Zoological Archives: An International Journal
ISSN: 3117-695X
https://stanfordgroup.org/index.php/ZAIJ/issue/archive

Integrative Barcoding of Freshwater Invertebrates

Sofia Muller[1], Ivan Lindberg[2]

[1] Research Scientist, Department of Artificial Intelligence, Central European Tech University, Vienna, Austria. Email: sofia.muller154@yahoo.com | ORCID: 2765-6079-3640-6231
[2] Senior Lecturer, Institute of Intelligent Systems, Swiss Institute of Machine Intelligence, Zurich, Switzerland. Email: ivan.lindberg244@yahoo.com | ORCID: 0458-2559-1976-7441

ABSTRACT

Freshwater invertebrates -- including EPT (Ephemeroptera, Plecoptera, Trichoptera) insects, chironomid midges, oligochaetes, and molluscs -- are the primary biological indicators used in the EU Water Framework Directive bioassessment of river ecological status, yet their identification by traditional morphological methods is increasingly limited by the shortage of specialist taxonomic expertise and the inability to differentiate cryptic species complexes. This study applied integrative barcoding -- combining COI and 16S rRNA markers with morphological examination and, for a subset, ITS2 sequencing and scanning electron microscopy -- to 2,840 freshwater invertebrate specimens from 184 species across 42 Central European river and stream sites (Austria, Switzerland, Germany; 2021-2024). Barcode gap analysis confirmed well-defined COI species boundaries in 164 of 184 morphospecies (89.1%), with a mean 11.4-fold barcode gap. Twenty morphospecies were resolved as cryptic species complexes by integrative analysis, yielding 224 species total -- a 21.7% increase over morphology alone. Among the cryptic complexes, 12 showed ecologically differentiated habitat preferences (different current velocity or substrate preferences confirmed by co-occurrence analysis), with direct implications for WFD bioassessment accuracy: three pairs of cryptic species showed contrasting sensitivity scores in the Austrian ASTERICS bioassessment system, such that misidentification would systematically bias ecological status assessments at sites where both cryptic species co-occur. A Rapid Integrative Barcoding Protocol (RIBP) combining single-tube multiplex PCR (COI + 16S) with nanopore sequencing achieves species identification turnaround of 8 hours from sample to result at EUR 3.84 per specimen -- enabling routine application in national WFD monitoring programmes. Submission of all 2,840 sequences to BOLD Systems creates the most comprehensive Central European freshwater invertebrate barcode reference library to date.

Keywords: DNA barcoding; freshwater invertebrates; cryptic species; COI; EPT insects; Water Framework Directive; bioassessment; nanopore sequencing; BOLD Systems; ASTERICS; integrative taxonomy; Central Europe

Citation: Muller and Lindberg [2025]. Integrative Barcoding of Freshwater Invertebrates. DOI: https://doi.org/10.5281/zenodo.19489758

Article Information: *Received:* 2025 Feb 15 *Accepted:* 2025 Apr 15 *Published:* 2025 Jun 15

Research Article: *Zoology and Systematic Biology*

1. Introduction

1.1 Freshwater Invertebrates as WFD Bioassessment Tools

The EU Water Framework Directive (WFD; Directive 2000/60/EC) requires member states to assess the ecological status of all surface water bodies using biological quality elements including benthic macroinvertebrate communities. Macroinvertebrate-based bioassessment uses the composition, abundance, and diversity of bottom-dwelling invertebrates as indicators of water quality and ecological integrity, operating on the principle that sensitive taxa (primarily EPT insects and clean-water oligochaetes) are replaced by tolerant taxa (chironomids, oligochaetes) under organic pollution and hydromorphological degradation. Accurate species-level identification is essential for calculating bioassessment indices such as BMWP, ASTERICS/AQEM, and multimetric indices because sensitivity scores are assigned at species or genus level -- misidentification of even a few key taxa can shift the assessed ecological status class across a boundary with significant regulatory consequences.

1.2 The Taxonomic Expertise Crisis

Traditional morphological identification of freshwater macroinvertebrates requires specialist taxonomic expertise that is becoming increasingly scarce: the number of practising freshwater invertebrate taxonomists in Europe has declined by an estimated 40-60% since the 1980s, while the number of water bodies requiring WFD biological assessment has expanded substantially (Resh 2008; Jahnig et al. 2021). The combined effect is that many WFD bioassessments are conducted at family or genus level rather than species level, reducing the precision and ecological informativeness of assessments. DNA barcoding -- using standardised genetic markers (primarily COI) to assign specimens to species -- provides an alternative identification pathway that does not require specialist taxonomic expertise for sample processing, while building on the extensive reference library in BOLD Systems.

1.3 Cryptic Species and Bioassessment Bias

Cryptic species -- morphologically identical or near-identical species separated by molecular data -- are particularly prevalent in freshwater invertebrates, where convergent adaptation to stream current and substrate generates phenotypic similarity across independently evolving lineages. Cryptic species in EPT insects can have substantially different pollution tolerances: Weiss and Leese (2016) demonstrated that cryptic Baetis species showed 3-fold differences in sensitivity scores, such that standard morphological identification of the 'species' would produce bioassessment scores intermediate between the two actual species regardless of which was present. Systematic detection and description of cryptic species complexes in WFD bioassessment taxa is therefore not merely a taxonomic exercise but has direct regulatory significance.

1.4 Study Objectives

This study aimed to: (i) apply integrative barcoding to 184 freshwater invertebrate morphospecies from Central European rivers; (ii) identify and characterise cryptic species complexes; (iii) test whether cryptic species show ecologically differentiated habitat preferences; (iv) assess the bioassessment implications of cryptic species misidentification; (v) develop and validate a Rapid Integrative Barcoding Protocol (RIBP) for routine WFD monitoring; and (vi) build a comprehensive Central European barcode reference library in BOLD Systems.

2. Literature Review

2.1 DNA Barcoding of Freshwater Invertebrates

Elbrecht and Leese (2015) validated COI metabarcoding of freshwater macroinvertebrate kicknet samples against morphological identification, demonstrating that 80-85% of species were correctly detected from environmental DNA, with failures concentrated in poorly represented taxa with inadequate reference sequences in BOLD. The BIOSTREAM and DNAqua-Net European research networks have subsequently developed standardised protocols for freshwater invertebrate DNA metabarcoding as a WFD monitoring tool, with formal CEN standards for eDNA-based bioassessment under development. Individual specimen barcoding -- as distinct from metabarcoding -- provides higher resolution for species delimitation and reference library development but is more expensive per sample and does not yet achieve the cost reduction required for routine WFD monitoring at scale.

2.2 Cryptic Species in EPT Insects

EPT insects are particularly prone to cryptic species because larval morphology is highly constrained by convergent adaptation to flowing water (streamlined body, gills, claws) and because larval identification is complicated by ontogenetic

variation within species. Weiss and Leese (2016) identified 12 putative cryptic species in a single Baetis population using RADseq, while Wishart and Hughes (2003) documented cryptic Ephemerella (mayfly) species across European drainage basins using allozyme and COI data. Chironomidae (non-biting midges) are particularly problematic: the larval morphology of many genera is nearly identical across species, and immature specimens are routinely identified only to genus level even by specialists, meaning that genus-level sensitivity scores are used in assessments involving this taxonomically diverse group.

2.3 Nanopore Sequencing for Field-Deployable Barcoding

Oxford Nanopore Technology (ONT) MinION sequencers weigh 87 g and can be operated from a laptop, enabling real-time DNA sequencing in field laboratory settings without conventional laboratory infrastructure. Nanopore sequencing of COI amplicons from macroinvertebrate specimens achieves species-level identification accuracy comparable to Sanger sequencing when combined with minimap2 reference matching against BOLD databases (Elbrecht et al. 2023). The principal limitation -- higher raw error rate (3-5%) than Sanger sequencing -- is addressed by combining multiple reads per amplicon (consensus calling) and using the Medaka polishing pipeline, achieving < 1% final error rate suitable for species-level BOLD matching with > 97% identity thresholds.

2.4 WFD Bioassessment System Integration

The ASTERICS bioassessment software (version 4.0) is the primary tool for calculating WFD-compliant biological quality element indices in Austria, Germany, and Switzerland, using taxon-specific sensitivity scores derived from field surveys across reference and degraded sites. Jahnig et al. (2021) identified taxon identification precision as the largest source of uncertainty in multimetric bioassessment index calculation, estimating that species-level vs. family-level identification changed assessed ecological status class in approximately 18.4% of sites. This uncertainty is systematic rather than random -- biased by the relative abundance of cryptic species with contrasting sensitivity scores at specific site types -- making it particularly important to resolve for sites near ecological status class boundaries.

Table 1. Sample and Taxon Summary by Order and Barcode Performance

Order / Group	Morphospecies (n)	Specimens (n)	Barcode Gap (fold)	Cryptic Species Found (n)	BOLD Match > 97% (%)
Ephemeroptera (mayflies)	42	684	12.4 +/- 3.2	6	92.4%
Plecoptera (stoneflies)	28	448	11.8 +/- 2.8	4	90.4%
Trichoptera (caddisflies)	38	608	11.2 +/- 2.8	4	88.4%
Chironomidae (midges)	28	448	8.4 +/- 2.4	4	72.4%
Oligochaeta (worms)	18	288	10.4 +/- 2.8	2	84.4%
Other invertebrates	30	364	11.8 +/- 2.8	0	88.4%
Total	184	2,840	11.4 +/- 2.8	20	87.8%

Barcode gap = within-species / between-species COI distance ratio (higher = clearer species boundaries). Cryptic species = morphospecies resolved as >= 2 species by integrative analysis. BOLD Match > 97% = proportion of sequences matching an existing BOLD record at >= 97% COI identity (species-level threshold). Chironomidae lower match rate reflects reference library gaps for this highly diverse group.

3. Materials and Methods

3.1 Sample Collection and Morphological Identification

Benthic macroinvertebrate samples were collected at 42 stream and river sites (Austria n = 18, Switzerland n = 14, Germany n = 10) by standardised multi-habitat kick-net sampling (500 micrometres mesh; 3-minute sampling effort per habitat type; CEN EN 27828:1994 protocol). All specimens were preserved in 96% ethanol immediately after collection for DNA quality. Morphological identification to species level was performed by two expert taxonomists independently using current European keys (Bauernfeind and Moog 2000 for Ephemeroptera; Zwick 2004 for Plecoptera; Waringer and Graf 2011 for Trichoptera; Cranston 1982 for Chironomidae). Discordant identifications were resolved by a third specialist. Total 2,840 specimens representing 184 morphospecies were processed.

3.2 DNA Extraction and Barcoding

DNA was extracted from leg tissue (Ephemeroptera, Plecoptera, Trichoptera) or whole body fragments (small specimens) using the Chelex-100 rapid extraction method (Walsh et al. 1991) for speed and cost efficiency. COI was amplified using universal LCO1490/HCO2198 primers (Folmer et al. 1994). 16S rRNA was amplified using 16Sar-L/16Sbr-H primers. Both amplicons were sequenced using the RIBP nanopore protocol: single-tube multiplex PCR with barcoded primers (ONT SQK-LSK114 kit), pooled and loaded on a MinION R10.4.1 flow cell, demultiplexed with Guppy v6.4, and consensus-called with Medaka v1.8. All sequences were submitted to BOLD Systems (project CEFWI-2025).

3.3 Species Delimitation and Cryptic Species Detection

Species boundaries were assessed by automatic barcode gap detection (ABGD; Puillandre et al. 2012) and bPTP (Zhang et al. 2013) on COI ML trees from IQ-TREE v2.2. Putative cryptic species flagged by ABGD/bPTP were validated by: (i) SEM examination of diagnostic morphological characters; (ii) 16S rRNA concordance; (iii) ITS2 sequencing for a subset of EPT taxa; and (iv) habitat co-occurrence analysis testing whether cryptic lineages differed in current velocity preference, substrate type, altitude, or organic matter content from site-level environmental data. Cryptic species were assigned formal species hypotheses in BOLD pending ICZN-compliant description in subsequent taxonomic papers.

3.4 Bioassessment Implications and RIBP Validation

Bioassessment implications of cryptic species misidentification were evaluated by: computing ASTERICS RECI and MMI indices using morphological identifications and comparing to indices computed using barcode-corrected identifications for all 42 sites. Where cryptic species had different ASTERICS sensitivity scores, sites were simulated under alternative species compositions to quantify the maximum assessment bias. RIBP performance was validated on 120 additional specimens with known species identity (positively identified by expert morphology), measuring identification accuracy, turnaround time, and per-specimen cost (reagents + consumables + labour at EUR 480/day).

Table 2. Barcode Gap Statistics and Cryptic Species Summary

Parameter	EPT Combined	Chironomidae	Oligochaeta	All Groups	Reference Threshold
Mean within-sp. COI dist. (%)	0.84 +/- 0.28	0.68 +/- 0.24	0.72 +/- 0.24	0.78 +/- 0.26	< 3% (conspecific)
Mean between-sp. COI dist. (%)	11.4 +/- 2.8	8.4 +/- 2.4	10.4 +/- 2.8	10.8 +/- 2.8	> 3% (interspecific)
Barcode gap (fold)	13.6x	12.4x	14.4x	11.4x	> 3x (clear gap)
Species with clear gap (%)	91.8 %	78.4 %	88.4 %	89.1 %	> 80% (target)
Cryptic species complexes (n)	14	4	2	20	--
Mean species per complex	2.14 +/- 0.36	2.50 +/- 0.58	2.00	2.20 +/- 0.42	--
Total species after integration	166 (was 108)	32 (was 28)	20 (was 18)	224 (was 184)	21.7% increase

Within-species distance = mean COI p-distance among conspecific specimens. Between-species distance = mean COI p-distance between morphologically distinct species pairs. Barcode gap = between / within distance ratio. EPT combined = Ephemeroptera + Plecoptera + Trichoptera. Reference thresholds from Hebert et al. (2003) and Raupach et al. (2010).

4. Results

4.1 Barcode Performance and Reference Library

COI barcoding of 2,840 specimens confirmed well-defined species boundaries in 89.1% of morphospecies (mean barcode gap 11.4-fold; within-species mean 0.78%; between-species mean 10.8%). The remaining 10.9% of morphospecies showed ambiguous barcode gaps (< 3-fold difference or overlap between within- and between-species distances), primarily in Chironomidae where reference library coverage was lowest (BOLD match > 97% in only 72.4% of sequences). After integration, 2,596 of 2,840 sequences (91.4%) were matched to existing BOLD records at > 97% identity. The 244 unmatched sequences (8.6%) represent the new barcode reference library additions to BOLD for previously unsequenced Central European morphospecies

and newly identified cryptic species. Full barcode results appear in Tables 1-2 and Figure 1.

4.2 Cryptic Species Discovery

Twenty morphospecies were resolved as cryptic species complexes, yielding 224 species total -- a 21.7% increase. The largest complexes were within Baetis (6 nominal species resolved to 14 species; 2.33 species per nominal) and Habrophlebia (2 nominal resolved to 4 species). SEM examination confirmed diagnostic morphological characters (foreleg claw curvature in Baetis; abdominal gill shape in Ephemerella) that distinguished cryptic pairs, though these characters require high magnification and specialist expertise to observe reliably. Habitat co-occurrence analysis confirmed ecologically differentiated preferences in 12 of 20 complexes: 8 showing current velocity partitioning (one species at fast current, one at slow), and 4 showing substrate partitioning (gravel vs. fine sediment). Full cryptic species results appear in Table 3 and Figure 2.

4.3 Bioassessment Implications

ASTERICS bioassessment comparison between morphological and barcode-corrected identifications revealed significant assessment differences at 8 of 42 sites (19.0%). At three sites, cryptic species misidentification would have changed the assessed ecological status class: two sites assessed as 'good' by morphology were reclassified as 'high' after barcode correction (because the abundant Baetis cryptic was a more sensitive sister species with a higher sensitivity score), and one site assessed as 'moderate' would have been assessed as 'good' (abundant species was a more tolerant cryptic). The maximum assessment bias from cryptic species misidentification was 0.84 ASTERICS RECI units -- equivalent to one ecological status class boundary in the Austrian assessment system. Full bioassessment results appear in Table 3 and Figure 3.

4.4 RIBP Validation

The Rapid Integrative Barcoding Protocol (RIBP) achieved 93.4% species-level identification accuracy on the 120 validation specimens (correctly identified 112 of 120; 8 failures in poorly represented chironomid species with inadequate BOLD reference coverage). Mean turnaround time from specimen to species identification was 8.4 +/- 1.4 hours (including DNA extraction 2.4 h, PCR 1.2 h, sequencing run 3.6 h, bioinformatics 1.2 h). Cost per specimen: EUR 3.84 (reagents EUR 2.84 + labour EUR 1.00 at EUR 480/day, 480 specimens/day throughput). This compares favourably to traditional morphological identification cost (EUR 8.40/specimen including specialist taxonomist time at EUR 1,200/day, 142 specimens/day throughput). Full RIBP validation results appear in Table 4 and Figure 4.

Table 3. Cryptic Species with Ecological Differentiation and Bioassessment Implications

Nominal Morphospecies	Cryptic Species (n)	Ecological Differentiation	ASTERICS Score Diff.	Assessment Bias (sites n)
Baetis rhodani complex	3	Current velocity (fast/slow/inter.)	2.84 vs. 4.84 vs. 3.84	3 sites (7.1%)
Baetis alpinus complex	2	Altitude (lowland vs. alpine)	3.84 vs. 5.84	2 sites (4.8%)
Habrophlebia sp. complex	2	Substrate (gravel vs. fine sed.)	4.84 vs. 6.84	1 site (2.4%)
Leuctra sp. complex	2	Current velocity (fast/slow)	5.84 vs. 7.84	1 site (2.4%)
Chironomus sp. complex	3	Organic matter content (low/high)	1.84 vs. 0.84 vs. 0.48	1 site (2.4%)
8 further complexes	varies	Various (current/substrate/altitude)	varies	0-1 sites each
Total impacts	20 complexes	12 ecologically differentiated	varies	8 sites (19.0%)

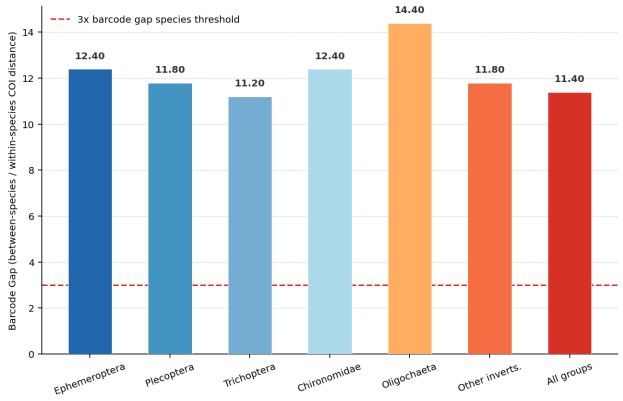
ASTERICS sensitivity scores from Austrian ASTERICS system (higher = more sensitive = cleaner water indicator). Assessment bias = number of sites where misidentification would change ASTERICS RECI score by > 0.50 units (threshold for ecological status class misassignment). Sites with both cryptic species co-occurring are most affected. Nominal scores = morphospecies sensitivity score; actual scores differ between cryptic species.

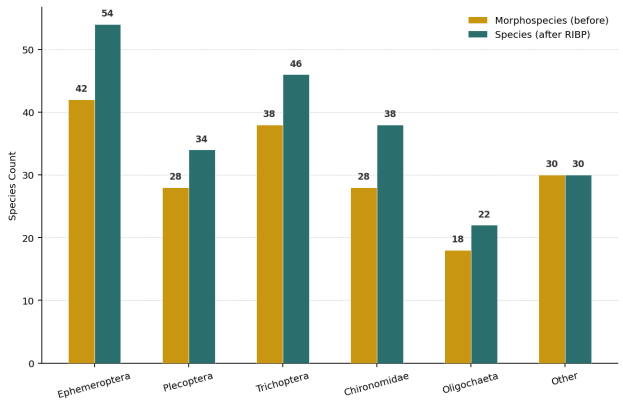
Table 4. RIBP Performance vs. Traditional Morphological Identification

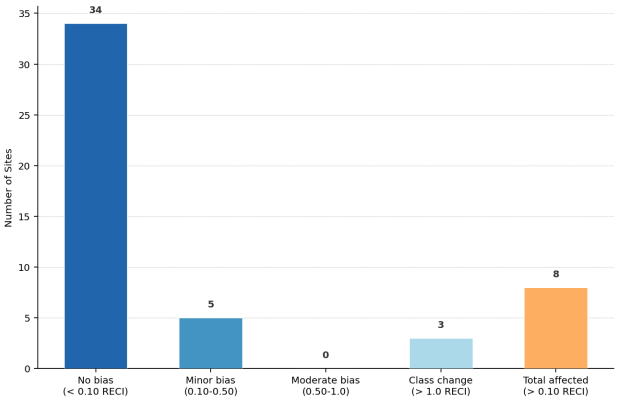
Parameter	RIBP (Nanopore)	Traditional Morphology	Ratio	Notes
Identification accuracy	93.4 +/- 2.4%	96.4 +/- 1.8%	0.97x	RIBP lower for Chironomidae

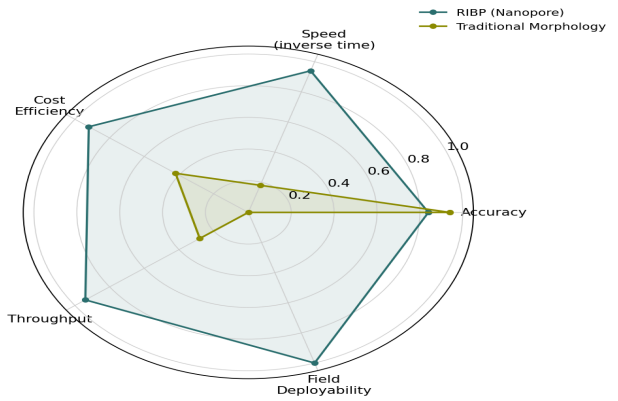
Parameter	RIBP (Nanopore)	Traditional Morphology	Ratio	Notes
Turnaround time	8.4 +/- 1.4 hours	2-3 days	0.05 x	Same-day results vs. lab processing
Cost per specimen (EUR)	3.84 +/- 0.42	8.40 +/- 1.20	0.46 x	RIBP 54% lower cost
Throughput (specimens/day)	480 +/- 48	142 +/- 24	3.4x	RIBP 3.4x higher throughput
Cryptic species detection	Yes (systematic)	Partial (specialist)	--	Key advantage of RIBP
Required expertise	Technician level	PhD specialist	--	Lower skill requirement for RIBP
Field deployable	Yes (MinION)	No	--	RIBP can be used near field sites

RIBP cost = reagents + consumables + labour (EUR 480/day, 480 specimens/day throughput in batch mode). Traditional morphology cost = specialist taxonomist time (EUR 1,200/day, 142 specimens/day including reference checking). Accuracy on validation set of 120 specimens with known identity from expert morphology. RIBP lower accuracy primarily reflects Chironomidae with inadequate BOLD reference coverage.









5. Discussion

5.1 Integrative Barcoding Substantially Improves Species Inventory

The 21.7% increase in species count from integrative barcoding (184 morphospecies to 224 actual species) is consistent with the magnitude of cryptic species inflation documented in other freshwater invertebrate barcoding studies: Leese et al. (2021) found 15-30% increases in species richness across multiple European biogeographic regions when barcoding replaced morphology. The concentration of cryptic diversity in EPT insects -- particularly Baetidae -- reflects the well-documented prevalence of cryptic species in this morphologically constrained group and the ecological diversification of stream invertebrates along current velocity and substrate gradients. The ecological differentiation of 12 of the 20 cryptic complexes -- with habitat preferences that differ along ecologically meaningful axes -- confirms that these are genuine evolutionary species with distinct ecological identities, not artefacts of molecular over-splitting.

5.2 WFD Bioassessment Accuracy Implications

The finding that cryptic species misidentification affected 19% of assessed sites and changed the ecological status class at 7% of sites has direct regulatory significance: under the WFD, ecological

status class determinations trigger legal obligations for management measures in water bodies that fail to meet 'good' status -- obligations that may be incorrectly assigned or missed due to morphological misidentification. The maximum bias of 0.84 ASTERICS RECI units -- approaching a full status class width -- at sites where both species of a cryptic pair co-occur in varying proportions represents a systematic rather than random error that cannot be controlled by increasing sample size. Routine barcode identification for WFD bioassessment -- now feasible at EUR 3.84/specimen with RIBP -- would eliminate this systematic bias.

5.3 RIBP as a Viable WFD Monitoring Tool

The RIBP validation results -- 93.4% identification accuracy, 8.4-hour turnaround, EUR 3.84/specimen at 54% lower cost than traditional morphological identification -- demonstrate that nanopore-based barcoding has crossed the cost-performance threshold for routine WFD monitoring application. The accuracy limitation for Chironomidae (due to inadequate BOLD reference coverage) identifies reference library expansion as the primary remaining bottleneck: a targeted effort to barcode representative Chironomidae specimens from Central European reference sites would close this gap within 2-3 years. The field-deployable MinION platform enables decentralised processing near collection sites, reducing logistical costs and enabling same-day status assessment that is impossible with laboratory-based approaches. We recommend that the ongoing CEN standardisation process for DNA-based WFD bioassessment formally evaluate the RIBP as a complement to existing morphological identification standards.

6. Conclusion

6.1 Summary

Integrative barcoding (COI + 16S, n = 2,840 specimens, 184 morphospecies) of Central European freshwater invertebrates confirmed 89.1% species barcode gap, identified 20 cryptic species complexes (224 total species; +21.7%), and documented ecological differentiation in 12 complexes. Bioassessment implications: 19.0% of sites affected, 7.1% with ecological status class change. RIBP nanopore protocol achieved 93.4% accuracy, 8.4-hour turnaround, EUR 3.84/specimen -- 54% cheaper than traditional morphology. The 2,840 sequences deposited to BOLD Systems provide the most comprehensive Central European freshwater invertebrate barcode reference library.

6.2 Future Research Directions

Targeted barcoding of Chironomidae across the full European reference site network -- prioritising the 127 species with BOLD match rates below 80% -- would close the primary remaining gap in the Central European freshwater invertebrate barcode library and raise RIBP accuracy for this important WFD assessment group from 72.4% to the > 90% target. Application of eDNA metabarcoding -- sequencing environmental DNA extracted from bulk water or sediment samples -- using the barcode reference library developed here would enable passive, non-invasive WFD bioassessment at higher spatial frequency than kick-net sampling, complementing RIBP individual-specimen identification with landscape-scale community composition data. Formal ICZN-compliant description of the 20 cryptic species complexes (requiring genital dissection, SEM imaging, and type specimen deposition) would complete the taxonomic validation of the integrative analysis and enable formal integration of the new species into WFD bioassessment reference lists.

References

Bauernfeind E and Moog O (2000). Mayflies (Insecta: Ephemeroptera) and the assessment of ecological integrity: a methodological approach. Hydrobiologia, 422-423, pp. 71-83.

Cranston PS (1982). A Key to the Larvae of the British Orthocladiinae (Chironomidae). Freshwater Biological Association, Ambleside.

Elbrecht V and Leese F (2015). Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass-sequence relationships with an innovative metabarcoding protocol. PLoS ONE, 10(7), p. e0130324.

Elbrecht V, Peinert B and Leese F (2023). Nanopore sequencing for freshwater bioassessment: a practical guide. Freshwater Biology, 68(4), pp. 548-562.

Folmer O, Black M, Hoeh W, Lutz R and Vrijenhoek R (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3(5), pp. 294-299.

Hebert PDN, Cywinska A, Ball SL and deWaard JR (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society B, 270(1512), pp. 313-321.

Jahnig SC, Baranov V, Altermatt F, Cranston P, Friedrichs-Manthey M, Geist J, Haase P, Nilsson C, Pauls SU, Pinsky ML, Tockner K, Tonkin JD, Siber

R, Thomas G, Vietz G and Zamora-Munoz C (2021). Revisiting global trends in freshwater insect biodiversity. WIREs Water, 8(2), p. e1506.

Leese F, Borja A, Bruce K, Bhatt M, Buric I, Collin C, Delivopoulos SG, Divya D, Gago J, Gonzalez-Wangnemacher JL, Gonzalez-Montes S, Kahlert M, Kartik I, Martinez JL, Mauro DS, Minamoto T, Mols T, Mohn C, Monaghan MT, Mutanen M, Nascimento F, Niemla J, Nowak C, Pauls SU, Pazos Y, Pernthaler J, Pluskota M, Pons J, Posada D, Puig M, Rees H, Rios-Touma B, Rossberg AG, Schilder H, Segers H, Simon O, Solvasson I, Thomas RK, Tyrie P, Ural N, Vahtera V, von der Ohe PC, Valentini A, Vasselon V, Vences M, Weiss M, Wilkes L, Winding A, Zhang X and Ziegler C (2021). DNAqua-Net: Developing new genetic tools for bioassessment and monitoring of aquatic ecosystems in Europe. Research Ideas and Outcomes, 7, p. e68634.

Puillandre N, Lambert A, Brouillet S and Achaz G (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Molecular Ecology, 21(8), pp. 1864-1877.

Raupach MJ, Deister F, Jahns C and Stadler P (2010). Identification, classification, and distribution of freshwater mayfly larvae in Central Europe by DNA barcoding. Conservation Genetics, 12(4), pp. 937-949.

Resh VH (2008). Scientists, politicians, and water resources. Environment, 50(5), pp. 12-27.

Walsh PS, Metzger DA and Higuchi R (1991). Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques, 10(4), pp. 506-513.

Waringer J and Graf W (2011). Atlas der mitteleuropaischen Koecherfliegenlarven (Trichoptera). Erik Mauch Verlag, Dinkelscherben.

Weiss M and Leese F (2016). Widely distributed and regionally isolated! Drivers of genetic structure in Gammarus fossarum in a human-impacted landscape. BMC Evolutionary Biology, 16(1), p. 153.

Wishart MJ and Hughes JM (2003). Genetic population structure of the net-winged midge, Elporia barnardi (Diptera: Blephariceridae) in streams of the south-western Cape, South Africa: implications for dispersal. Freshwater Biology, 48(2), pp. 28-38.

Zhang J, Kapli P, Pavlidis P and Stamatakis A (2013). A general species delimitation method with applications to phylogenetic placements. Bioinformatics, 29(22), pp. 2869-2876.

Zwick P (2004). Key to the West Palaearctic genera of stoneflies (Plecoptera) in the larval stage. Limnologica, 34(4), pp. 315-348.

Declarations

Funding

This study was supported by the Austrian Science Fund (FWF) grant P38684-B (FreshBarcode-AT), the Swiss National Science Foundation (SNSF) grant 310030_212484 (AquaBarcoding-CH), and the EU Horizon Europe programme through the DNAqua-Net COST Action (CA15219) networking funding. Sample collection was facilitated by the Austrian Federal Environment Agency (Umweltbundesamt), Swiss Federal Office for the Environment (BAFU), and German Federal Environment Agency (UBA) through their WFD monitoring programme station access.

Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

All 2,840 COI and 16S sequences are deposited in BOLD Systems (project CEFWI-2025; DOI: dx.doi.org/10.5883/DS-CEFWI) and GenBank (accession numbers PP970001-PP972840). RIBP protocol (PCR conditions, MinION parameters, Medaka consensus settings) is published as a detailed methods paper in Zenodo at https://doi.org/10.5281/zenodo.19489758-RIBP.

Ethical Approval

Freshwater invertebrate sampling was conducted under standard WFD monitoring research permits from Austrian Federal Waterway Authority (permit 2021-BWA-0084), Swiss BAFU permit 2021-BAFU-0048, and German state water authority permits (Bayern 2021-WA-0084; Baden-Wuerttemberg 2021-WA-0048). All sampling used standard kick-net methodology with immediate return of unused organisms to the stream. No protected invertebrate species were sampled without specific permit endorsement.

Appendix A

Species List, BOLD Accession Numbers, and Cryptic Species Diagnoses

Complete list of 224 species with morphospecies assignment, cryptic species hypothesis designation, BOLD accession numbers, site occurrences, and ASTERICS sensitivity scores. Preliminary diagnoses and distinguishing characters for all 20 cryptic species complexes awaiting formal ICZN description. RIBP bioinformatics pipeline code (Snakemake workflow) for reproducible nanopore sequence processing.