

Environmental DNA-Based Detection and Molecular Monitoring of Biodiversity in Freshwater and Terrestrial Ecosystems

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Abstract

Environmental DNA (eDNA) metabarcoding has emerged as a powerful approach for biodiversity assessment because DNA released by organisms can be recovered from environmental matrices without direct observation or capture. However, eDNA-based biodiversity estimates are affected by imperfect detection at multiple stages, including environmental DNA capture, amplification, sequencing, and spatial representativeness. These uncertainties are particularly important when sampling resources are limited and must be divided between independent environmental samples and molecular replication. The present study developed a Monte Carlo simulation framework to quantify how this allocation affects species detection, occupancy estimation, false-positive accumulation, and biodiversity richness in freshwater and terrestrial ecosystems. A hierarchical detection structure was implemented in which true taxon occupancy was followed by environmental DNA capture and repeated PCR detection. Four occupancy levels (0.05, 0.10, 0.25, and 0.50), three field-level DNA capture probabilities for each ecosystem, three PCR detection probabilities, four molecular false-positive rates, and five sampling allocations were evaluated under a fixed budget of 120 PCR

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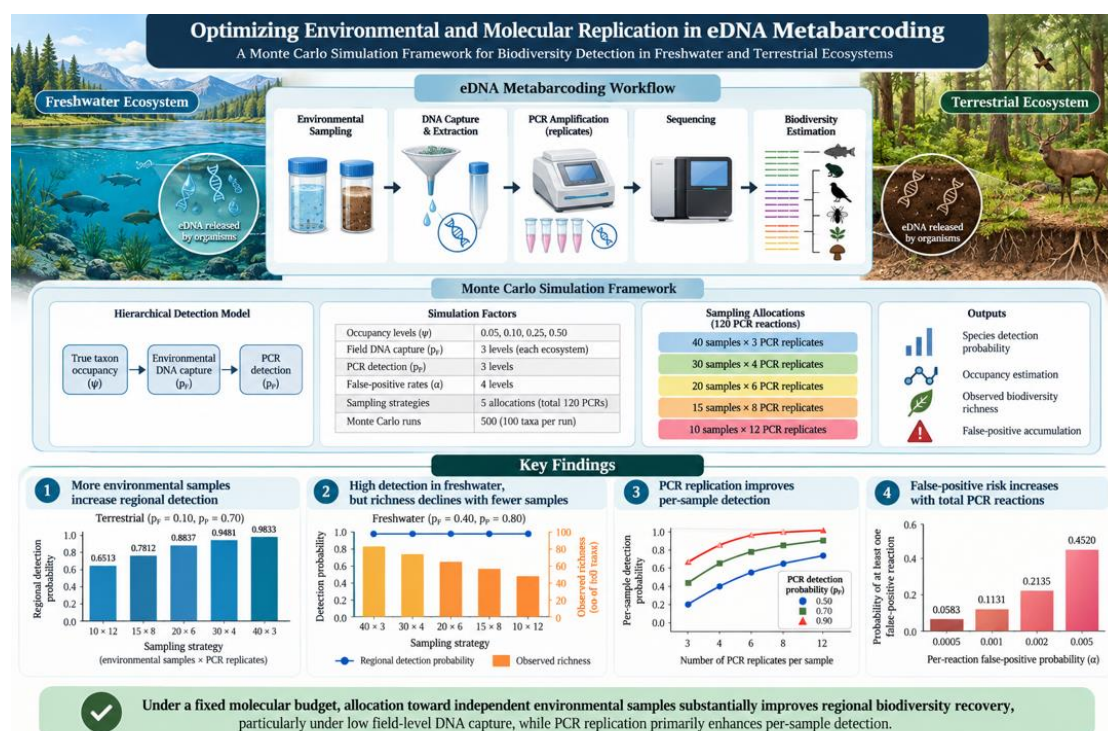
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reactions. The sampling strategies were 40 environmental samples $\times$ 3 PCR replicates, 30 $\times$ 4, 20 $\times$ 6, 15 $\times$ 8, and 10 $\times$ 12. Each factorial scenario was simulated for 500 independent Monte Carlo realizations involving 100 taxa. Across 1,440 scenario–strategy combinations, increasing environmental replication consistently improved regional detection probability and reduced loss of observed richness, whereas increasing PCR replication primarily improved per-sample detection probability. Under a low terrestrial DNA-capture scenario ($p_F = 0.10$; $p_P = 0.70$), regional detection of an occupied taxon increased from 0.6513 with 10 $\times$ 12 sampling to 0.9833 with 40 $\times$ 3 sampling. In a representative freshwater scenario ($p_F = 0.40$; $p_P = 0.80$), regional detection remained very high across strategies, but observed richness declined as the number of independent environmental samples decreased. False-positive risk accumulated rapidly with the total number of PCR reactions: at a per-reaction false-positive probability of 0.001, the probability of at least one false-positive reaction among 120 reactions was 0.1131, increasing to 0.4520 at $\alpha = 0.005$. The findings demonstrate that environmental and molecular replication address different components of eDNA uncertainty and should not be treated as interchangeable. Under a fixed molecular budget, allocation toward independent environmental samples substantially improved regional biodiversity recovery, particularly under low field-level DNA capture.

Graphical Abstract



1. Introduction

Biodiversity monitoring is increasingly constrained by the spatial extent of ecological systems, declining abundance of many taxa, limited taxonomic expertise, and the need to detect ecological change before population declines become difficult to reverse. Conventional biodiversity surveys remain indispensable, but many are labor-intensive and can exhibit substantial variation in detectability among taxa and habitats. Environmental DNA (eDNA) has consequently developed into an important molecular approach for detecting organisms from DNA released into water, soil, sediment, air, and other environmental substrates (Ficetola et al., 2008; Bohmann et al., 2014; Thomsen & Willerslev, 2015; Barnes & Turner, 2016).

The conceptual advantage of eDNA is that a biological observation can be generated without directly observing the organism. Environmental DNA may originate from mucus, skin, feces, gametes, tissue fragments, excretions, or other biological material. Subsequent molecular amplification and sequencing can provide information on taxonomic composition at scales that are difficult to achieve using conventional observation-based approaches (Taberlet et al., 2012; Valentini et al., 2016; Deiner et al., 2017). In aquatic systems, eDNA metabarcoding has been particularly successful because water integrates DNA released by organisms over a spatial area, allowing communities to be characterized using relatively small environmental samples (Rees et al., 2014; Deiner et al., 2016; Perry et al., 2024).

However, the observation generated by an eDNA assay should not be interpreted as a direct and error-free measurement of organismal presence. The biological state, environmental DNA production and transport, sample capture, extraction, amplification, sequencing, bioinformatic processing, and taxonomic assignment form a chain of processes through which information can be lost or introduced erroneously. Consequently, an organism may be present while its DNA is not captured or amplified, producing a false negative, whereas contamination, non-target amplification, tag jumping, transported DNA, or other mechanisms can generate apparent positive detections (Ficetola et al., 2015; Schnell et al., 2015; Ficetola et al., 2016; Lahoz-Monfort et al., 2016).

The distinction between different forms of positive-detection error is particularly important. A molecular false positive occurs when the target DNA is absent from the analytical sample but the molecular procedure produces a positive result. This differs from spatial discordance, in which DNA is genuinely present but has been transported from another location, and from statistical occupancy error arising when observations are incorrectly translated into ecological occurrence estimates (Darling et al., 2021). Rivers provide a particularly important example because DNA can be transported downstream and therefore integrate biodiversity information over space (Deiner et al., 2016).

False-negative errors are equally important. Environmental DNA concentrations may be low because organisms are rare, shedding rates are low, DNA degrades rapidly, environmental conditions reduce DNA persistence, or sampling fails to capture the relevant DNA fraction. Laboratory processes introduce additional uncertainty through inhibition, extraction efficiency, assay sensitivity, and amplification stochasticity (Klymus et al., 2020; Burian et al., 2021).

These problems naturally lead to occupancy modelling. Classical occupancy models were developed to separate the ecological process of species occurrence from the observation process responsible for imperfect detection (MacKenzie et al., 2002, 2003). The framework has subsequently been adapted for eDNA, including models that explicitly represent multiple levels of replication and false-positive detection (Lahoz-Monfort et al., 2016; Smith & Goldberg, 2020; Doi et al., 2019; Fukaya et al., 2022).

A major consequence is that eDNA sampling design cannot be reduced to simply increasing the number of PCR reactions. Environmental replication and PCR replication operate at different levels of the detection process. Increasing independent environmental samples increases the opportunity to capture DNA that is spatially heterogeneous, whereas increasing PCR replication increases the probability that DNA present in an environmental sample is detected analytically. Fukaya et al. (2022) demonstrated the importance of modelling these nested stages jointly and showed that study design can be optimized according to the available budget. More recently, the NeMO framework has extended this logic to flexible nested multispecies occupancy models incorporating environmental-sample and PCR replication (Macé et al., 2026).

Freshwater and terrestrial ecosystems present different molecular sampling challenges. Freshwater systems often facilitate DNA transport and spatial integration, whereas terrestrial surveys may require carefully selected substrates such as soil, leaf

litter, waterholes, or airborne particles. Terrestrial sampling therefore presents greater heterogeneity in both substrate and spatial structure (Taberlet et al., 2012; van der Heyde et al., 2020, 2022).

The present study addresses this design problem using a Monte Carlo simulation framework. The principal research question was: How should a fixed molecular sampling budget be allocated between independent environmental samples and PCR replication when eDNA detection is imperfect, false-positive risk is non-zero, and detection probabilities differ between freshwater and terrestrial ecosystems?

The study had four specific objectives: (1) to quantify the effects of environmental-sample and PCR replication on eDNA detection probability; (2) to compare detection performance between freshwater and terrestrial ecosystems; (3) to quantify the accumulation of false-positive risk under increasing molecular replication; and (4) to determine how imperfect detection distorts naïve occupancy estimates and observed biodiversity richness.

2. Materials and Methods

2.1 Study design and research framework

A stochastic hierarchical model was developed to represent biodiversity detection through an eDNA workflow. The model consisted of four conceptual levels: ecological state, environmental DNA capture, PCR detection, and false-positive generation. The design was based on established occupancy and eDNA detection frameworks, in which the biological occurrence process is separated from the observation process (MacKenzie et al., 2002; Lahoz-Monfort et al., 2016; Fukaya et al., 2022).

Strategy	Environmental samples (J)	PCR replicates (K)	Total PCR
S1	40	3	120
S2	30	4	120
S3	20	6	120
S4	15	8	120
S5	10	12	120

2.2 Monte Carlo simulation framework

The simulation used 500 independent Monte Carlo realizations for every scenario. The factorial design contained two ecosystems, four occupancy probabilities, three field-level DNA-capture probabilities per ecosystem, three PCR detection probabilities, four false-positive probabilities, and five sampling strategies, generating 1,440 scenario–strategy combinations and 720,000 independent survey realizations. A fixed pseudorandom seed of 20260928 was used for reproducibility.

2.3 Ecological occupancy model

For taxon i at environmental sampling unit j , true occupancy was represented as:

$$Z_{ij} \sim \text{Bernoulli}(\psi_i) \quad (1)$$

where $Z_{ij} = 1$ indicates true occurrence, $Z_{ij} = 0$ indicates absence, and ψ_i is the true occupancy probability. Four occupancy levels were investigated: $\psi \in \{0.05, 0.10, 0.25, 0.50\}$.

2.4 Environmental DNA detection model

Freshwater scenarios used $pF \in \{0.20, 0.40, 0.60\}$, whereas terrestrial scenarios used $pF \in \{0.10, 0.25, 0.40\}$. PCR detection probabilities were $pP \in \{0.70, 0.80, 0.90\}$.

$$PPCR(K) = 1 - (1 - pP)^K \quad (2)$$

The resulting probability of detecting an occupied taxon in one environmental sampling unit was:

$$pD = pF [1 - (1 - pP)^K] \quad (3)$$

2.5 False-positive model

The per-PCR false-positive rate was $\alpha \in \{0, 0.0001, 0.001, 0.005\}$. The probability of at least one false-positive PCR result among K replicates was:

$$PFP, sample = 1 - (1 - \alpha)^K \quad (4)$$

For a complete survey containing $J \times K$ PCR reactions:

$$PFP, survey = 1 - (1 - \alpha)^{JK} \quad (5)$$

For occupied and unoccupied sampling units, respectively:

$$q1 = 1 - (1 - pD)(1 - PFP, sample) \quad (6)$$

$$q0 = PFP, sample \quad (7)$$

2.6 Freshwater and terrestrial scenarios

Freshwater and terrestrial systems were represented as distinct detection environments rather than assuming that eDNA behaves identically across ecosystem types. Freshwater eDNA can be transported through flowing systems and may integrate information over distances larger than the exact position of an organism, whereas terrestrial eDNA is strongly influenced by substrate selection and spatial heterogeneity (Deiner et al., 2016; Perry et al., 2024; van der Heyde et al., 2020, 2022).

2.7 Sampling-effort allocation

The fixed molecular budget was constrained by:

$$J \times K = 120 \quad (8)$$

The five strategies represented a gradient from environmental-sample intensive to PCR-intensive sampling. PCR replication primarily improves analytical detection conditional on DNA being represented in the sample, whereas environmental replication increases the number of independent opportunities to capture heterogeneous DNA distributions.

2.8 Simulation procedure

Generate true occupancy states for 100 taxa.

Generate occupied environmental units according to the specified ψ .

Simulate environmental DNA capture using ecosystem-specific pF .

Simulate PCR detection using pP and K .

Introduce false-positive reactions according to α .

Summarize detection histories at the environmental-sample level.

Calculate naïve occupancy without correcting for imperfect detection.

Calculate true and observed regional richness.

Calculate occupancy bias and RMSE by comparing observed estimates with simulated truth.

2.9 Performance metrics

The probability of detecting an occupied taxon somewhere within J environmental samples was:

$$Pregional = 1 - (1 - q1)^J \quad (9)$$

Naïve occupancy was calculated as:

$$\psi_{naive} = \frac{\sum_{j=1}^J Y_j}{J} \quad (10)$$

Occupancy bias was:

$$Bias = \hat{\psi} - \psi \quad (11)$$

Root mean square error was:

$$RMSE = \sqrt{[(1/n) \sum (\hat{\psi}_r - \psi)^2]} \quad (12)$$

2.10 Statistical analysis and uncertainty assessment

All reported numerical values were obtained from the Monte Carlo simulations. Means were calculated over 500 independent survey realizations per scenario. Simulation uncertainty was assessed from the empirical distribution of survey-level estimates. The simulation represents a controlled sensitivity analysis rather than empirical biodiversity measurements from a particular geographic region.

3. Results

3.1 Overall simulation performance

The complete factorial experiment comprised 1,440 scenario–strategy combinations and 500 Monte Carlo realizations per combination. The fixed 120-reaction budget produced a clear separation between the effects of PCR replication and environmental replication. Increasing PCR replication generally increased per-sample sensitivity, whereas increasing independent environmental samples produced substantially greater regional detection and reduced community-level richness loss, especially under low field-level DNA capture.

Allocation	Me per-sample sensitivity	Me an regional detection	Me an absolute occupancy bias	Me an occupancy RMSE	Me an richness bias
10 × 12	0.3 371	0.9 252	0.1 386	0.1 394	−23 .80
15 × 8	0.3 331	0.9 636	0.1 415	0.1 421	−23 .56
20 × 6	0.3 310	0.9 805	0.1 437	0.1 441	−22 .63
30 × 4	0.3 280	0.9 937	0.1 465	0.1 467	−20 .26
40 × 3	0.3 242	0.9 978	0.1 485	0.1 487	−17 .96

3.2 Effect of environmental sampling replication

The influence of environmental replication was most apparent under low field-level DNA capture. In the representative terrestrial scenario $\psi = 0.10$, $pF = 0.10$, $pP = 0.70$, and $\alpha = 0$, the regional detection probability was 0.9833 for 40 × 3 sampling but only 0.6513 for 10 × 12 sampling. Thus, increasing PCR replication could not compensate for the loss of independent environmental sampling opportunities.

Allocation	Pe r-sample detection	Regi onal detection	Na ïve occupanc y	T rue richness	Obse rved richness
40 × 3	0.0 973	0.98 33	0.0 098	98 .53	32.49
30 × 4	0.0 992	0.95 64	0.0 100	95 .84	26.06
20 × 6	0.0 999	0.87 82	0.0 102	88 .05	18.55
15 × 8	0.1 000	0.79 41	0.0 102	79 .44	14.16
10 × 12	0.1 000	0.65 13	0.0 103	65 .29	9.85

3.3 Effect of PCR replication

Increasing PCR replication produced a rapid increase in per-sample molecular detection probability, particularly when pP was moderate. Under $pF = 0.10$ and $pP = 0.70$, increasing PCR replication from three to six reactions increased per-sample detection from approximately 0.0973 to 0.0999, after which additional replication produced little further gain. This illustrates the diminishing-return structure of repeated molecular amplification.

3.4 Freshwater versus terrestrial detection

Freshwater scenarios generally produced higher regional detection under the parameter ranges used in the simulation because their field-level DNA-capture probabilities were higher. In the representative freshwater scenario $\psi = 0.25$, $pF = 0.40$, $pP = 0.80$, and $\alpha = 0$, regional detection remained approximately 1.0000 under 40×3 and approximately 0.9940 under 10×12 . Nevertheless, community-level richness still declined as independent environmental sampling decreased.

Allocation	Per-sample detection	Regional detection	Naïve occupancy	True richness	Observed richness
40×3	0.3 968	1.00 00	0.0 994	10 0.00	98.53
30×4	0.3 994	1.00 00	0.1 001	99. 97	95.99
20×6	0.4 000	1.00 00	0.1 002	99. 66	87.83
15×8	0.4 000	0.99 95	0.1 002	98. 65	79.40
10×12	0.4 000	0.99 40	0.1 002	94. 45	65.01

3.5 False-positive accumulation

Because every strategy contained exactly 120 PCR reactions, the probability of at least one false-positive reaction was identical across strategies at a given per-reaction false-positive rate.

Per-PCR α	Probability of ≥ 1 false-positive among 120 reactions
0	0.0000
0.0001	0.0119
0.001	0.1131
0.005	0.4520

3.6 Occupancy estimation

Naïve occupancy estimates were strongly downward biased when true occupancy was moderate but detection probability was low. In the representative freshwater scenario with true occupancy $\psi = 0.25$ and detection probability near 0.40, naïve occupancy was approximately 0.10. This illustrates why raw eDNA detection frequency should not automatically be interpreted as true occurrence probability. When false positives were introduced, the negative bias was partially offset because positive observations could arise at unoccupied sites.

3.7 Biodiversity richness and community-level consequences

The largest difference among strategies emerged at the community level. Under low terrestrial DNA capture, the 40×3 strategy recovered approximately 32.5 taxa compared with approximately 9.9 taxa under 10×12 sampling in the representative $\psi = 0.10$ scenario. The corresponding true richness across the sampled environmental units was approximately 98.5 and 65.3 taxa, respectively. This indicates that observed richness depends jointly on the number of ecological units sampled and the effectiveness of molecular analysis within each unit.

4. Discussion

The present Monte Carlo analysis demonstrates that environmental replication and PCR replication should not be regarded as interchangeable forms of eDNA replication. They address different stages of the observation process and consequently

produce different effects on biodiversity monitoring.

The principal result was that increasing independent environmental samples generally produced larger gains in regional biodiversity recovery than increasing PCR replication under a fixed 120-reaction budget. This effect was particularly strong when field-level DNA capture was low. In contrast, increasing PCR replication improved detection probability within individual environmental samples and produced smaller incremental benefits once molecular detection was already high.

This distinction is consistent with the hierarchical structure of eDNA detection. The probability of detecting an occupied taxon can be represented as $pD = pF[1 - (1 - pP)^K]$, where pF represents environmental capture and the second term represents molecular detection. Increasing K can only improve the molecular component. It cannot increase the probability that the relevant DNA was physically represented in the environmental sample.

The results are consistent with Fukaya et al. (2022), who developed a multispecies occupancy framework that explicitly represented the nested stages of eDNA metabarcoding and evaluated study-design trade-offs under limited budgets. The present simulation extends that conceptual framework by comparing freshwater and terrestrial field-capture scenarios and explicitly incorporating false-positive accumulation.

The false-positive results provide an additional reason not to interpret PCR replication as an unlimited solution to detection uncertainty. With 120 reactions, a per-reaction false-positive probability of 0.001 generated an 11.31% probability of at least one false-positive reaction. At $\alpha = 0.005$, this increased to 45.20%. The probability of at least one false-positive reaction is not equivalent to the proportion of taxa incorrectly classified as present; it is an error-opportunity metric.

Darling et al. (2021) emphasized the importance of distinguishing molecular false positives from other forms of mismatch between molecular detection and ecological occurrence. This distinction is particularly relevant in freshwater systems, where genuine DNA transported from elsewhere can generate a detection that is molecularly correct but spatially discordant.

The occupancy results further demonstrate why raw eDNA detection frequency should not automatically be interpreted as true occurrence probability. Classical occupancy models were developed specifically because species can be present but undetected (MacKenzie et al., 2002, 2003). When false positives are absent, naïve occupancy is attenuated toward zero as detection probability declines. When false positives occur simultaneously, the observed estimate becomes the result of two opposing error processes.

The simulation also has implications for biodiversity richness metrics. Richness is especially sensitive to false negatives because rare taxa are represented by fewer opportunities for detection. A taxon with low occupancy and low environmental capture probability can be missed throughout a survey even when PCR replication is extensive.

The findings therefore support a hierarchical interpretation of eDNA survey design: ecological occurrence → DNA availability → environmental capture → PCR detection → sequence assignment. Errors at each stage can propagate to the final biodiversity estimate.

A particularly important implication is that more sequencing is not synonymous with better biodiversity monitoring. Increasing PCR replication can compensate for some analytical uncertainty, but it cannot recover information that was never captured in the environmental sample.

The appropriate allocation therefore depends on which component of the detection process is limiting. When pF is low and pP is already reasonably high, environmental replication becomes especially valuable. When pF is high but pP is low, additional PCR replication can provide substantial gains. When both are high, additional replication may yield diminishing returns.

The present study has several limitations. The model assumes conditional independence among taxa and does not explicitly represent ecological interactions, spatial autocorrelation, abundance-dependent shedding, directional freshwater transport, substrate-specific terrestrial processes, or correlated contamination. The parameter ranges are scenario values for sensitivity analysis rather than universal empirical estimates. Future models should incorporate these processes and fit full hierarchical occupancy models to simulated detection histories.

5. Future Perspectives

The future development of environmental DNA (eDNA)-based biodiversity monitoring is likely to move from relatively static species-detection surveys toward integrated, spatially explicit, temporally resolved, quantitative, and predictive biodiversity surveillance systems. The Monte Carlo framework developed in this study demonstrates that sampling allocation is not merely a logistical consideration but a determinant of inference quality. Future methodological development should therefore focus on integrating environmental sampling, molecular replication, ecological modelling, and computational prediction within a unified framework.

A major priority will be the development of fully hierarchical and multispecies occupancy models capable of simultaneously representing ecological occurrence, environmental DNA availability, sample-level capture, PCR detection, sequencing uncertainty, and false-positive processes. Existing occupancy frameworks have already demonstrated the value of separating ecological and observation processes (MacKenzie et al., 2002, 2003; Lahoz-Monfort et al., 2016), while eDNA-specific developments have extended these concepts to replicated metabarcoding surveys and nested detection probabilities (Ficetola et al., 2015; Doi et al., 2019; Fukaya et al., 2022). Future models should move beyond fixed detection probabilities and allow taxon-, habitat-, season-, substrate-, and environmental-condition-specific detection parameters. Recent flexible modelling frameworks such as NeMO provide a foundation for this development by enabling nested multispecies occupancy analyses and optimization of eDNA study designs (Macé et al., 2026). Integrating these approaches with simulation-based experimental design could allow researchers to determine sampling configurations before fieldwork begins, thereby reducing inefficient allocation of molecular resources.

The next generation of eDNA monitoring should also become increasingly spatially explicit. Freshwater systems present a particularly important opportunity because DNA can be transported through rivers and streams, meaning that an observed molecular signal may represent organisms located upstream rather than at the exact sampling point (Deiner et al., 2016). Consequently, future models should couple eDNA observations with hydrological transport, DNA degradation, dilution, sedimentation, discharge, and flow velocity. Recent work demonstrating the value of integrated spatio-temporal riverine eDNA observations highlights the potential of using eDNA not simply as a site-level presence–absence tool but as a spatial biodiversity-information system (Perry et al., 2024). Such models could ultimately reconstruct probable source areas and distinguish local biodiversity signals from downstream transport.

Terrestrial eDNA monitoring requires parallel methodological development. Soil, leaf litter, water bodies, airborne particles, and other terrestrial substrates differ considerably in DNA persistence, extraction efficiency, spatial heterogeneity, and susceptibility to environmental degradation. Terrestrial ecological restoration studies have already emphasized the importance of substrate and sampling decisions in determining metabarcoding performance. Future research should therefore develop substrate-specific detection models rather than transferring aquatic eDNA assumptions directly to terrestrial systems. Combining spatially replicated terrestrial sampling with occupancy models could substantially improve detection of rare, cryptic, or otherwise difficult-to-observe taxa.

Another important direction is the transition from presence–absence detection toward quantitative and abundance-informed eDNA monitoring. Although eDNA

metabarcoding is highly effective for determining community composition, translating sequence information into organismal abundance remains challenging because DNA production, transport, degradation, extraction, amplification, and sequencing introduce multiple sources of nonlinearity (Thomsen & Willerslev, 2015; Klymus et al., 2020). Future studies should integrate molecular concentration, quantitative PCR or digital PCR, sequencing read abundance, organismal biomass, and independent ecological observations within hierarchical models. Such approaches could allow eDNA to provide estimates of relative or absolute abundance rather than simply binary detection.

False-positive control and error decomposition will remain central to reliable molecular biodiversity surveillance. Molecular false positives, contamination, tag switching, non-specific amplification, and spatially displaced DNA should be explicitly distinguished rather than treated as one generic error term (Schnell et al., 2015; Ficetola et al., 2016; Darling et al., 2021). Future computational frameworks should therefore model different error mechanisms separately. This is particularly important because increasing PCR replication can increase the opportunity for both successful detection and erroneous positive reactions. The present simulation showed that even a relatively small per-reaction false-positive probability can accumulate substantially across a molecular survey. Future experimental designs should consequently combine replication with negative controls, field blanks, extraction controls, positive controls, assay validation, and statistical false-positive correction.

The increasing scale of biodiversity monitoring will also require automation and artificial intelligence. Large eDNA datasets contain complex taxonomic, sequence, spatial, temporal, and environmental information that increasingly exceeds what can be efficiently interpreted manually. Machine-learning approaches could be used to classify sequence reads, identify anomalous molecular signals, predict detection probability, identify contamination patterns, and estimate biodiversity under environmental uncertainty. AI-based systems could also integrate eDNA observations with remote sensing, climate variables, hydrological information, land-use data, and conventional biodiversity surveys. Rather than replacing ecological models, such approaches should be incorporated into transparent hierarchical frameworks in which predictions remain biologically interpretable.

A related future opportunity is the development of near-real-time and autonomous eDNA surveillance. Current eDNA workflows often require field collection followed by laboratory filtration, extraction, amplification, sequencing, and bioinformatic processing. Advances in portable molecular technologies and automated environmental samplers could shorten this pipeline substantially. Autonomous sampling platforms deployed in rivers, lakes, wetlands, or protected terrestrial landscapes could repeatedly collect environmental material and transmit molecular observations to centralized analytical systems. Combined with statistical change detection, these systems could provide early warnings of invasive species, biodiversity loss, pathogen emergence, or ecosystem disturbance.

Future eDNA research should also emphasize long-term temporal monitoring. Many existing studies provide snapshots of biodiversity rather than continuous measurements. However, biodiversity responses to climate change, pollution, habitat degradation, restoration, invasive species, and extreme environmental events are inherently dynamic. Repeated eDNA surveys could generate time series capable of detecting changes in community composition before conventional surveys reveal substantial ecological deterioration. Temporal occupancy models and dynamic state-space approaches could be integrated with eDNA detection models to estimate colonization, extinction, persistence, and seasonal turnover while accounting for imperfect observation (MacKenzie et al., 2003; Smith & Goldberg, 2020).

Standardization and interoperability will become increasingly important as eDNA datasets expand across geographical regions. Differences in filtration methods, sample volumes, DNA extraction protocols, primer selection, PCR replication,

sequencing depth, bioinformatic pipelines, taxonomic databases, and detection thresholds can make direct comparison between studies difficult. Future international monitoring programs should therefore establish standardized metadata requirements, reporting standards, quality-control procedures, reference databases, and reproducible analytical workflows. Such harmonization would facilitate large-scale biodiversity assessments and allow independent datasets to be combined into regional and global biodiversity observatories.

The integration of eDNA with conventional ecological observations represents another important research frontier. eDNA should not necessarily be viewed as a replacement for trapping, visual surveys, acoustic monitoring, electrofishing, camera trapping, or morphological identification. Instead, complementary data streams can be combined within hierarchical models. Conventional observations can provide independent information about abundance or life-history characteristics, whereas eDNA can increase spatial and taxonomic coverage. Joint models could therefore estimate biodiversity while explicitly accounting for the different detection processes associated with each monitoring method.

The framework developed here could also be extended to climate-change and ecosystem-change forecasting. Future simulations could incorporate changing temperature, precipitation, hydrology, habitat fragmentation, and environmental DNA persistence to predict how detection probabilities themselves may change under future environmental conditions. This would allow researchers to distinguish a genuine decline in biodiversity from a decline in detectability caused by environmental changes affecting DNA production, persistence, or transport. Such modelling will be particularly important for long-term monitoring programs in which environmental conditions may differ substantially between baseline and future surveys.

Finally, future research should move toward adaptive eDNA survey design, in which sampling effort is dynamically modified according to information obtained during earlier sampling rounds. Rather than fixing the number of environmental samples and PCR replicates before fieldwork, sequential designs could identify locations or taxa with high uncertainty and allocate additional sampling accordingly. The Monte Carlo framework presented in this study provides a foundation for such optimization because it explicitly quantifies how alternative allocations of a fixed molecular budget influence detection and biodiversity recovery. Future extensions could combine Monte Carlo simulation, Bayesian decision theory, occupancy modelling, machine learning, and real-time molecular observations to create adaptive monitoring systems that continuously optimize sampling effort.

Overall, the future of eDNA-based biodiversity monitoring lies in moving beyond simple molecular detection toward integrated ecological inference. The combination of multispecies occupancy modelling, spatial and temporal process models, quantitative molecular measurements, AI-assisted analysis, autonomous sampling, rigorous error control, and adaptive experimental design could transform eDNA from a molecular survey technique into a comprehensive biodiversity-monitoring infrastructure. Such development should remain grounded in transparent uncertainty quantification: greater sequencing depth or computational complexity should not automatically be interpreted as greater ecological accuracy. Instead, future monitoring systems should explicitly identify the dominant source of uncertainty and allocate field, molecular, and computational resources to the component that most strongly limits reliable biodiversity inference.

6. Conclusion

This Monte Carlo study demonstrates that eDNA biodiversity monitoring is fundamentally a nested detection problem in which ecological occurrence, environmental DNA capture, PCR amplification, and false-positive processes contribute differently to the final observation. Under a fixed molecular budget of 120 PCR reactions, environmental-sample replication and PCR replication produced

distinct benefits. Increasing PCR replication improved the probability of detecting DNA within individual environmental samples, whereas increasing independent environmental samples produced substantially greater gains in regional biodiversity recovery when field-level DNA capture was low. False-positive risk also accumulated with molecular effort, reinforcing the need to combine replication with contamination control, assay validation, negative controls, and statistical treatment of false-positive errors. The principal methodological implication is that eDNA survey design should identify the dominant source of uncertainty and allocate effort accordingly rather than treating sequencing depth as a universal solution. Future work should extend the framework by incorporating continuous abundance-dependent DNA production, spatially explicit freshwater transport, terrestrial substrate-specific detection, temporal dynamics, taxon-specific detection heterogeneity, correlated contamination, and full hierarchical occupancy-model fitting.

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