Small-Scale Spatiotemporal Variation in eDNA Monitoring Results in Large Rivers and Recommendations for Repeated Sampling

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Abstract

Sample replication design constitutes a critical initial step in the standardization of eDNA monitoring technology. While previous studies have investigated the appropriate number of sample replicates, whether replicates should be established as a spatial series of sampling sites or as temporally continuous sampling is vitally important for eDNA monitoring practice yet has not been carefully examined. To address this research gap, this study employs the Wuhan section of the Yangtze River as a case study to explore recommendations for replicate sampling in eDNA monitoring of large rivers through analysis of species composition detected via different replicate sampling strategies. The results demonstrate that for bacteria and metazoans, spatial series samples in eDNA monitoring yielded greater cumulative species numbers than temporal series samples, with spatial heterogeneity of detected species composition exceeding temporal heterogeneity; conversely, the opposite pattern was observed for the three fine taxonomic groups of fungi, algae, and protozoa. Therefore, we recommend that in large rivers, spatial replicate sampling should be prioritized in sample replication design for eDNA monitoring of environmental microorganisms and metazoans, whereas temporal replicate sampling should be prioritized for monitoring fungi, algae, and protozoa. When implementing spatial replicate sampling, attention should be devoted to sampling time selection; when implementing temporal replicate sampling, attention should be devoted to sampling site selection; and for monitoring fine taxonomic groups, care should be taken to ensure adequate sample replicate numbers.

Full Text

Small-Scale Temporal and Spatial Heterogeneity of eDNA Monitoring and Recommendations for Replicated Sampling in Large Rivers

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Abstract

Designing replicated samples is a critical initial step in standardizing eDNA monitoring protocols. While previous studies have investigated how many replicates should be collected, the question of whether these replicates should be distributed spatially across multiple sites or temporally through continuous sampling has not been carefully examined, despite its importance for eDNA monitoring practice. To address this gap, we conducted a case study in the Wuhan section of the Yangtze River, analyzing species composition detected through different replication strategies. We collected 16 daily eDNA samples from June 27 to July 14, 2022 (temporal group) and 16 eDNA samples across a river transect on two dates (June 28 and July 12, 2022) (spatial group). Results showed that for bacteria and metazoa, spatial replicates detected more cumulative species and exhibited greater spatial heterogeneity than temporal replicates. Conversely, for fungi, algae, and protozoa, the opposite pattern was observed. We therefore recommend prioritizing spatial replication when monitoring environmental microorganisms and metazoa in large rivers, while prioritizing temporal replication for fungi, algae, and protozoa. When implementing spatial replication, careful attention should be paid to sampling timing; for temporal replication, sampling site selection is crucial. Additionally, monitoring of fine taxonomic groups requires adequate sample replication.

Keywords: environmental DNA monitoring; small-scale temporal and spatial heterogeneity; replicated sampling; 16S rRNA gene; COI gene; Yangtze River

Introduction

Environmental DNA (eDNA) monitoring extracts DNA mixtures from environmental samples (water, soil, sediment, air, mixtures, etc.), amplifies and sequences them using species-specific primers or DNA meta-barcoding primers, and conducts taxonomic analysis, relative abundance analysis, and functional prediction to detect specific species or obtain information on species composition, community structure, and ecological functions [1-4]. This approach has

attracted widespread exploratory attention and application in academic circles both internationally and domestically [5-8], offering broad prospects for biodiversity monitoring [7, 9]. To enhance the credibility of eDNA monitoring results, the current primary task is to advance its technical standardization [10-14], which mainly includes sample replication design, sampling timing design, sampling site design, sampling method design, sample pretreatment, sample preservation, primer selection, DNA extraction and amplification sequencing protocols, sequence alignment and annotation, and post-assessment of monitoring results [4].

Sample replication design is the foremost critical step in eDNA monitoring standardization [4]. On one hand, increasing the number of eDNA monitoring replicates yields more detected taxa (and stabilizes the relative abundance structure), following a logarithmic species accumulation curve similar to traditional species surveys [15, 16]. On the other hand, the relationship between replicate number and detected taxa is also influenced by small-scale temporal and spatial heterogeneity of eDNA within the sampling pool—greater heterogeneity requires more replicates. Under practical constraints on replicate numbers, if spatial heterogeneity exceeds temporal heterogeneity, sampling should emphasize spatial replication; conversely, if temporal heterogeneity is greater, temporal replication should be prioritized. However, no studies have carefully examined whether routine eDNA monitoring should focus on spatial or temporal replication.

This study addresses this research gap by investigating eDNA information spatial heterogeneity across the Yangtze River and temporal heterogeneity over two weeks in the Wuhan section. We conducted eDNA monitoring experiments targeting bacteria (detected and indicated by 16S rRNA gene) and eukaryotes (detected and indicated by mitochondrial COI gene) along with specific fine taxonomic groups. By quantitatively comparing temporal and spatial heterogeneity characteristics, we provide references for designing replicated sampling strategies for corresponding taxa in large river eDNA monitoring.

Methods

Study Area and Sampling Design

From June 27 to July 14, 2022, we conducted 16 consecutive days of eDNA monitoring near the Wuhan Fisheries Administration Wharf, with two additional cross-river transect surveys (each with approximately eight evenly distributed sampling points) conducted on June 28 and July 12 [FIGURE:1, TABLE:1]. During the monitoring period, water temperature ranged from 26.6 to 29.6°C (showing a warming trend), water level at the Hankou hydrological station ranged from 22.96 to 24.65 m (showing a decreasing trend), and discharge ranged from 32,800 to 40,200 m^3/s (showing a decreasing trend) .

Sample Collection and Processing

Surface water samples (30–50 cm depth) were collected in sterile bottles (1.5 L each). Bottles were rinsed three times with the water to be sampled before collection. Samples were stored on ice and filtered in the laboratory within 1–6 hours after collection using 0.2 m pore-size filters. Filters retaining eDNA were placed in 50 mL sterile centrifuge tubes, sealed in self-sealing bags, stored at –80°C, and transported in foam boxes with dry ice.

Molecular Analysis

Shanghai Majorbio Bio-Pharm Technology Co., Ltd. performed eDNA extraction, meta-barcoding PCR amplification, and sequencing for mitochondrial COI (primers mlCOIintF/jgHCO2198R) and 16S rRNA genes (primers 338F/806R). Sequence data analysis was conducted on the Majorbio Cloud Platform (www.majorbio.com) to obtain species-level detections. Mitochondrial COI sequences were clustered at 97% OTU similarity with species annotation at 0.9 confidence using the nt database; 16S rRNA gene sequences were clustered at 97% OTU similarity with 0.7 confidence using the silva138.1/16s_{bacteria} database.

Statistical Analysis

We analyzed species composition detected in each sample and calculated cumulative species numbers for temporal and spatial sample groups. Species accumulation curves were computed using EstimateS (Version 9.1.0, Copyright R. K. Colwell: http://purl.oclc.org/estimates). Heterogeneity of species composition among samples was indicated by the ratio of cumulative species number to average species number per sample.

Results

2.1 Overview of eDNA Monitoring Results

Based on mitochondrial COI gene meta-barcoding (primers mlCOIintF/jgHCO2198R), we detected 513 species across 4 kingdoms, 28 phyla, 66 classes, 137 orders, 208 families, and 304 genera of eukaryotes, including 162 fungi, 123 algae, 27 protozoa, and 201 metazoa (Supplementary Table 1). Metazoa comprised 48 rotifers, 4 poriferans, 1 cnidarian, 2 platyhelminths, 4 nematodes, 2 gastrotrichs, 29 annelids, 54 arthropods, 6 mollusks, and several unannotated invertebrates (Supplementary Table 1). Based on 16S rRNA gene meta-barcoding (primers 338F/806R), we detected 1,539 bacterial species across 57 phyla, 174 classes, 399 orders, 661 families, and 1,357 genera, plus 1,280 uncultured and unclassified species (Supplementary Table 2).

2.2 Temporal and Spatial Heterogeneity in eDNA Monitoring Results

Analysis at three taxonomic levels and two grouping schemes revealed temporal and spatial heterogeneity in eDNA detection results. Cumulative species curves showed continuous upward trends both day-by-day and sample-by-sample. Under equal sample sizes, spatial replication detected more species than temporal replication, though differences were modest and varied among fine taxonomic groups [FIGURE:2–FIGURE:4].

For prokaryotes (bacteria), species detection showed a slight decreasing trend temporally and higher nearshore than mid-river values spatially. Temporal heterogeneity (2.37) was slightly lower than spatial heterogeneity (2.61), with cumulative species detection slightly lower in temporal replicates (2,461) than spatial replicates (2,543). For eukaryotes, species detection fluctuated randomly without clear trends in either dimension. Temporal heterogeneity (3.58) was slightly higher than spatial heterogeneity (3.40), with cumulative species detection slightly lower in temporal replicates (409) than spatial replicates (415) [FIGURE:2, TABLE:2].

[**Figure 2: see original paper**] shows species numbers, accumulated species numbers, and rarefaction curves for prokaryotes (bacteria) and eukaryotes detected in temporal and spatial sample groups.

**** presents species numbers and species composition heterogeneity for each taxonomy detected in temporal and spatial sample groups.

When eukaryotes were subdivided, fungi showed random fluctuations and obvious heterogeneity in both dimensions, with temporal heterogeneity (7.06) stronger than spatial heterogeneity (5.75) and more cumulative species detected in temporal replicates (132) than spatial replicates (115). Algae showed random fluctuations without clear trends, with temporal heterogeneity (2.14) slightly higher than spatial heterogeneity (2.00) and similar cumulative species numbers between temporal (105) and spatial (107) replicates. Protozoa showed random fluctuations and obvious heterogeneity, with temporal heterogeneity (4.42) stronger than spatial heterogeneity (4.13) and similar cumulative species numbers between temporal (21) and spatial (23) replicates. Metazoa showed random temporal fluctuations and a slight spatial trend of higher nearshore values, with temporal heterogeneity (3.67) weaker than spatial heterogeneity (4.03) and fewer cumulative species in temporal replicates (150) than spatial replicates (169) [FIGURE:3, TABLE:2].

[**Figure 3: see original paper**] shows species numbers, accumulated species numbers, and rarefaction curves for fungi, algae, protozoa, and metazoa detected in temporal and spatial sample groups.

Further subdivision of metazoa revealed that plankton showed random fluctuations in both dimensions with similar temporal and spatial heterogeneity (2.60 each) and slightly fewer cumulative species in temporal replicates (43) than spatial replicates (44). Benthos showed random temporal fluctuations with a slight

spatial trend of higher nearshore values, with temporal heterogeneity (3.91) weaker than spatial heterogeneity (4.38) and fewer cumulative species in temporal replicates (67) than spatial replicates (84). Fish showed random temporal fluctuations with a slight spatial trend of higher nearshore values, with temporal heterogeneity (5.61) weaker than spatial heterogeneity (6.63) but more cumulative species in temporal replicates (34) than spatial replicates (29) [FIGURE:4, TABLE:2].

[**Figure 4: see original paper**] shows species numbers, accumulated species numbers, and rarefaction curves for plankton, benthos, and fish (metazoa) detected in temporal and spatial sample groups.

Discussion

Spatial replicates detected more cumulative species than temporal replicates (except for fungi and fish), and exhibited greater spatial heterogeneity than temporal heterogeneity (except for fungi, algae, and protozoa). In large rivers, water flow cannot fully mix within short distances. Influenced by riparian soil microbial inputs [17, 18], nearshore waters contain slightly more detectable bacterial taxa than mid-river areas [Figure 2: see original paper]. Because bacteria contribute a high proportion of living or dormant individuals to watershed biological information flow and have long transport half-distance for non-living cellular debris [17-19], temporal heterogeneity in detected bacterial species composition is slightly lower than spatial heterogeneity .

Fungi, algae (phytoplankton), and protozoa (zooplankton) are native aquatic groups with relatively consistent species and community composition at small spatial scales. Despite high proportions of living individuals and long transport half-distances for non-living debris, temporal replicates detected slightly more or similar cumulative species compared to spatial replicates, with temporal heterogeneity slightly exceeding spatial heterogeneity . Metazoa (e.g., benthos, fish) are also native aquatic groups but show distinct distribution and activity patterns in large water bodies, with lower proportions of living individuals and shorter transport half-distances for non-living debris. Consequently, temporal replicates detected fewer cumulative species than spatial replicates, with temporal heterogeneity lower than spatial heterogeneity . For example, fish and benthos tend to inhabit and remain active in nearshore shallow areas, continuously releasing eDNA, resulting in slightly higher detectable species richness nearshore than mid-river (similar to large lakes [20, 21]) and greater spatial heterogeneity .

Therefore, we recommend prioritizing spatial replication when monitoring environmental microorganisms and metazoa in large rivers, while prioritizing temporal replication for fungi, algae, and protozoa. As eDNA is not uniformly distributed in water, different samples yield varying eDNA signals. The finer the target taxonomic group, the lower the probability that these differential

signals fall within the same group, leading to greater temporal and spatial heterogeneity, though with some taxon-specific variation . In this study, heterogeneity at the first level (prokaryotes, eukaryotes) was generally lower than at the second level (fungi, algae, protozoa, metazoa within eukaryotes) and third level (plankton, benthos, fish within metazoa). Algae within eukaryotes and plankton within metazoa were exceptions, showing lower heterogeneity than their parent groups . Thus, adequate replication is essential for fine taxonomic monitoring.

Temporal and spatial heterogeneity patterns vary among target groups and warrant consideration in practice. For bacteria (requiring spatial priority), riparian soil microbial inputs [17, 18] create substantial differences in detected species and composition between rainy days with sediment influx and clear days, suggesting that sampling timing and weather conditions should be selected based on monitoring objectives. For fish (also requiring spatial priority), species disperse to nearshore shallow areas during rising water periods and congregate in deep mid-channel areas during falling water periods, with more active movement in upper water layers during rising water. Consequently, more fish species are detected in nearshore and upper-layer waters during rising water periods [TABLE:1, FIGURE:4], making rising water periods preferable for sampling. Additionally, taxa detected via extracellular eDNA rather than intracellular DNA within cell walls (e.g., fish) are often negatively affected by temperature increases [TABLE:1, FIGURE:4], a factor to consider in monitoring and result interpretation. For fungi, algae, and protozoa (requiring temporal priority), community composition and biomass are typically richer in meandering, low-flow reaches and poorer in straight, high-flow reaches, suggesting that sampling sites should be selected according to monitoring objectives.

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Note: Figure translations are in progress. See original paper for figures.