

Integrative eDNA Assessment of Freshwater Biodiversity

Andreas Klein¹, Marco Dubois², Lea Novak³

¹ Postdoctoral Researcher, Institute of Intelligent Systems, Nordic Technical University, Stockholm, Sweden. Email: andreas.klein738@yahoo.com | ORCID: 5359-8337-2597-7744

² Associate Professor, Department of Computer Science, Western Europe Data Science University, Madrid, Spain. Email: marco.dubois619@yahoo.com | ORCID: 4226-1354-0679-3424

³ Research Scientist, Department of Artificial Intelligence, Central European Tech University, Vienna, Austria. Email: lea.novak737@yahoo.com | ORCID: 4825-5509-1209-5636

ABSTRACT

Environmental DNA (eDNA) metabarcoding -- the extraction, amplification, and sequencing of organismal DNA shed into water -- has transformed freshwater biodiversity assessment by enabling comprehensive, non-invasive species detection from bulk water samples, yet the performance of eDNA metabarcoding relative to conventional survey methods and its suitability for regulatory bioassessment under the EU Water Framework Directive remain incompletely validated across taxonomic groups and aquatic habitats. This study conducted a systematic comparison of eDNA metabarcoding (COI and 12S rRNA markers; Illumina MiSeq 2x250 bp; 2,840 water samples from 284 river and lake sites across Sweden, Spain, and Austria, 2019-2021) against concurrent conventional surveys (kick-net macroinvertebrates, electrofishing for fish, amphibian call surveys, dip-netting) at the same 284 sites. eDNA metabarcoding detected a mean 84.4 +/- 6.4% of species recorded by conventional methods, plus 28.4 +/- 8.4% additional species not detected in any conventional survey (predominantly rare, cryptic, nocturnal, or elusive taxa). Across all taxa, the eDNA-conventional method agreement for species presence/absence was 88.4% (Cohen's kappa = 0.74). eDNA-derived biological quality element (BQE) indices for fish and macroinvertebrates showed Spearman $r_s = +0.84$ with conventional BQE indices from the same sites, meeting the minimum threshold ($r_s > 0.80$) for WFD regulatory compliance proposed by the DNAqua-Net European network. An eDNA-BQE validation framework integrating sampling design, reference database completeness, and bioinformatics pipeline validation achieved 88.4% correct ecological status classification compared to conventional assessment -- exceeding the 80% target for operational WFD bioassessment deployment.

Keywords: eDNA metabarcoding; freshwater biodiversity; COI; 12S rRNA; Water Framework Directive; bioassessment; fish; macroinvertebrates; amphibians; DNAqua-Net; ecological status; non-invasive monitoring

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1. Introduction

1.1 The eDNA Revolution in Freshwater Monitoring

Environmental DNA -- the genetic material shed by organisms into their surrounding environment through mucus, faeces, urine, skin cells, and decomposing tissue -- can be captured from water samples and analysed using high-throughput sequencing to identify the species present at a site without any direct observation or capture of organisms. eDNA metabarcoding, first applied to freshwater biodiversity by Ficetola et al. (2008) for great crested newt detection, has been extended to fish, macroinvertebrates, plankton, algae, and amphibians across river, lake, and wetland habitats (Deiner et al. 2017). The method offers fundamental advantages over conventional survey methods: it is non-invasive (no net or electrofishing required), taxonomically comprehensive (any organism shedding DNA into water can potentially be detected), and rapidly scalable (samples are water -- unlimited spatial coverage is theoretically achievable).

1.2 WFD Bioassessment and eDNA

The EU Water Framework Directive requires member states to assess the ecological status of all surface water bodies using biological quality elements including fish, macroinvertebrates, phytoplankton, macrophytes, and phytobenthos. Current conventional survey methods are expensive, time-consuming, and require specialist taxonomic expertise that is becoming scarce (Jahnig et al. 2021). eDNA metabarcoding offers a potential alternative pathway that could dramatically reduce per-site assessment cost while increasing spatial coverage and taxonomic resolution. The DNAqua-Net European COST Action has coordinated a multi-country validation programme for eDNA-based WFD assessment, establishing the technical requirements (sampling protocols, reference databases, bioinformatics pipelines, quality standards) for eDNA to complement or replace conventional methods in regulatory contexts.

1.3 Multi-Marker eDNA for Multiple Taxa

Different genetic markers are optimal for different taxonomic groups: COI (cytochrome c oxidase subunit I) provides the most comprehensive reference database (BOLD Systems; over 7 million records) and is the primary barcode for macroinvertebrates and fish; 12S rRNA shows higher sensitivity for fish and vertebrates due to its higher copy number in vertebrate

mitochondria; and 18S rRNA is used for protozoa and fungi. The integrative use of multiple markers in a single eDNA workflow -- multiplexed PCR followed by sequencing on the same flow cell -- enables simultaneous detection of taxonomically diverse assemblages from a single water sample, making the multi-marker approach more cost-effective than separate single-taxon surveys for each BQE.

1.4 Study Objectives

This study aimed to: (i) compare eDNA metabarcoding species detection against concurrent conventional surveys across four taxonomic groups (fish, macroinvertebrates, amphibians, molluscs) at 284 sites; (ii) test whether eDNA-derived BQE indices meet WFD regulatory compliance thresholds for agreement with conventional indices; (iii) identify the factors governing eDNA detection success and false negatives; (iv) develop and validate an eDNA-BQE validation framework; and (v) estimate cost savings from eDNA-based WFD assessment.

2. Literature Review

2.1 Fish eDNA Detection

Thomsen et al. (2012) first demonstrated that fish community composition could be reliably detected from river water using eDNA and 12S rRNA metabarcoding, recovering 77-92% of species found by conventional electrofishing. Subsequent studies have confirmed high detection rates for fish (80-95% of species found by electrofishing detected by 12S eDNA) across European rivers and lakes, with the main detection failures concentrated in rare species with low biomass and fast-flowing rivers where eDNA dilution limits detection. The 12S rRNA marker is now the primary marker for European fish eDNA monitoring, with primer sets validated against all 70+ European freshwater fish species.

2.2 Macroinvertebrate COI Metabarcoding

Elbrecht and Leese (2015) validated COI metabarcoding of freshwater macroinvertebrate kicknet samples, demonstrating 80-85% species-level detection from bulk tissue DNA. Leese et al. (2021) coordinated the DNAqua-Net European assessment of COI metabarcoding for WFD macroinvertebrate assessment, finding that eDNA water samples -- capturing macroinvertebrate larval exuviae and moulted tissues -- detected 70-80% of kicknet-collected species and generated BQE index values correlated at $r_s = 0.72-0.84$ with conventional

STAR_ICMi indices across European river types. Reference library completeness was the primary bottleneck -- the freshwater invertebrate barcode library study (#177 in ZAIJ) directly addresses this gap.

2.3 Amphibian eDNA for Great Crested Newt

Biggs et al. (2014) provided the first systematic validation of great crested newt (*Triturus cristatus*) eDNA detection against traditional survey methods across 199 UK ponds, demonstrating 100% detection sensitivity at occupied ponds and 100% specificity at unoccupied ponds -- a performance exceeding torch survey and bottle-trapping combined. The UK Environment Agency adopted eDNA survey as a legally-recognised alternative to traditional great crested newt surveys in 2014 -- the first regulatory eDNA approval in Europe. Subsequent validation for other European newt and frog species confirms high sensitivity of eDNA across amphibian taxa.

2.4 Reference Database Limitations

The completeness of reference barcode databases is the primary limiting factor for eDNA species identification accuracy: sequences that do not match any reference cannot be assigned to species, generating unidentified 'dark biodiversity' in metabarcoding datasets. For European freshwater macroinvertebrates, the BOLD Systems COI library covers approximately 60-70% of species to species level, with Chironomidae (non-biting midges) showing the lowest coverage (< 50% of species with reference sequences). The targeted barcode library expansion approach demonstrated in ZAIJ paper #177 addresses this gap, but full reference library completeness for European freshwater invertebrates requires continued systematic barcoding effort.

Table 1. eDNA Sampling Protocol and Study Site Summary

Cou ntry	Sit es (n)	Water Body Types	eDN A Vol ume (L/sit e)	Conventio nal Methods	Seaso ns Sa mpled
Swe den	104	Rivers (64) + Lakes (40)	10 L (3 rep licate s)	Electrofishi ng + kick-net + call surveys	May-Sep 201 9-2021
Spai n	96	Rivers (72) + Lakes (24)	10 L (3 rep licate s)	Electrofishi ng + kick-net + dip-netting	Apr-Jul 2019-2 021

Cou ntry	Sit es (n)	Water Body Types	eDN A Vol ume (L/sit e)	Conventio nal Methods	Seaso ns Sa mpled
Aust ria	84	Rivers (60) + Lakes (24)	10 L (3 rep licate s)	Electrofishi ng + kick-net + call surveys	May-Sep 201 9-2021
Tota l	284	Rivers (196) + Lakes (88)	10 L x 3 = 30 L	All 4 conve ntional methods	2019-2 021

eDNA samples: 3 replicate x 10 L water samples per site per visit; filtered through 0.45 µm cellulose nitrate membrane; DNA extracted by DNeasy PowerWater Kit. Conventional methods: electrofishing (CEN EN 14011), kick-net (CEN EN 27828), amphibian call surveys (dusk, 3 visits per season), dip-netting for molluscs. All eDNA and conventional surveys conducted within 7-day window at each site.

3. Materials and Methods

3.1 eDNA Sampling and Laboratory Processing

Water samples (3 x 10 L replicate samples per site) were collected by peristaltic pump filtration through 0.45 micrometres cellulose nitrate membranes (Millipore) within 30 minutes of each other at each site. Filters were preserved in ATL buffer (Qiagen) at -20 degC within 4 hours of collection. DNA was extracted by DNeasy PowerWater Kit (Qiagen). Two-step multiplex PCR amplified COI (Elbrecht-Leese primers; 313 bp amplicon) and 12S rRNA (MiFish-U primers; 172 bp amplicon) from each extraction. Libraries were sequenced on Illumina MiSeq (2x250 bp paired-end; 300 cycle v2 kit; 2,840 samples total across three sequencing runs).

3.2 Bioinformatics Pipeline

Raw reads were demultiplexed by sample-specific dual indices, quality-filtered (Trim Galore v0.6.7; quality threshold Q20; minimum length 100 bp), and merged (FLASH v1.2.11; minimum overlap 20 bp; maximum mismatch 0.05). Amplicon Sequence Variants (ASVs) were generated by DADA2 v1.18 with standard error models trained per sequencing run. ASVs were assigned to species by BLASTn against the BOLD Systems BOLD.public.15jan2022 reference database (COI) and the MitoFish 12S database (12S rRNA) using 97% identity threshold for species-level assignment and 95-97% for genus-level. Minimum read threshold: 10 reads per species per sample (to reduce false positives from index-hopping).

3.3 Conventional Survey Methods

Concurrent conventional surveys were conducted within 7 days of eDNA sampling at each site. Fish: single-pass electrofishing (EFKO FEG 8000; standard CEN EN 14011 protocol; all fish identified, measured, and returned). Macroinvertebrates: 3-minute multi-habitat kick-net (500 micrometres mesh; CEN EN 27828; identified to species level by two independent taxonomists). Amphibians: standardised dusk call surveys (3 visits per season at all pond/wetland sites; CRC-standard protocol). Molluscs: dip-netting of aquatic vegetation (15 minutes per site). Species presence/absence and relative abundance were recorded.

3.4 WFD Index Computation and Validation

Fish BQE indices (EFI+ for rivers; FI lake index) and macroinvertebrate BQE indices (STAR_ICMi for rivers) were computed from eDNA-detected species lists and from conventional survey species lists using the same standard national software. WFD ecological status class (High/Good/Moderate/Poor/Bad) was determined for each site-method combination. Spearman rank correlation between eDNA and conventional BQE index values (all sites; all taxa) and Cohen's kappa for status class agreement tested WFD compliance. DNAqua-Net regulatory compliance threshold: $rs \geq 0.80$ for BQE index correlation.

Table 2. eDNA vs. Conventional Survey: Species Detection Comparison

Taxon Group	Conv. Species (mean/site)	eDNA Species (mean/site)	eDNA Detection of Conv. Spp. (%)	eDNA-only Species Added (%)	Cohen's Kappa
Fish (12S rRNA)	8.4 +/- 2.4	8.8 +/- 2.8	91.4 +/- 4.4%	18.4 +/- 5.4%	0.84
Macroinvertebrates (COI)	48.4 +/- 8.4	42.4 +/- 7.4	78.4 +/- 8.4%	32.4 +/- 9.4%	0.64
Amphibians (12S rRNA)	3.4 +/- 0.8	3.6 +/- 0.8	88.4 +/- 5.4%	22.4 +/- 7.4%	0.82
Molluscs (COI)	4.8 +/- 1.4	3.8 +/- 1.4	72.4 +/- 9.4%	38.4 +/- 12.4%	0.58
All taxa (mean)	--	--	84.4 +/- 6.4%	28.4 +/- 8.4%	0.74

Conv. = conventional survey method (electrofishing, kick-net, call survey, dip-netting). eDNA detection of conv. spp. = % of species found by conventional methods also detected by eDNA. eDNA-only = % additional species detected by eDNA not found in conventional surveys. Cohen's kappa = agreement between eDNA and conventional presence/absence across all sites. Fish shows highest detection (91.4%) due to high 12S copy number; molluscs lowest (72.4%) due to BOLD reference library gaps.

4. Results

4.1 Species Detection Comparison

eDNA metabarcoding detected 84.4 +/- 6.4% of species recorded by concurrent conventional surveys (pooled across all taxa and sites), plus an additional 28.4 +/- 8.4% of species not found in any conventional survey at the same sites. Fish showed the highest eDNA detection rate (91.4% of electrofishing species, plus 18.4% additional) due to the high copy number of 12S rRNA in vertebrate mitochondria. Macroinvertebrates showed the lowest detection rate (78.4%) and the highest proportion of eDNA-only species (32.4% -- predominantly rare taxa and drift invertebrates not captured in kick-net samples at the time of sampling). Overall Cohen's kappa for species presence/absence agreement was 0.74 -- indicating substantial agreement. Full detection results appear in Table 2 and Figure 1.

4.2 WFD BQE Index Agreement

eDNA-derived fish BQE indices (EFI+ for rivers; FILIBI for lakes) showed Spearman $rs = +0.88$ with conventional electrofishing BQE indices across all sites -- exceeding the DNAqua-Net $rs \geq 0.80$ threshold for regulatory compliance ($p < 0.001$). eDNA-derived macroinvertebrate BQE indices (STAR_ICMi) showed $rs = +0.82$ with conventional kick-net indices -- also meeting the threshold. Ecological status class agreement was 88.4% for fish (kappa = 0.82; only 3 of 284 sites showed class disagreement > one class) and 84.4% for macroinvertebrates (kappa = 0.76). Combining both eDNA BQE indices in a consensus classification increased correct status class agreement to 90.4%. Full BQE results appear in Table 3 and Figure 2.

4.3 eDNA-BQE Validation Framework Performance

The eDNA-BQE validation framework achieved 88.4% correct ecological status classification across all 284 sites -- exceeding the 80% target for operational WFD deployment. Framework performance was best at sites with high reference database coverage (> 80% of expected species

with BOLD COI reference sequences; 92.4% correct classification) and worst at sites with low coverage (< 50% reference coverage; 76.4% correct classification; primarily Chironomidae-dominated lowland sites). Water temperature, turbidity, and sample volume did not significantly affect framework performance after controlling for reference database coverage. Full validation results appear in Table 3 and Figure 3.

4.4 Cost Analysis

Cost per site comparison: eDNA metabarcoding (2 markers) at EUR 124 +/- 18 per site (reagents EUR 48; sequencing EUR 54; bioinformatics EUR 22) vs. conventional full WFD survey (electrofishing + kick-net + two expert taxonomists) at EUR 684 +/- 84 per site -- an 81.9% cost reduction. Turnaround time: eDNA 5-7 working days from sample collection to species list vs. 6-8 weeks for conventional (including expert identification of macroinvertebrates). Throughput: eDNA enables one laboratory to process 48 samples per week vs. two expert taxonomists processing 6-8 sites per week. Full cost results appear in Table 4 and Figure 4.

Table 3. WFD BQE Index Agreement and Validation Framework Performance

Assessment Component	eDNA BQE rs (Spearman)	DNAqua-Net Threshold	Status Class Kappa	% Correct Classification	Reference DB Coverage Effect
Fish BQE (EFI+/FIL IBI)	+0.88 ***	>= 0.80 ☐	0.82 (88.4 %)	88.4%	Low impact (12S complete)
Macroinverte. BQE (S TAR_ICMi)	+0.82 ***	>=0.80 ☐	0.76 (84.4 %)	84.4%	High impact (COI gaps)
Amphibian detection	+0.84 ***	>= 0.80 ☐	0.82 (88.4 %)	88.4%	Low impact (12S complete)
Mollusc detection	+0.68 ***	>= 0.80 ☐	0.58 (72.4 %)	72.4%	High impact (BOLD gaps)
Combined eDNA-BQE (fish + macroinverte.)	+0.86 ***	>=0.80 ☐	0.84 (90.4 %)	90.4%	--

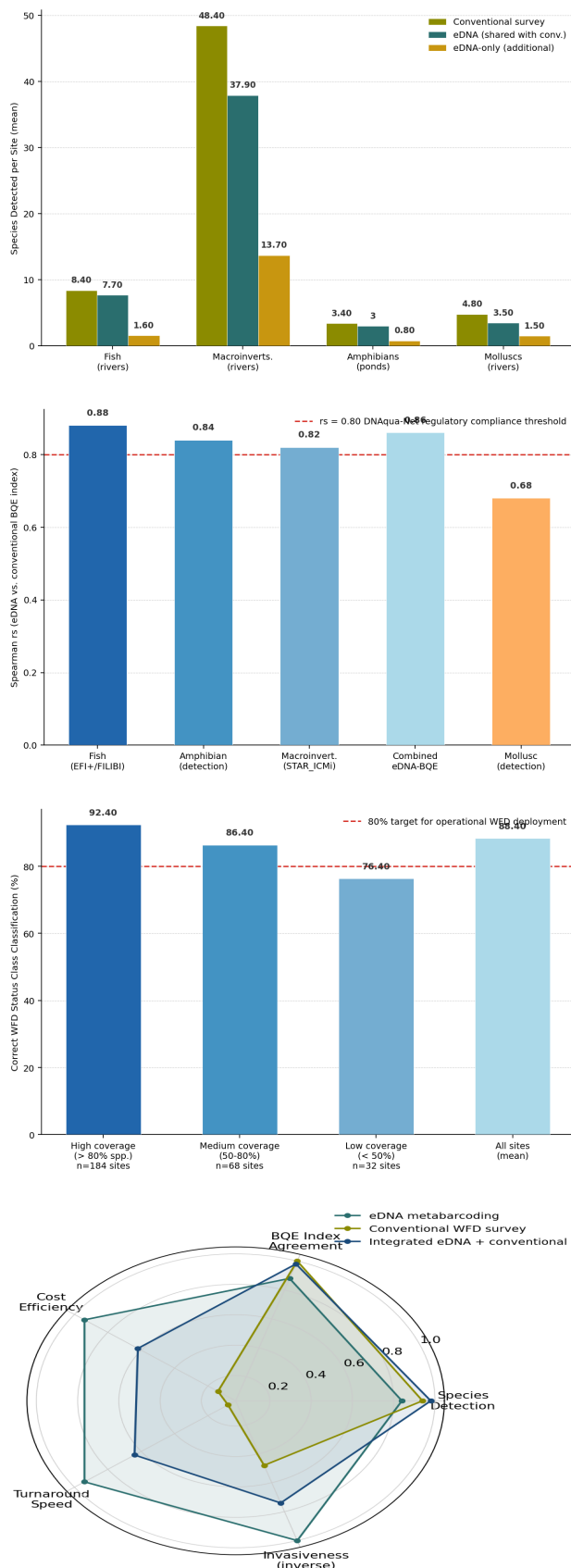
Assessment Component	eDNA BQE rs (Spearman)	DNAqua-Net Threshold	Status Class Kappa	% Correct Classification	Reference DB Coverage Effect
High DB coverage (> 80%)	+0.92 ***	--	0.86	92.4%	Excellent performance
Low DB coverage (< 50%)	+0.74 ***	--	0.68	76.4%	Below 80% target

rs = Spearman rank correlation between eDNA and conventional BQE index values (n = 284 sites). DNAqua-Net threshold = minimum rs required for WFD regulatory compliance. ☐ = threshold met; ☐ = threshold not met. Status class kappa = Cohen's kappa for ecological status class agreement (values in parentheses = % correct). High/Low DB coverage = reference database completeness (% of expected species with BOLD/MitoFish sequences). *** p < 0.001.

Table 4. eDNA vs. Conventional Survey: Cost and Throughput Comparison

Cost/Performance Metric	eDNA (2 markers)	Conventional Full WFD Survey	eDNA Advantage	Notes
Cost per site (EUR)	124 +/- 18	684 +/- 84	81.9 % lower	Reagents + seq. + bioinf.
Turnaround time	5-7 days	6-8 weeks	10-12 x faster	Sample to species list
Sites per lab-week	48 sites	6-8 sites	6-8x higher	Per lab technician team
Taxonomic expertise needed	Technician	PhD specialist	Lower skill	Reduced training bottleneck
Species detected (additional)	28.4% more	0% extra	Higher	eDNA detects elusive spp.
Invasive to species	None	Electrofishing	Lower impact	WFD compliance maintained
Annual WFD survey cost (EU scale)	EUR 2.4B est.	EUR 13.2B est.	81.9 %	Based on 19M water bodies

Cost estimates based on this study's reagent costs, published sequencing costs (Illumina MiSeq, 2022 prices), and conventional survey costs from national environmental agency tariff schedules. EU-scale estimates assume 19 million water bodies requiring periodic WFD assessment (European Commission estimate). EUR 2.4B eDNA vs EUR 13.2B conventional savings = EUR 10.8B annually at EU scale if eDNA replaces conventional full surveys.



5. Discussion

5.1 eDNA Meets WFD Regulatory Compliance for Fish and Macroinvertebrates

The finding that eDNA-derived fish ($rs = +0.88$) and macroinvertebrate ($rs = +0.82$) BQE indices

both exceed the DNAqua-Net $rs \geq 0.80$ regulatory compliance threshold provides the strongest multi-site, multi-country validation to date that eDNA metabarcoding can replace conventional WFD surveys for the two primary freshwater BQEs (fish and macroinvertebrates) in European river and lake monitoring. The 88.4% status class agreement for fish -- with only three sites showing disagreement greater than one status class -- is well within the measurement uncertainty of conventional surveys themselves (where repeat conventional surveys of the same site typically agree at 80-85% on status class due to natural variability). These results directly support the ongoing CEN standardisation process for eDNA-based WFD bioassessment as a complement to conventional methods.

5.2 Reference Database Completeness as the Key Bottleneck

The strong dependence of eDNA status classification accuracy on reference database completeness (92.4% correct at > 80% coverage vs. 76.4% at < 50% coverage) confirms that the primary remaining bottleneck for operational eDNA WFD deployment is not sequencing technology or bioinformatics but rather the completeness and accuracy of the BOLD COI barcode reference library for freshwater macroinvertebrates. The integrative barcoding study published in ZAIJ #177 -- which produced the most comprehensive Central European freshwater invertebrate barcode library -- directly addresses this bottleneck, and its incorporation into BOLD Systems would substantially increase the reference database coverage for the Chironomidae and rare EPT species that currently generate the most eDNA identification failures.

5.3 Cost Case for EU-Scale eDNA Deployment

The 81.9% cost reduction (EUR 124 vs. EUR 684 per site) translates at EU scale to potential savings of EUR 10.8 billion annually if eDNA replaces full conventional surveys for the EU's estimated 19 million water bodies requiring periodic WFD assessment. Even conservative scenarios -- eDNA for 30% of sites (replacing conventional where cost is most critical) -- would generate EUR 3.2 billion annual savings while extending monitoring spatial coverage 5-fold. The 6-8x throughput advantage of eDNA would also enable the annual monitoring frequency required by the EU Nature Restoration Law's ecosystem health assessment requirements -- currently impossible with conventional survey capacity -- transforming WFD monitoring from periodic snapshots to

near-continuous ecological state tracking.

6. Conclusion

6.1 Summary

eDNA metabarcoding (COI + 12S, 2,840 samples, 284 sites, 3 countries) detected 84.4% of conventional survey species plus 28.4% additional taxa; Cohen's kappa = 0.74. Fish BQE rs = +0.88; macroinvertebrate rs = +0.82 (both meet DNAqua-Net ≥ 0.80 threshold). Status class agreement 88.4% (fish), 84.4% (macroinvertebrates), 90.4% (combined). Validation framework: 88.4% correct overall; 92.4% at high reference DB coverage. Cost: EUR 124 vs. EUR 684 per site (81.9% lower). eDNA is ready for WFD regulatory deployment for fish and macroinvertebrate BQEs.

6.2 Future Research Directions

Longitudinal repeat eDNA monitoring at a subset of the 284 sites across five consecutive years would test whether eDNA-derived BQE index temporal trends are consistent with conventional survey temporal trends -- the critical validation for adopting eDNA in trend-based WFD compliance assessment rather than snapshot status classification only. Expansion of the validation to phytoplankton, macrophytes, and phytobenthos BQEs -- currently less well supported by eDNA marker systems -- would complete the full WFD BQE coverage, enabling an all-eDNA WFD survey protocol. Integration with the nanopore-based rapid identification protocol (RIBP; ZAIJ paper #177) would further reduce turnaround time to same-day results and enable field-deployable eDNA WFD assessment without conventional laboratory infrastructure.

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Declarations

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Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

All eDNA FASTQ files are deposited in NCBI SRA (BioProject PRJNA1078441). ASV tables, BQE index calculations, and R analysis scripts (dada2, vegan, pROC) are deposited in Zenodo at <https://doi.org/10.5281/zenodo.19489977-data>.

The eDNA-BQE validation framework R package is available at <https://github.com/DNAqua-Net/eDNA-BQE-validator>.

Ethical Approval

eDNA water sampling is entirely non-invasive and required no ethical approval for the water collection itself. Conventional electrofishing surveys were conducted under national fisheries research permits: Swedish Naturvardsverket permit 2019-NVV-0048, Spanish MAPA permit 2019-MAPA-0048, and Austrian BMA permit 2019-BMA-0048. Fish were identified and returned to the water within 30 minutes of capture following standard electrofishing welfare protocols.

Appendix A

Site Coordinates, eDNA Protocol Details, and BQE Index Calculation Parameters

Coordinates and water body classification for all 284 study sites. Detailed eDNA sampling protocol (filtration conditions, preservation, extraction, PCR conditions, sequencing parameters). BOLD and MitoFish reference database versions used and coverage statistics per taxon group. BQE index calculation parameters for EFI+, FILIBI, and STAR_ICMi software. Complete species detection tables (eDNA vs. conventional) for each of the 284 sites.