fixed tidal state; strata defined by salinity	1
Resuspended sediment	Signal from fish no longer present	Mid-water sampling away from the bed; record turbidity	4
Gaps in the reference library	Species unassigned or misassigned	Audit against the regional checklist; sequence vouchers	2
Low template	Patchy amplification among replicates	Multiple PCR replicates analysed with occupancy models	6, 8

3. The Proposed Procedure

The procedure has eight steps, shown in Figure 1. The first three are preparatory and are done once for an estuary; the remaining five are repeated at every survey. A return loop from the pilot allows the filtration and replication to be revised before routine sampling begins.

3.1. Step 1: Set the objective and the sampling strata

The design follows from the question. A species inventory calls for wide spatial coverage; trend monitoring calls for fixed stations revisited under the same conditions. In either case the estuary should be divided into strata along the salinity gradient, conventionally the oligohaline, mesohaline and polyhaline reaches, with stations in the main channel and in marginal habitats within each. To limit the influence of tidal transport, all stations should be sampled at the same tidal state. Low water slack has the advantage that the water at a station has spent longest within the estuary, although it is also when turbidity near the bed tends to be highest.



3.2. Step 2: Audit the reference library

A sequence can only be named if the species has been sequenced for the marker in use. Before any sampling, the regional checklist of estuarine and coastal fishes should be compared with the public sequence databases for the chosen marker, and the proportion of species covered recorded. Species that are missing, and groups of close relatives that share an identical sequence over the marker, should be listed so that their absence from the results is not mistaken for absence from the estuary. Where resources allow, tissue from locally caught voucher specimens should be sequenced to fill the gaps.

3.3. Step 3: Pilot the filtration

No single filter suits every estuary, so a short pilot is proposed at the most and least turbid stations. Candidate arrangements include a fine membrane used alone, a coarser membrane, and a pre-filter ahead of a fine membrane. For each, the volume passed before the flow stalls and the total DNA recovered are recorded. Work on particle size indicates that pore sizes somewhat above those usual in clear water still retain most microbial eDNA while passing far more water (Turner et al., 2014). Enclosed capsule filters reduce handling and the contamination that goes with it (Spens et al., 2017). Where clogging is severe, several filters from the same water sample can be extracted and pooled. The arrangement chosen is then fixed for the programme.

3.4. Step 4: Sample with field controls

At each station, at least three replicate water samples are taken from mid-water, avoiding disturbance of the bed, with single-use or bleach-sterilised equipment. Turbidity, salinity, temperature and the volume filtered are recorded for every replicate, since each will be needed to interpret detection rates. A field blank of clean water is filtered at every sampling occasion with the same equipment. Filters are preserved at once, either by freezing or in a lysis buffer that is stable at ambient temperature, an option that suits remote sites (Renshaw et al., 2015).

3.5. Step 5: Extract and test for inhibition

Extraction should use a kit or protocol that includes an inhibitor-removal stage, with an extraction blank

in every batch. Inhibition is then tested directly and not assumed to be absent. A known quantity of a synthetic or non-native control template is added to an aliquot of each extract and amplified by quantitative PCR; a delay in amplification compared with the control in clean water indicates inhibition. Affected extracts are cleaned again or diluted, and the test repeated (McKee *et al.*, 2015). The outcome of the test is reported for each sample.

3.6. Step 6: Amplify with replicates

Short fragments of the mitochondrial 12S ribosomal RNA gene are the usual markers for fishes because degraded DNA still carries them. The MiFish primers (Miya *et al.*, 2015) and the shorter teleo primers (Valentini *et al.*, 2016) are both widely used; the choice should follow the library audit in Step 2. Each extract is amplified in several independent PCR replicates, because detection of rare templates is uneven and a single reaction understates what is present (Ficetola *et al.*, 2015). Negative PCR controls and a positive control made up of species that cannot occur locally are carried through sequencing. Samples are labelled with unique combinations of indexes so that reads transferred between samples during library preparation can be recognised (Schnell *et al.*, 2015).

3.7. Step 7: Denoise, assign and filter

Reads are trimmed of primers, quality filtered and resolved into exact sequence variants with a denoising algorithm such as DADA2 (Callahan *et al.*, 2016). Variants are assigned to species only when the match to the reference is close and unambiguous, and otherwise to genus or family. Thresholds for accepting a detection are set from the controls: the read counts that appear in blanks and the rate at which positive-control species appear in other samples define the level below which a detection is discarded. Non-target reads from humans and from food fish, which are common in urban estuaries through sewage, are flagged and not silently removed.

3.8. Step 8: Model detection and report

The data that result are detections and non-detections of each species in each PCR replicate of each water sample at each station. This nested structure suits site occupancy models, which estimate the probability that a species is present separately from the probability of detecting it when present (MacKenzie *et al.*, 2002), and which have been adapted for eDNA surveys (Schmidt *et al.*, 2013). Turbidity and volume filtered enter as covariates of detection, so their effect is estimated and not ignored. Read counts are reported but are not interpreted as abundance. The report states the controls run and their results, the coverage of the reference library and the taxa that could not be resolved.

4. Fitting the Procedure to a Monitoring Programme

A programme adopting the procedure would need to settle several practical matters. Cost is driven mainly by the number of PCR replicates and by sequencing depth, and the pilot gives the information needed to choose both. Field teams need little specialised training, but the laboratory must separate its pre-amplification and post-amplification work physically, as recommended in general guidance (Goldberg *et al.*, 2016). Consistency between years depends on holding the marker, the filter arrangement, the tidal state and the bioinformatic thresholds constant, and on archiving extracts so that samples can be re-run if methods change.

The method complements netting and does not replace it. eDNA gives no information on size, age, condition or reproductive state, which estuarine assessments often require. A sensible arrangement is to use eDNA for broad and frequent coverage of species occurrence and to keep a reduced netting programme for the demographic information that only specimens provide.

5. Limitations and What Remains to Be Shown

The procedure is proposed and has not been validated here. Its components are each supported by published work, but most of that work was done in fresh or clear coastal water, and their combined performance in highly turbid estuaries is untested. A validation would pair eDNA sampling with multi-gear netting at the same stations and tides over at least one annual cycle, and would report the

species detected by each method, the detection probabilities estimated for eDNA, and how both vary with turbidity.

Some uncertainties cannot be designed away. The spatial resolution of a detection in a tidal system is coarse, and a species recorded at a station may have been some distance upstream or downstream. The contribution of resuspended sediment to the signal has not been quantified for estuaries. Closely related species, for example among mullets, gobies and some clupeids, may not be separable with a short 12S fragment. Users of the results should be told of these limits in plain terms.

6. Conclusion

Turbidity does not rule out eDNA metabarcoding as a tool for monitoring estuarine fish, but it changes what a sound protocol looks like. The eight steps proposed here place a filtration pilot, an explicit inhibition test, replicated amplification and occupancy modelling at the centre of the design, and tie sampling to salinity and tide. Whether the procedure delivers reliable species lists in practice remains to be demonstrated by comparison with conventional surveys.

Conflict of interest

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