UNIVERSIDADE FEDERAL DO PARANÁ

VILMAR BIERNASKI

BEHAVIORAL DYNAMICS OF ENVIRONMENTAL DNA (eDNA) DISPERSION IN THE WATER COLUMN

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Orientador: Prof. Dr. Giorgi Dal Pont

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RESUMO

O DNA ambiental (eDNA) emergiu como ferramenta poderosa para o monitoramento da biodiversidade aquática, permitindo a detecção não invasiva e altamente sensível de diferentes grupos biológicos. No entanto, seu comportamento de distribuição vertical e dinâmica de dispersão na coluna d'água ainda são pouco compreendidos, limitando a precisão das avaliações ecológicas e estratégias de monitoramento ambiental. O objetivo desse trabalho é investigar o movimento vertical do eDNA e esclarecer os fatores que influenciam sua distribuição, por meio da análise de dados pretéritos (revisão sistemática) e de experimentos laboratoriais controlados. A revisão analisou 87 estudos sobre a dinâmica vertical do eDNA, revelando variações significativas na concentração e composição do eDNA em diferentes profundidades. Os resultados destacam o impacto de fatores abióticos, como profundidade do ambiente estudado, estratificação térmica e correntes, além de influências biológicas, incluindo o comportamento das espécies e as taxas de degradação do eDNA, ressaltando, em alguns casos, a necessidade de protocolos padronizados de amostragem em múltiplas profundidades para maior precisão na detecção. Complementando essa revisão, experimentos laboratoriais avaliaram a dispersão do DNA extracelular livre (f-exDNA) e do eDNA multi-fração em uma coluna d'água lêntica. O f-exDNA se dispersou rapidamente, tornando-se homogêneo em até 30 minutos, enquanto o eDNA multi-fração apresentou padrões mais lentos e distintos de dispersão. Esses achados evidenciam que a fração de eDNA influencia diretamente sua dispersão, reforçando a necessidade de considerar sua composição ao definir estratégias de amostragem. Este estudo contribui para a compreensão da verticalidade do eDNA e propõe diretrizes para otimizar amostragens, aprimorando sua aplicação no biomonitoramento ambiental e na conservação.

Palavras-chave: coluna d'água; DNA ambiental; dispersão de eDNA; distribuição vertical; ecossistemas aquáticos; monitoramento da biodiversidade.

ABSTRACT

Environmental DNA (eDNA) has emerged as a powerful tool for monitoring aquatic biodiversity, enabling the non-invasive and highly sensitive detection of various biological groups. However, its vertical distribution behavior and dispersion dynamics in the water column remain poorly understood, limiting the accuracy of ecological assessments and environmental monitoring strategies. This study aims to investigate the vertical movement of eDNA and clarify the factors influencing its distribution through the analysis of previous data (systematic review) and controlled laboratory experiments. The review analyzed 87 studies on the vertical dynamics of eDNA, revealing significant variations in eDNA concentration and composition at different depths. The results highlight the impact of abiotic factors, such as study site depth, thermal stratification, and currents, as well as biological influences, including species behavior and eDNA degradation rates, underscoring in some cases the need for standardized multi-depth sampling protocols for greater detection accuracy. Complementing this review, laboratory experiments assessed the dispersion of free extracellular DNA (f-exDNA) and multi-fraction eDNA in a lentic water column. The f-exDNA dispersed rapidly, becoming homogeneous within 30 minutes, while the multi-fraction eDNA exhibited slower and distinct dispersion patterns. These findings demonstrate that the eDNA fraction directly influences its dispersion, reinforcing the need to consider its composition when defining sampling strategies. This study contributes to the understanding of eDNA verticality and proposes guidelines to optimize sampling, enhancing its application in environmental biomonitoring and conservation.

Keywords: aquatic ecosystems; biodiversity monitoring; eDNA dispersion; environmental DNA; vertical distribution; water column.

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GENERAL PRESENTATION

The conservation of biodiversity is one of the most pressing environmental challenges today (SEDDON *et al.*, 2016). This issue is particularly critical in aquatic ecosystems, which face increasing pressures from both natural factors, such as climate change, and human activities, including pollution, dam construction, and overfishing (ARTHINGTON *et al.*, 2016; BAI *et al.*, 2024; LAPOINTE *et al.*, 2013; MANTYKA-PRINGLE *et al.*, 2014; REID *et al.*, 2019). The complexity of ecological interactions in these environments and the interdependence among organisms make it essential to monitor aquatic biodiversity to understand ecological dynamics, assess environmental impacts, and support sustainable management strategies (GOLPOUR *et al.*, 2022). Ichthyofauna, benthic invertebrates, phytoplankton, and zooplankton are the main groups of monitored aquatic organisms, each group playing fundamental roles in ecosystems.

Historically, the identification and monitoring of aquatic organisms have been based on morphological taxonomic methods, which classify species according to their external characteristics (GOLPOUR *et al.*, 2022). While these approaches have been fundamental to advancing biodiversity knowledge, they present significant limitations (IKNAYAN *et al.*, 2014). The accuracy of these analyses depends on highly trained professionals and specialized reference materials, in addition to requiring considerable time and resources for sample collection, sorting, and identification (RADINGER *et al.*, 2019). Furthermore, traditional monitoring methods, such as trawl nets, traps, and direct sampling, tend to be selective, favoring the detection of certain groups while underestimating smaller organisms with different life strategies or at specific ontogenetic stages. Cryptic species, those with low population densities, or those associated with hard-to-access microhabitats may also be underrepresented. Another limiting factor is the invasive nature of these approaches, which often involve the direct handling of organisms, potentially causing stress, injury, or even removal from their natural habitats. Additionally, these techniques require substantial logistical investment in terms of time, personnel, and equipment,

making traditional monitoring costly and, in some cases, limited in terms of representativeness and spatial coverage (SEYMOUR *et al.*, 2021).

To overcome these challenges, molecular techniques have emerged as promising alternatives for environmental monitoring (MANFRIN *et al.*, 2019). Among these approaches, environmental DNA (eDNA) analysis stands out, as it involves detecting genetic material released by organisms into the environment through cells, feces, mucus, or skin fragments (WANG *et al.*, 2019). This DNA can be collected directly from water in a non-invasive manner and analyzed to infer species presence, providing a more efficient strategy with minimal impact on natural populations. eDNA-based techniques can be categorized into two main groups: targeted methods, such as quantitative PCR (qPCR) and droplet digital PCR (ddPCR), which enable the detection and quantification of specific species with high sensitivity; and broad-spectrum approaches, such as metabarcoding, which utilizes next-generation sequencing to identify multiple taxa simultaneously from a single sample (COBLE *et al.*, 2019). Metabarcoding, for example, has proven to be a powerful tool for studying complex biological communities, as it combines speed, high sensitivity, and the ability to generate comprehensive biodiversity assessments (ZHANG *et al.*, 2023).

The use of eDNA for monitoring aquatic biodiversity has expanded rapidly, establishing itself as an effective tool to overcome the limitations of traditional approaches. This growth reflects not only the reliability and efficiency of the technique but also the increasing recognition of its applicability in different ecological contexts. Takahashi et al. (2023) demonstrated that the number of publications on eDNA in aquatic environments has increased significantly in recent years, particularly since 2016. Most of these studies focus on freshwater ecosystems and fish detection, but there has also been a notable rise in the application of this technique in marine environments and for other taxonomic groups, such as invertebrates and amphibians. This progress highlights the central role of eDNA in improving biodiversity monitoring strategies, making it an increasingly adopted method for ecological research and the development of conservation and environmental management policies.

Despite the significant growth in eDNA use, the technique is still relatively recent in the study of aquatic macroorganisms. The first study to apply it in this context, conducted by Ficetola et al. (2008), was published just over a decade

ago, emphasizing the emerging nature of this tool. Consequently, many questions about its application remain unanswered, particularly given the diversity and complexity of aquatic environments. Factors such as degradation, transport, and the retention time of genetic material in water vary widely across different systems and can directly influence data interpretation (BARNES and TURNER, 2016; HUANG *et al.*, 2022; TURNER, UY and EVERHART, 2015). In particular, the processes of eDNA transport and dispersion require further understanding, as they are affected by variables such as currents, stratification, sedimentation, and degradation, making it difficult to establish clear patterns for its detection (HARRISON, SUNDAY and ROGERS, 2019). Therefore, deepening knowledge of these mechanisms is essential for refining sampling and analysis protocols, ensuring more robust and reliable ecological inferences.

The horizontal dispersion of eDNA has been widely studied, with research focusing on quantifying the distances traveled by genetic material and the mechanisms influencing its transport across different aquatic ecosystems (BAETSCHER *et al.*, 2024; JANE *et al.*, 2015; JERDE *et al.*, 2016; LAPORTE *et al.*, 2022; LAPORTE *et al.*, 2020; LI *et al.*, 2019; MURAKAMI *et al.*, 2019; NEVERS *et al.*, 2021; PERRY *et al.*, 2024; SHEA *et al.*, 2022; VAN DRIESSCHE *et al.*, 2023; VAN DRIESSCHE *et al.*, 2024). Some of these studies have developed mathematical models to predict species distribution based on eDNA transport patterns, aiding in the interpretation of environmental data (CARRARO and ALTERMATT, 2024; CARRARO, BLACKMAN and ALTERMATT, 2023; JO and YAMANAKA, 2022; PONT, 2024). However, the vertical dispersion of eDNA remains largely underexplored. Few studies have investigated how genetic material behaves across different depth layers, and these knowledge gaps hinder the accurate interpretation of environmental data. Robinson et al. (2023) demonstrated that, in certain contexts, the vertical perspective can be even more relevant than the horizontal one, reinforcing the need for studies that directly address this dimension of eDNA transport.

Given the identification of this gap and its importance for interpreting environmental data, this dissertation aims to investigate the vertical dispersion patterns of eDNA, expanding the understanding of its dynamics in the water column and its implications for aquatic biodiversity monitoring.

In the first chapter, we conducted a systematic literature review to examine aspects related to eDNA verticality in different aquatic environments. As previously mentioned, few studies have primarily focused on the vertical dynamics of eDNA. In most cases, the transport and vertical dispersion of eDNA are addressed only secondarily, limited to brief discussions within studies with other objectives. To fill this gap, our research comprehensively compiled and analyzed information from various types of studies. Based on the analysis of 87 scientific documents, we explored key issues related to vertical eDNA transport, including stratification and mixing in the water column, sedimentation and resuspension, the significance of suspended eDNA signals, ecological responses obtained through sampling at multiple depth layers, among other factors.

In the second chapter, we conducted, for the first time, a series of controlled environment experiments to study the vertical dispersion of eDNA. To this end, we developed and built three experimental units simulating a 5-meter-deep lentic aquatic environment. These setups allowed for measurements along the water column at 1-meter intervals, as well as the injection of eDNA from target species at different depths. We selected two target species (*Oreochromis niloticus* and *Limnoperna fortunei*) to prepare concentrated eDNA solutions containing two distinct types of genetic material: free extracellular DNA (f-exDNA) and multi-fraction DNA. This enabled us to measure eDNA dispersion throughout the water column, both from the surface to the bottom and vice versa, while also evaluating how the properties of free and complexed-eDNA particles influence its transport.

The findings of this dissertation may contribute to improving eDNA sampling protocols and data interpretation, providing valuable insights to enhance the effectiveness of this tool in aquatic biodiversity monitoring. With a deeper understanding of vertical eDNA dispersion, we aim to strengthen its applicability in ecological studies and conservation strategies.

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1. CHAPTER 1 - FROM SURFACE TO BOTTOM: A SYSTEMATIC REVIEW OF THE VERTICAL DISTRIBUTION OF ENVIRONMENTAL DNA (EDNA) IN AQUATIC SYSTEMS†

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ABSTRACT

The use of environmental DNA (eDNA) for monitoring aquatic species has become a highly effective, non-invasive, and versatile tool. However, the vertical dynamics of eDNA in the water column remain poorly understood, particularly regarding the factors influencing its dispersion and detectability at different depths. This systematic review consolidates and analyzes existing studies on the vertical distribution of eDNA in aquatic environments, focusing on how physical factors, such as thermal gradients, currents, and salinity stratification, as well as biological aspects, including species behavior, affect eDNA transport, persistence, and distribution. The review also discusses methodological limitations associated with surface-only sampling, emphasizing the need for multi-depth sampling strategies to improve the accuracy and representativeness of aquatic biodiversity assessments. Conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) methodology, this study analyzed 87 scientific documents, revealing that eDNA composition and concentration vary significantly across vertical layers, particularly in environments with well-defined thermal and halocline stratification. The thermocline was identified as a major constraint on eDNA vertical transport, while the halocline played a key role in genetic material segregation in estuarine and fjord systems. Additionally, species vertical mobility, eDNA particle size, and sedimentation emerged as critical factors shaping eDNA distribution. Given these findings, this review provides guidelines for improving eDNA sampling strategies, contributing to a better understanding of eDNA ecology and enhancing its application in environmental monitoring and biodiversity conservation.

Keywords: Aquatic environment; eDNA; fresh water; marine environment; species monitoring; vertical dynamics.

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1.1 INTRODUCTION

Environmental DNA (eDNA) techniques have been extensively used as an alternative or complement to traditional strategies for monitoring aquatic species, standing out for their efficacy and versatility (CORNELIS *et al.*, 2024; FENG *et al.*, 2022; JAQUIER *et al.*, 2024; SHELTON *et al.*, 2022; WILCOX *et al.*, 2016; WILLBANKS *et al.*, 2023). Introduced nearly two decades ago (FICETOLA *et al.*, 2008), this approach enables the acquisition of information about aquatic organisms through DNA traces present in water samples. In recent years, the use of eDNA has gained remarkable popularity in scientific literature. From 2012 to 2021 the annual number of eDNA publications increased from 4 to 121 (TAKAHASHI *et al.*, 2023). This evolution reflects the transformative potential of eDNA while also highlighting technical and conceptual challenges that remain unresolved.

Despite the growing prominence of eDNA techniques, the methods are not yet fully established, standardized, or thoroughly understood (BHENDARKAR and RODRIGUEZ-EZPELETA, 2024; RAMÍREZ-AMARO *et al.*, 2022). Advances in the use of this technique underscore the need to deepen knowledge about the so-called "ecology of eDNA," which encompasses factors related to the origin, state, transport, and fate of target species' DNA in the environment (BARNES and TURNER, 2016). Among these factors, transport plays a central role as it directly affects the spatial and temporal distribution of eDNA in aquatic ecosystems. Once released by organisms, eDNA interacts with the surrounding environment, influenced by physical, chemical, and biological processes that determine its dispersion and persistence. These interactions shape the dynamics of eDNA, influencing its integrity, detectability, and, consequently, the interpretation of obtained data.

From a horizontal perspective, many studies have explored the distances and transport patterns of eDNA from known sources, analyzing how genetic material disperses in different aquatic environments (BAETSCHER *et al.*, 2024; JANE *et al.*, 2015; JERDE *et al.*, 2016; LAPORTE *et al.*, 2022; LAPORTE *et al.*, 2020; LI *et al.*, 2019; MURAKAMI *et al.*, 2019; NEVERS *et al.*, 2021; PERRY *et al.*, 2024; SHEA *et al.*, 2022; VAN DRIESSCHE *et al.*, 2023; VAN DRIESSCHE

et al., 2024). Some of these studies have developed predictive models that estimate the distribution of species based on eDNA transport (CARRARO and ALTERMATT, 2024; CARRARO, BLACKMAN and ALTERMATT, 2023; JO and YAMANAKA, 2022; PONT, 2024). However, approaches focusing on the vertical perspective remain limited, especially regarding experiments with known sources of eDNA to directly explore transport and distribution patterns across depth layers.

Information on the vertical distribution of eDNA often emerges as complementary data in studies with other objectives, where sampling at multiple depths reveals relevant ecological patterns (CAI *et al.*, 2024; SUZUKI *et al.*, 2024; ZHANG *et al.*, 2024). Nevertheless, the vertical dynamics of eDNA have gained prominence in scientific literature, with studies exploring variations among layers (ADAMS *et al.*, 2023; CANALS *et al.*, 2021; FENG *et al.*, 2022; FUKUMORI *et al.*, 2024; GOVINDARAJAN *et al.*, 2023; JEUNEN *et al.*, 2020; LITTLEFAIR *et al.*, 2021). Additionally, studies on particle sizes and the association of eDNA with suspended materials provides valuable insights into processes such as sedimentation and resuspension, contributing to understanding its persistence and distribution in aquatic environments (BRANDÃO-DIAS, HALLACK*, et al.*, 2023; BRANDÃO-DIAS, TANK*, et al.*, 2023; JERDE *et al.*, 2016; TURNER *et al.*, 2014; TURNER, UY and EVERHART, 2015).

Understanding these factors has the potential to transform studies involving environmental DNA, particularly regarding the limitations of sampling exclusively from the water's surface layer. Although widely used for its simplicity and lack of need for specific equipment (e.g., Van Dorn bottles, Niskin bottles, depth samplers), this approach may fail to capture representative information about the studied environment (EICHMILLER, BAJER and SORENSEN, 2014; JEUNEN *et al.*, 2020; LITTLEFAIR *et al.*, 2021; TURNER, UY and EVERHART, 2015). In freshwater and especially marine environments, species segregation across different vertical layers, influenced by environmental and behavioral factors, can affect the distribution of the eDNA signal (FUKUMORI *et al.*, 2024; LITTLEFAIR *et al.*, 2021; LIU *et al.*, 2024; PENG *et al.*, 2023; YAMAMOTO *et al.*, 2016). Thus, studies aiming to provide a comprehensive overview of an environment's diversity or detect species from deeper habitats may produce incomplete results due to unrepresentative sampling.

This review synthesizes current scientific literature examining the vertical dynamics of environmental DNA (eDNA) in aquatic ecosystems. We analyze how physical parameters, including water currents and thermal stratification, interact with biological factors such as species-specific behaviors to influence the vertical distribution of eDNA throughout the water column. Our investigation addresses critical methodological constraints inherent to surface-water sampling protocols and examines how eDNA transport mechanisms and persistence patterns affect data interpretation. Through a comprehensive analysis of existing research, we identify significant knowledge gaps and propose future research directions to advance our understanding of eDNA behavior in aquatic environments.

1.2 METHODS

This study was conducted based on the PRISMA methodology (MOHER *et al.*, 2009). The review began with the construction of a search string consisting of three categories of terms to identify articles on the transport and distribution of eDNA in aquatic environments. The first category, restricted to titles, included the terms “environmental DNA” and “eDNA,” focusing on studies explicitly related to the topic. The second and third categories were, respectively, applied to the abstract section of each document, to select studies from aquatic environments (e.g., “lake,” “river,” “sea,” and “waterbodies”) and presented data related to eDNA dispersal (e.g., “resuspension,” “stratification,” “depths,” and “spatial distribution”) (APPENDIX 1 - Table S1.). The terms within each category were connected using the Boolean operator “OR” while the categories were combined using the operator “AND.”

Scientific articles and reviews published in English between 2008 and July 2024 were included, with the lower limit established by the study on the use of Ficetola et al. (2008) on the use of eDNA for the detection of aquatic macroorganisms. The databases Scopus, Web of Science, and PubMed were chosen for their scientific relevance and robust search functionalities. The search strings were adjusted to meet the specific requirements of each database (APPENDIX 2 - Table S2.).

After defining the search strategy, it was carried out through institutional access at the Universidade Federal do Paraná (UFPR) using the Portal de Periódicos of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), linked to the Ministério da Educação of Brasil. The number of articles retrieved from Scopus, Web of Science, and PubMed were, 551, 394, and 236, respectively. The total number of documents were 1,181. From these searches, files in formats (.bib and .nbib) containing the article information were generated and loaded into the StArt software (v. 3.3 Beta 03, LaPES-UFSCar) for organization, categorization, and removal of duplicates.

After removing duplicates (n=594), a total of 587 documents underwent full-text reading. We chose not to conduct a pre-selection based solely on titles and abstracts to ensure that relevant information about the processes associated with eDNA in the water column, potentially present in other sections of the manuscripts, was not overlooked. The exclusion criteria included: unavailability of the full text, non-English language, publication outside the stipulated period, lack of primary study characteristics, exclusive focus on microorganisms, significant methodological limitations, or lack of relevant considerations on the topic. A document was considered accepted if it did not meet any of these exclusion criteria and presented any discussion about eDNA dynamics in aquatic environments, regardless of whether the perspective analyzed was vertical, longitudinal, latitudinal, or another. At the end of this screening, 204 articles were accepted.

Then, the documents underwent a second evaluation to identify those that addressed aspects of eDNA in the water column from a vertical perspective. The selection criteria included: sampling at different depth layers, with results analyzed and discussed; abiotic factors of vertical segregation (e.g., thermocline stratification) and/or homogenization (e.g., tides and currents); aspects related to species behavior and eDNA in the water column; comparisons between water and sediment sampling; and considerations on processes of eDNA sedimentation and resuspension. At the end of this stage, 87 articles were accepted and selected for metadata extraction and analysis in this study. Figure 1 presents the flowchart based on the PRISMA methodology, outlining the selection steps followed in this work.

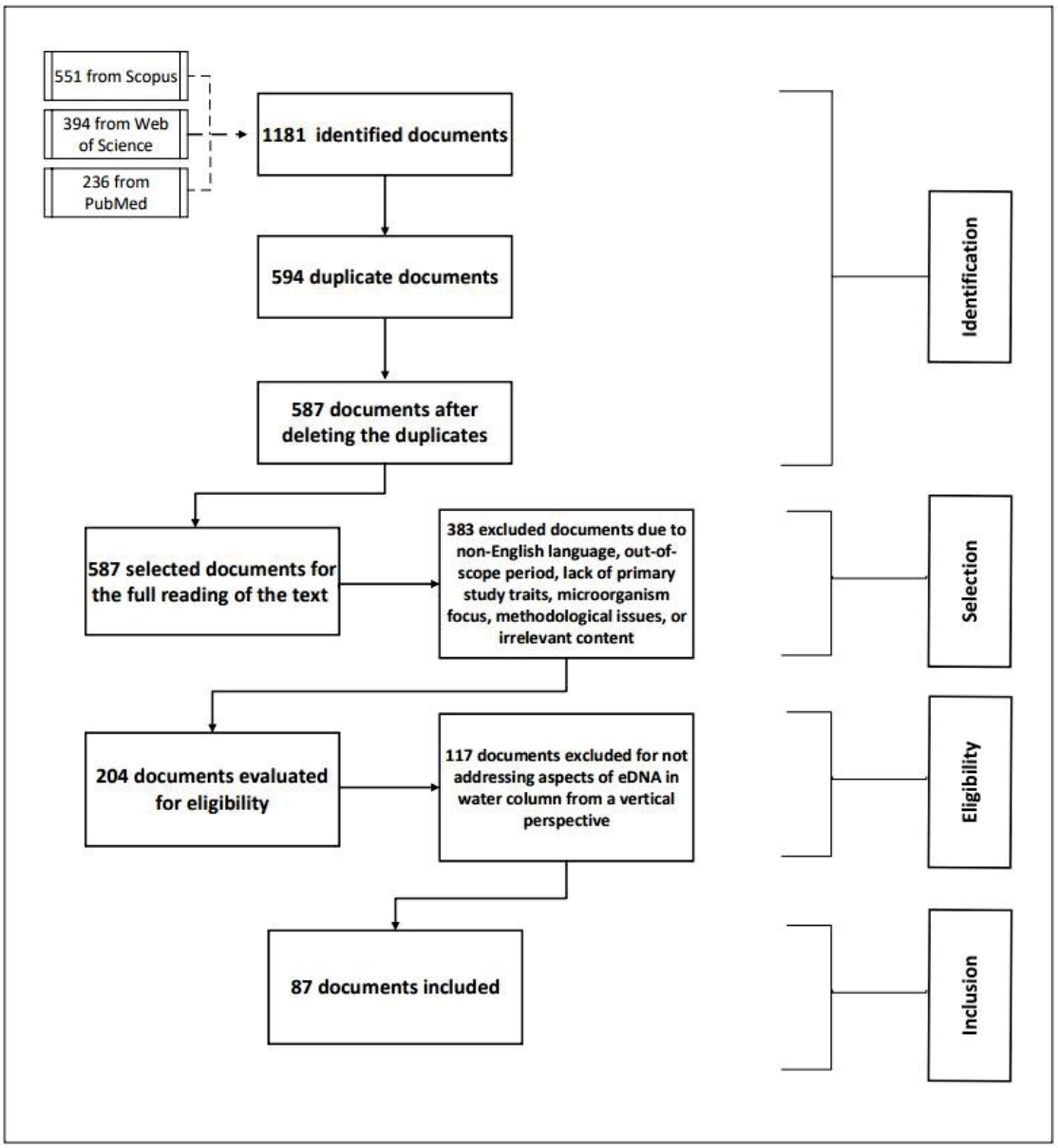

Figure 1 Flowchart based on the PRISMA methodology, illustrating the steps followed in this work for the selection and inclusion of relevant articles on eDNA in the water column, with a focus on the vertical perspective.

For each selected article, we evaluated and extracted 78 metadata items, including bibliographic and publication data, study location and environment, physical and environmental parameters, methods and methodologies, questions related to eDNA dynamics, unique and preferential patterns of taxon distribution inferred from eDNA, and additional information. A detailed description of all items is available in Supplementary Table S3 (APPENDIX 3 - Table S3.).

In this study, we hypothesized that the key factor for understanding the vertical dynamics of eDNA and its representativeness is the sampling of multiple

vertical layers of the water column. To test this, we conducted an additional analysis on the selected documents, evaluating the number of samples collected per sampling point or water body, their distribution, and their relation to the total depth of the location. To be included in this analysis, the study needed to meet the following criteria: having at least one dataset containing the maximum depth (in meters) of the sampling point or of the water body, sampling two or more different depth layers, and providing a sufficient description for identifying the sampled layers. Fifteen studies (17.24%) did not meet these requirements and were excluded from the analysis, being relevant only for other discussions. On the other hand, 72 studies (82.76%) met the established criteria and had data collected from texts, tables, figures, and supplementary materials, totaling 554 datasets.

To achieve relative standardization, we divided the water collum for each dataset in ten layers of equal height, numbering then from 1 to 10 – from the surface (layer 1) to the bottom (layer 10). Using the sampling depth data, we assigned each sample to the corresponding layer by dividing the sampling depth by the total depth. Also, to improve data interpretation, we categorized the water bodies based on their maximum depth and water type (freshwater, marine, and mixed/brackish), with particular consideration of differences between freshwater and marine environments. The water bodies were classified into the categories “very shallow”, “shallow”, “medium”, “deep”, and “very deep” (with “very deep” excluded for mixed/brackish environments), using the following maximum depth thresholds: 1, 3, 20, 50, and >50 m for freshwater; 10, 50, 200, 1000, and >1000 m for marine water; and 1, 5, 20, and >20 m for mixed/brackish water.

1.3 RESULTS AND DISCUSSION

1.3.1 Characterization of the analyzed studies

Among the 87 studies analyzed, a heterogeneous geographical distribution was observed across both continental regions and oceanic basins. Continental analysis revealed Asia as the predominant research location (n=26), followed by North America (n=23) and Europe (n=13). The contribution by

Australia/Oceania, Africa, South America, and Antarctica, collectively contributed fewer studies (n=10). Other studies were exclusively considered oceanic and therefore were not associated with any continent (n=15). In marine environments, the Pacific Ocean dominated the research landscape with 28 studies, while the Atlantic Ocean (n=8), the Arctic Ocean (n=5), the Indian Ocean (n=3), and the Mediterranean Sea (n=2) showed comparatively lower research intensity. Analysis of study distribution by country indicated that the United States led in research output with 21 publications, followed by China (n=15) and Japan (n=10). Canada demonstrated substantial contribution with 5 publications. European representation included France (n=4), while Australia and New Zealand contributed equally (n=3 each). Spain, Belgium, and the United Kingdom each produced 2 publications. Single contributions were documented from Thailand, Chile, Norway, the Netherlands, Indonesia, Tanzania, and Italy.

Analysis of study distribution by aquatic environment revealed that 54 studies (62.0%) were conducted in marine systems, 26 (29.9%) in freshwater, and 7 (8.1%) in brackish or mixed-water environments such as estuarine zones (Figure 2). In contrast, Takahashi et al. (2023), in their meta-analysis of macroorganisms biomonitoring using aquatic eDNA, reported that most studies were conducted in freshwater environments (65.4%), with marine systems representing only 24.8% of the total. This disparity in environmental focus may be attributed to the greater vertical dimensionality of marine ecosystems. Given that this review specifically examinates the vertical dynamics of eDNA, the prevalence of marine environments studies is consistent with the increased significance of depth-stratified sampling in these deeper ecosystems. On the other hand, in freshwater environments, which typically exhibit shallower depths, vertical sampling strategies are often considered less critical, with collection efforts primarily concentrated in surface waters (EICHMILLER, BAJER and SORENSEN, 2014).

Analysis of sampling distribution showed that 55 studies (63.2%) focused on a single aquatic environment, while 32 studies (36.8%) investigated multiple environments, such as integrated coastal-bay systems or river-estuary continua. Environmental categorization demonstrated that coastal zones constituted the highest proportion of study sites (24.79%), followed by open ocean systems (19.83%), lacustrine environments (10.74%), bay ecosystems (9.09%), riverine

systems (7.44%), and estuarine habitats (5.79%). The remaining study sites were distributed among other freshwater (9.92%), marine (9.09%), and transitional water environments (3.31%).

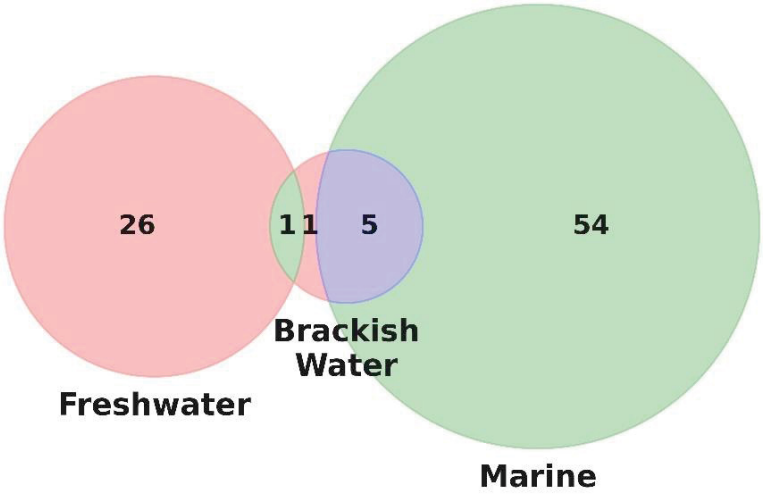

Figure 2 Venn diagram showing the distribution of the analyzed studies, categorized by water types: “Freshwater”, “Brackish Water”, and “Marine”. The numbers represent the number of studies in each category and their intersections.

The analysis of the 554 datasets, regarding the relationship between environmental maximum depth and sampling depth frequency, revealed that marine environments dominated the sampling distribution (n=456, 82.31%), followed by freshwater (n=84, 15.16%) and mixed/brackish water environments (n=14, 2.53%). Examination of vertical sampling resolution showed that protocols using two-depth sampling strategies were most prevalent (42.96% of datasets). Three- and four-depth sampling protocols represented 18.41% and 22.92% of datasets, respectively, while five-depth sampling accounted for 11.01%. More intensive vertical sampling (≥6 depths) constituted only 4.69% of the analyzes data (APPENDIX 4 - Table S4.). Vertical distribution analysis indicated that surface layer sampling (layer 1) predominated, occurring in 90.43% of datasets, likely due to accessibility and minimal equipment requirements (EICHMILLER, BAJER and SORENSEN, 2014; MOYER *et al.,* 2014; UTHICKE, LAMARE and DOYLE, 2018). The benthic-adjacent layer (layer 10) was the second most frequently sampled stratum, represented in 47.65% of datasets. Notably, sampling strategies targeting exclusively the surface and benthic layers (layers 1 and 10) comprised 19.86% of all sampling events.

In freshwater environments, the maximum depths ranged from extremely shallow, such as 0.419 m in a controlled mesocosm experiment (KAMOROFF and GOLDBERG, 2018) and shallow streams of 0.7 m (KATANO *et al.*, 2017; SHAW *et al.*, 2016), to a deep lake with locations where the maximum depth was 91 m (WU *et al.*, 2019). Most of the data were obtained from environments classified as medium, deep, and very deep, representing 35.71%, 28.57%, and 26.19%, respectively. Sampling in shallow and very shallow environments (≤ 3 m) represented only 9.52% of the freshwater data, indicating that in these shallow environments, multi-depth sampling is uncommon, likely due to the assumption that in these environments, eDNA homogenization in the water column is sufficient to justify sampling only surface water (CURRIER *et al.*, 2018; KATANO *et al.*, 2017). Indeed, for 100% of the freshwater sampling data, at least one sample was collected at the shallowest layer (layer 1), highlighting the preference for sampling at this layer. For more than half of the data (66.67%), sampling was performed at only two depths, even in some environments classified as deep and very deep (JANOSIK *et al.*, 2021; KLOBUCAR, RODGERS and BUDY, 2017; WU *et al.*, 2019). Of these two-depth sampling events, 73.21% adopted sampling protocol in the extreme layers (layers 1 and 10). Events in which 3 or 4 depths were sampled accounted for 23.81%. Sampling of 5 or more depths was less frequent in freshwater environments (9.52%) and is mainly associated with very deep environments (HÄNFLING *et al.*, 2016) or studies focused on vertical distributions (LITTLEFAIR *et al.*, 2021).

Marine environments exhibited a broader range of depth variation compared to freshwater. The shallowest depth for this type of environment was 1.5 m (UTHICKE, LAMARE and DOYLE, 2018), while the greatest depth reached 4600 m, which also coincides with the deepest depth collected in our data (KIM, JU and SUH, 2024). Most marine environments were classified into the “shallow” (28.51%) and “medium” (46.71%) categories. Sampling with four depths was predominant in “medium” and “very deep” environments (45.07% and 34.69%, respectively), indicating that in marine environments, where depths are greater, a significant portion of studies acknowledges the need to increase the number of sampled layers for better environmental characterization. However, even in environments deeper than 1000 m, 46.94% of the samples were taken from 2 or

3 depths, which, depending on the study's goal, may indicate under-representation of the environment.

There were no data for very shallow environments in brackish or mixed waters, and only one study (MARQUES *et al.*, 2024) classified this type of environment as shallow, with a depth of 3 m. Medium and deep environments represent 21.43% and 71.43% of the data, respectively. The greatest depth recorded for brackish/mixed environments was 350 m, in Doubtful Sound Fjord, New Zealand (JEUNEN *et al.*, 2020). Only sampling protocols with 2 (71.43%) and 3 (28.57%) depths were reported at this environment.

In addition to data generated exclusively from water samples, we also identified studies that combined water column sampling with sediment samples. These studies sought to answer questions such as whether eDNA in sediment accumulates more information than that in water (NEVERS *et al.*, 2021; PICARD *et al.*, 2023; TURNER, UY and EVERHART, 2015; WILLBANKS *et al.*, 2023), whether sampling one or the other is more efficient (ALEXANDER *et al.*, 2023; CLARKE *et al.*, 2021; SHAW *et al.*, 2016; SHEN *et al.*, 2024; ZHOU *et al.*, 2023), or whether sediment enhances the detection of benthic species (PAINE, HURT and MATTINGLY, 2021). We believe this approach is essential for discussing aspects related to the eDNA signal and the dynamics of sedimentation and resuspension. Seven studies in freshwater environments and four in marine environments met this criterion.

1.3.2 Influence of sampling depth on eDNA detection

Depending on the objective of the eDNA study, especially when the aim is to conduct a representative survey of the biodiversity of an environment, sampling effort becomes a crucial factor for obtaining meaningful results (JEUNEN *et al.*, 2020; LITTLEFAIR *et al.*, 2021; ROBINSON *et al.*, 2023). From a horizontal perspective, several studies indicate that increasing the distance between sampling points reflects changes in organism communities based on eDNA data, both in freshwater (EICHMILLER, BAJER and SORENSEN, 2014; ZHANG, Y. *et al.*, 2023) and marine environments (INOUE *et al.*, 2022; KIM, JU and SUH, 2024; MONUKI, BARBER and GOLD, 2021; WANG, LU, ZHAO,

YANG*, et al.*, 2021; WANG *et al.*, 2022). Therefore, to obtain more comprehensive data, it is necessary sampling at a sufficiently spaced distances, considering the characteristics of each environment. However, does this principle also apply to vertical distances? Or is sampling from a single point, such as the surface, sufficient to capture all the diversity present in the water column?

In our results, 37 studies indicate that sampling at different depths can, in fact, reveal significant differences in the composition of organism communities, sometimes even more pronounced than those observed between horizontal points (ROBINSON *et al.*, 2023). Most of these studies (n=28 – 75,6%) were conducted in marine environments, 13 (35,1%) of which refer to sites with depths greater than 200 meters. In environments with such characteristics, it is expected that differences in communities will be observed, since the increase in vertical distance and the more evident segregation between layers of the water column are important factors for species segregation and, consequently, for distinguishing eDNA signals. Liao et al. (2023) observed differences in fish composition between surface and deep layers of the ocean, with deep samples generally differing from surface samples at the same sites. This pattern is consistent with the results of Muff et al. (2023), who also identified variations in the composition of fish communities across different depths, stating that each depth has its own set of molecular operational taxonomic units (MOTUs). However, Diao et al. (2023) did not observe significant variation in the fish assemblage according to depth, possibly due to the greater adaptive capacity of these organisms to different depths, but they noted a significant influence of depth on the detection of phytoplankton and invertebrates. Differences in the detection of lower trophic organisms according to depth were also reported by Kim et al. (2024), Feng et al. (2022), Zhang et al. (2020), and Liu et al. (2019).

Even in marine environments with relatively shallow depths, some studies indicate differences between layers. For example, Alexander et al. (2023) collected samples near the Kwinana Bulk Jetty, off the coast of Western Australia, at depths of up to 8 meters and observed differences between the surface (0 m) and bottom (8 m), with variations in small-scale communities. Similarly, Robinson et al. (2023) investigated environments ranging from 8 to 25 meters in depth and found contrasts between surface samples, which were dominated by salmon (*Oncorhynchus spp.*) and rotifer DNA, and deeper layers, where Pacific herring,

copepods, and mussels predominated. Even in a shallow and dynamic coastal environment (10 m), where tides and waves could homogenize eDNA in the water column and potentially mask the segregation of communities at different depths, Monuki et al. (2021) found evidence of fish community signatures along a depth gradient of 4–5 meters, with results aligning with the expected behavior of species in the water column. Notably, an 8-meter maximum depth was the shallowest threshold in our research where differences between marine communities were reported.

In freshwater environments, the shallowest depth at which differences in communities were identified was 6 meters, with the overall environment depth ranging from 6 to 44 meters, as reported by Zhang et al. (2023). Only six studies recognized that there may be differences between communities at different depths based on eDNA research (FUKUMORI *et al.*, 2024; HÄNFLING *et al.*, 2016; LITTLEFAIR *et al.*, 2021; LIU *et al.*, 2024; ZHANG, Y. *et al.*, 2023; ZHOU *et al.*, 2023). However, the low number of studies on the topic may indicate that this factor has often been neglected in freshwater environments, rather than being truly irrelevant. This hypothesis is reinforced by the fact that, in two of these studies, the vertical distribution of eDNA was the primary focus of the research. Fukumori et al. (2024), who conducted sampling at four depths in Lake Yunoko, Japan (with depths ranging from 10 to 13 meters), and Littlefair et al. (2021), who sampled six depths in the IISD Experimental Lakes Area, Ontario, Canada (with depths ranging from 13.2 to 30.4 meters), observed significant patterns in fish community composition between vertical layers in stations where the lakes were under thermal stratification.

Regarding brackish/mixed environments, three studies pointed out differences between the communities (JEUNEN *et al.*, 2020; SEVELLEC *et al.*, 2024; WANG *et al.*, 2024). A common feature in estuarine environments is the pronounced hydrological stratification, where seawater remains in the deeper layer, while freshwater from the land runs through the upper layer (KASAI *et al.*, 2010; PRANDLE, 2009).

Some studies (n=13) also indicated which layers had the highest taxon diversity, as obtained through eDNA sampling (APPENDIX 5 - Table S5.). However, we did not identify a general pattern established to determine which layer would be best for revealing greater taxon richness. This occurs because,

even in studies with the same assay and target taxon, different locations may present distinct patterns of higher diversity between the layers (SEVELLEC *et al.*, 2024).

Only four studies reported that sampling at different depths did not reveal vertical differences in communities. Dukan et al. (2024), analyzed samples from the Belgian part of the North Sea (depths from 8 to 31 meters) and found no differences between samples collected at 1 meter below the surface and 1 meter above the bottom. The authors attributed this result to the dynamic and well-mixed waters of the region, allowing pelagic species such as *Sardina pilchardus* to be detected in deep samples, while demersal species such as *Cyclopterus lumpus* appeared in surface waters. Van Driessche et al. (2024) also observed a marginal impact of depth on fish composition in the Scheldt estuary, highlighting that eDNA dilution and homogenization dynamics influenced the results. Similarly, Saenz-Agudelo et al. (2022) observed little variation in communities along the Chilean coast, both vertically and horizontally, between sites separated by 16 km, suggesting a homogeneity in the assemblages. Liu et al. (2022), while monitoring elasmobranchs in the Western English Channel, also found no differences between surface and deep samples, justifying the eDNA homogeneity by the vertical mobility of shark species and benthic species migrations, in addition to water mixing dynamics in the region.

In addition to community differentiation, 17 studies in marine environments, 8 in freshwater, and 3 in brackish/mixed water environments indicated that sampling at different depths could reveal differences in eDNA concentrations. Of these studies, four estimated concentration differences through metabarcoding. Ye et al. (2024) estimated layer preferences: surface for *Aurelia coerulea*, middle-lower for *Nemopilema nomurai*, and middle-upper for *Cyanea nozakii*, based on the relative abundance of metabarcoding reads. McClenaghan et al. (2020), Govindarajan et al. (2022), and Dan et al. (2024), using metabarcoding to study marine biodiversity, found higher eDNA concentrations in shallower waters, especially at depths less than 200 meters, with emphasis on areas such as the maximum chlorophyll layer (DCM) in Govindarajan et al. (2022) research. Two other studies made semi-quantitative conclusions from conventional PCR. Burgoa Cardás et al. (2020) observed that eDNA from *Anguilla anguilla* was approximately four times higher in bottom

samples, while Janosik et al. (2021) demonstrated that bottom sampling resulted in a higher number of positive detections for *Scaphirhynchus suttkusi*. However, most of these studies (n=22) are related to research focused on quantitative eDNA data for specific species, obtained through techniques such as qPCR and/or ddPCR, some of which also use additional metabarcoding. A synthesis of these studies, including the target species and the layers where the eDNA of these species was most concentrated, is presented in Table 1.

Table 1 List of studies presenting the layers where the eDNA of target species was found in higher concentrations.

Research	Water Type	Species	Layer(s)
Moyer et al. 2014	Freshwater	*Hemichromis letourneuxi*	Shallow
Carim et al. 2016	Freshwater	*Mysis diluviana*	Deep
Yamamoto et al. 2016	Marine	*Trachurus japonicus*	Shallow / Deep[1]
Klobucar et al. 2017	Freshwater	*Salvelinus alpinus*	Deep (in Summer)
Minamoto et al. 2017	Marine	*Chrysaora pacifica*	Deep
Stewart et al. 2017	Freshwater	*Neophocaena asiaeorientalis asiaeorientalis*	Deep
Uthicke et al. 2018	Marine	*Acanthaster cf. solaris*	Deep
Murakami et al. 2019	Marine	*Pseudocaranx dentex*	Shallow
Takasu et al. 2019	Marine	*Chrysaora pacifica*	Intermediate
Wu et al. 2019	Freshwater	*Palaemon paucidens*	Deep
Harper et al. 2020	Marine	*Chelonia mydas*	Deep
Lor et al. 2020	Freshwater	*Margaritifera monodonta*	Deep
Wang et al. 2020	Marine	*Larimichthys polyactis*	Intermediate
Wang et al. 2021	Marine	*Larimichthys crocea*	Intermediate and Deep
Sasano et al. 2022	Marine; Brackish Water	*Acanthopagrus schlegelii*	Shallow
Shelton et al. 2022	Marine	*Merluccius productus*	Intermediate
Wu et al. 2022	Marine	*Larimichthys crocea*	Shallow and Deep
Zhang et al. 2022	Marine	*Pampus echinogaster*	Shallow and Intermediate
Peng et al. 2023	Marine	*Aurelia coerulea*	Deep
Baetscher et al. 2024	Marine	*Oncorhynchus keta*	Shallow
Fukumori et al. 2024	Freshwater	*Oncorhynchus nerka*	Deep

Research	Water Type	Species	Layer(s)
Fukumori et al. 2024	Freshwater	*Oncorhynchus mykiss*	Uniformly distributed
Fukumori et al. 2024	Freshwater	*Oncorhynchus masou*	Below the detection limit
Marques et al. 2024	Marine; Brackish Water	*Pinna nobilis*	Deep

[1]The highest concentrations varied between shallower and deeper layers depending on the sampling point.

Fukumori et al. (2024) were the only ones to present qPCR assays for more than one species in the same study, which yielded an interesting result for our discussion. By applying assays for three species of the genus Oncorhynchus, the authors were unable to obtain quantifications above the detection limit for one of the species (*O. masou*). However, for the other two species, different patterns in the distribution of eDNA were observed. While eDNA from *O. nerka* was clearly more concentrated in the deeper layers, eDNA from *O. mykiss* was evenly distributed throughout the water column, demonstrating that, for the same environment, the eDNA signal can be more restricted or dispersed depending on the species and its behavior.

In addition to the result from Fukumori et al. (2024) for *O. mykiss*, six studies did not detect differences in eDNA concentration between depths. In marine environments, both Wang et al. (2022) and Zhang et al. (2022) reported that eDNA concentrations for *Sciaenops ocellatus* and *Acanthopagrus schlegelii*, respectively, were lower in deeper layers. However, these studies acknowledged that the differences in concentrations between layers were not statistically significant. For the other four studies conducted exclusively in freshwater, we observed that the environments were very shallow, with a maximum depth of 4 meters (EICHMILLER, BAJER and SORENSEN, 2014). Three of these studies (CURRIER *et al.*, 2018; KATANO *et al.*, 2017; PAINE, HURT and MATTINGLY, 2021) were conducted in lotic environments with depths ≤ 1 meter, characteristics that favor a strong homogenization of the water column and, consequently, of the eDNA signal (CURRIER *et al.*, 2018; PAINE, HURT and MATTINGLY, 2021). Furthermore, for these studies, the vertical distance between the analyzed samples was at most 0.9 meters, which considerably reduces the chances of these studies presenting different results.

1.3.3 Mechanisms of vertical segregation and mixing of eDNA in the water column

Based on the data presented earlier, we can observe that shallower environments, in general, do not present differences across depths, neither in eDNA concentration or in the structuring of communities detected. In these environments, factors such as wind (KERIMOGLU and RINKE, 2013; XU *et al.*, 2023) promote water mixing, preventing the stratification of the water column into layers. In contrast, in environments with intermediate and deep depths, the water column is often segregated into distinct layers, separated by gradients of temperature, salinity, light, and other factors (HENDERSON-SELLERS and DAVIES, 1989; WETZEL, 2001).

In stratified environments, fine particles tend to remain confined to specific layers due to limited vertical mixing, especially under conditions of high-density variation (FISCHER *et al.*, 2013; IMBERGER and HAMBLIN, 1982). Considering that eDNA behaves similarly to fine particulate organic matter (FPOM) (WILCOX *et al.*, 2016), it is expected that stratification limits the vertical transport of this material, causing it to remain mostly restricted to the layer in which it was released (FUKUMORI *et al.*, 2024; LITTLEFAIR *et al.*, 2021; LIU *et al.*, 2024; PENG *et al.*, 2023; YAMAMOTO *et al.*, 2016).

The stratification of the water column, driven by the thermocline, has been identified as the most relevant abiotic factor limiting the vertical transport of eDNA. In marine environments, for example, Yamamoto et al. (2016) suggest that the thermal stratification observed in June restricts the vertical transport of eDNA from *Trachurus japonicus*, so that the detection layer reflects the species' actual distribution. Similarly, studies conducted in Yantai Sishili Bay (PENG *et al.*, 2023) and the Bohai Sea (YE *et al.*, 2024) have demonstrated that the presence of thermal stratification limits the vertical dispersion of eDNA, highlighting the occupation of different thermal layers by jellyfish species.

However, to elucidate the relationship between eDNA dynamics in the water column and the influence of the thermocline, studies conducted in freshwater lakes have proven particularly relevant. Research by Littlefair et al. (2021), Fukumori et al. (2024), and Klobucar et al. (2017) investigated lakes with

depths ranging from 13.2 to 30.4 m (with six sampling depths), 10 to 13 m (with four sampling depths), and 9.9 to 21 m (with two sampling depths), respectively. These studies demonstrated that, during stratification periods — such as in summer — the water column is segmented into distinct microhabitats (ANDRUSZKIEWICZ *et al.*, 2017; KLOBUCAR, RODGERS and BUDY, 2017; LITTLEFAIR *et al.*, 2021).

Under these conditions, cold-water stenothermic fish, such as *Salvelinus alpinus*, *Oncorhynchus nerka*, and *Salvelinus namaycush*, tend to concentrate in the deeper, colder layers just below the thermocline. Consequently, the eDNA of these species is predominantly detected in deep waters, while detection in surface layers is significantly reduced or absent. Similarly, other species, such as *Pseudaspius hakonensis* and *Gnathopogon elongatus*, may be restricted to upper layers during stratification — a fact corroborated by Fukumori et al. (2024), who found the eDNA of these species exclusively in waters above the thermocline. In contrast, species with more plastic behavior, capable of moving across all depth layers, such as *Oncorhynchus mykiss*, had their eDNA detected throughout the water column, even during stratification periods (FUKUMORI *et al.*, 2024).

In addition to sampling conducted during stratification periods (summer), Klobucar et al. (2017) and Littlefair et al. (2021) also collected samples during autumn turnover, when deep waters mix with surface waters. During this period, the eDNA signal appears homogenized due to intense water column mixing, preventing the identification of stratified layers. This finding suggests that the season in which sampling is conducted may determine whether collections at multiple depths are necessary. During turnover, the exchange between deep and surface waters reduces the need for differentiated sampling; however, eDNA detected during this period may not accurately reflect the recent presence of species, as recirculating currents can resuspend genetic material previously deposited in sediments (BLABOLIL *et al.*, 2022; KLOBUCAR, RODGERS and BUDY, 2017).

Halocline-driven stratification has been identified as the second most relevant factor in eDNA signal segregation. Environments with significant salinity variations in the water column are typical of regions where continental freshwater meets marine saltwater, such as estuaries and fjords. In these locations, as

previously mentioned, freshwater can flow for several meters in the surface layer without mixing with deeper, saltier waters. As a result, sampling from surface layers tends to reveal the eDNA of freshwater species, while deeper layers predominantly contain the eDNA of marine species. This pattern has been observed in both fjords (JEUNEN *et al.*, 2020; ROBINSON *et al.*, 2023) and estuaries (LACOURSIÈRE-ROUSSEL *et al.*, 2018; SEVELLEC *et al.*, 2024) areas. In locations where a halocline is present, it is essential to collect samples at different depths to obtain a comprehensive view of local biodiversity, as eDNA transport between these layers is considered unlikely (SASANO *et al.*, 2022).

Picnocline-driven stratification was highlighted by Takasu et al. (2019), who observed that the eDNA concentration of *Chrysaora pacifica* peaked near or below the pycnocline during summer (between June and September). According to the authors, a strong pycnocline may limit the vertical migration of jellyfish due to buoyancy resistance, which hinders movement between layers with significant density differences. Even when the pycnocline is weak, the distribution of eDNA appears to be affected, suggesting that it is more sensitive to stratification conditions than the physical movement of the jellyfish themselves. In contrast, Closek et al. (2019) and Guri et al. (2024) reported little influence of the pycnocline on the eDNA signal. Closek et al. (2019) stated that the taxon assemblages above and below the pycnocline did not show significant differences, possibly due to the diel vertical migration of mesopelagic species, which alternate their presence between layers. Similarly, Guri et al. (2024) identified differences in communities between subsurface samples (10 m) and deep samples (~80 m) in subarctic fjords but found no significant variations when comparing these layers with samples taken from the pycnocline (~50 m). According to these authors, sampling at the pycnocline may be unnecessary, as this layer represents only a smooth transition between the surface and the bottom.

All these types of stratifications are particularly relevant because they create physical barriers that prevent or hinder the vertical transport of eDNA between layers. Conversely, other forms of water column segregation — such as differentiation of layers by light incidence — do not physically restrict this transport but may influence the distribution and aggregation of certain species,

leading to a higher concentration of eDNA in specific layers. This factor will be explored later.

Before concluding this section, however, it is important to address the mechanisms governing eDNA distribution when there is no physical segregation between the layers of the water column. In situations where no barrier separates vertical layers, eDNA can follow two distinct trajectories, driven by environmental dynamics: it can remain suspended and be evenly distributed throughout the column, or it can settle and accumulate on the substrate due to gravitational forces.

In high-energy environments that promote upward and downward movements in the water column, eDNA tends to homogenize throughout the column. In addition to the deep-to-surface water circulation events observed in lentic environments during turnover periods, flows in riverine systems (CURRIER *et al.*, 2018; PAINE, HURT and MATTINGLY, 2021) and currents and tides in marine systems (DOWELL *et al.*, 2024; DUKAN *et al.*, 2024; VAN DRIESSCHE *et al.*, 2024) also contribute to the vertical movement of eDNA. However, caution is needed when concluding that eDNA is completely homogenized, even in highly dynamic and non-stratified environments (MONUKI, BARBER and GOLD, 2021). For example, Van Driessche et al. (2024), although not observing significant differences in the vertical distribution of eDNA due to dilution and particle homogenization, identified that a bottom-specialist species, *Pholis gunnellus*, was detected exclusively in samples from the lower layers.

Regarding more lentic systems, there is a consensus — though not an absolute rule — that once released by an organism, eDNA remains suspended in the water column for a short period before tending to settle under low water movement conditions. Studies such as those by Turner et al. (2014) and Wilcox et al. (2015) have already demonstrated that a significant portion of the eDNA present in the water column is found in particles ranging from 1 to 10 µm in size, dimensions that do not allow these particles to remain suspended indefinitely (TURNER, UY and EVERHART, 2015).

It is important to emphasize, however, that size alone does not determine whether a particle will settle. Intrinsic characteristics — such as the material's origin and electrical charges — also influence its behavior, conferring properties of either sedimentation or buoyancy (JERDE *et al.*, 2016). For example, collected

eDNA may include pelagic eggs (SASANO *et al.*, 2022; WANG, LU, ZHAO, DU*, et al.*, 2021; WANG, LU, ZHAO, YANG*, et al.*, 2021; WU *et al.*, 2022), which, despite typically exceeding 10 µm in size, possess characteristics that enhance their buoyancy. Nevertheless, some studies reinforce that the particle size composition of eDNA is fundamental to understanding the persistence of the signal in the water column (BAETSCHER *et al.*, 2024; BRANDÃO-DIAS, HALLACK*, et al.*, 2023; BRANDÃO-DIAS, TANK*, et al.*, 2023; CANALS *et al.*, 2021) and that sedimentation plays a crucial role in the removal of eDNA from the water (BRANDÃO-DIAS, TANK*, et al.*, 2023; NEVERS *et al.*, 2021).

Like water sampling, eDNA collection from sediments reveals important information about the communities present in an environment. However, it is essential to understand that data obtained from these two sample types are distinct, as demonstrated in 11 studies that compared water and sediment samples. First, eDNA tends to be found in higher concentrations in sediments than in the water column (MURRAY *et al.*, 2024; PAINE, HURT and MATTINGLY, 2021; PENG *et al.*, 2023; TURNER, UY and EVERHART, 2015). This is largely because eDNA deposited in sediments is less susceptible to degradation (MURRAY *et al.*, 2024; PENG *et al.*, 2023; SHEN *et al.*, 2024; TURNER, UY and EVERHART, 2015). However, a higher concentration of eDNA in sediments does not necessarily imply a higher probability of detection compared to water samples. Factors such as the lower presence of PCR inhibitors in water samples and the ability to process larger volumes of water compared to solid sediment often result in superior detection rates in water sampling (EICHMILLER, BAJER and SORENSEN, 2014; SHAW *et al.*, 2016; WILLBANKS *et al.*, 2023; ZHOU *et al.*, 2023).

The most relevant aspect when comparing these sample types is that the greater preservation of eDNA in sediments allows for a more extended temporal perspective. In other words, sedimented eDNA reflects species' presence over longer periods, whereas eDNA in the water column tends to indicate more recent organism presence. This pattern is primarily due to the higher susceptibility of suspended eDNA to degradation — especially by microbial activity (CORINALDESI, BEOLCHINI and DELL'ANNO, 2008; MURAKAMI *et al.*, 2019; POTÉ, ACKERMANN and WILDI, 2009) — and to sedimentation processes (EICHMILLER, BAJER and SORENSEN, 2014; FENG *et al.*, 2022; HARPER *et*

al., 2020; KAMOROFF and GOLDBERG, 2018; MURAKAMI *et al.*, 2019; NEVERS *et al.*, 2021).

1.3.4 Ecological and taxonomic information derived from eDNA in the water column

Since eDNA is subject to constant processes of removal from the water column, influenced by factors such as the sedimentation of larger particles and the microbial degradation of smaller or free-floating fragments, the genetic material detected at a given depth generally represents a localized and transient signal - closely associated with the recent presence of species in that layer (EICHMILLER, BAJER and SORENSEN, 2014; FENG *et al.*, 2022; HARPER *et al.*, 2020; KAMOROFF and GOLDBERG, 2018; MURAKAMI *et al.*, 2019; NEVERS *et al.*, 2021). Considering this factor in relation to the specific characteristics of different environments, especially those associated with water column stratification, we can infer that the layer where eDNA is detected tends to reflect its point of release (FUKUMORI *et al.*, 2024; KAWAKAMI *et al.*, 2023; MARQUES *et al.*, 2024; MINAMOTO *et al.*, 2017; ROBLET *et al.*, 2024). Thus, sampled eDNA may indeed indicate the preferred layer of each species.

In our review, 36 studies indicated that the detected eDNA reflected the organisms' preference for the layer where sampling occurred. For example, Harper et al. (2020) observed higher concentrations of eDNA from the turtle *Chelonia mydas* in deeper waters, which aligns with the species' resting and foraging areas in San Diego Bay, California. Similarly, Marques et al. (2024) found significantly higher concentrations of eDNA from *Pinna nobilis* near the bottom, consistent with its benthic and sessile behavior. Roblet et al. (2024), by collecting samples from both the surface and near the bottom, not only demonstrated differences in communities across depths but also identified cryptobenthic species exclusively in the deeper samples and pelagic species in the surface samples.

Although eDNA typically reflects the preferred layer of the organism, this relationship is not always straightforward. For example, Hoban et al. (2023) detected 11 species of fish at depths of up to 15 meters beyond their previously

recorded maximum limits. The authors suggest that these species may be expanding or adapting to deeper habitats, or that the known patterns do not reflect the actual distribution limits of these species. However, we cannot discard the hypothesis that the detections are the result of eDNA particles in the process of sedimentation, as, in marine environments, eDNA is rarely detected above the depth at which it was released, but it can be found below that depth (CANALS *et al.*, 2021).

In other cases, when eDNA is found dispersed across different layers, it may not indicate the transport and dispersion of particles containing genetic material, but rather the mobility of the species itself across these layers, especially in taxonomically more complex species. Studies like those of Diao et al. (2023) and Zhang et al. (2020) were clear in demonstrating that higher trophic organisms, such as fish and aquatic mammals, tend to have their eDNA more evenly distributed due to their greater vertical mobility. In contrast, these same studies, along with others (FENG *et al.*, 2022; LIU *et al.*, 2019), emphasized the strong segregation of lower trophic organisms, such as bacteria, protozoa, algae, and invertebrate metazoans, whose communities vary significantly between different depths. For these organisms, in addition to size limitations and swimming structures that are insufficient to overcome vertical physical barriers (thermocline, halocline, pycnocline), vertical dispersion is restricted by the ecological stability of the community in a specific layer, influenced by factors that make the environment optimal for the group. For example, Diao et al. (2023) highlight that light availability in the more superficial layers (< 10 m) makes this layer suitable for the detection of phytoplankton communities, photosynthetic organisms that depend heavily on light. As depth increases and light decreases, the eDNA of these organisms becomes undetectable. Although more complex organisms may have greater vertical mobility, it is important to recognize that, even in these groups, it is common to observe specializations, such as pelagic or benthic tendencies, which can lead to the vertical segregation of eDNA. The fact is that the tendency of eDNA to remain in the layer in which it was released, or at least in its proximity, reinforces the idea that sampling from a single depth may not capture all species in an environment.

From the analysis of the studies, we identified 170 species with reports of preferred layers (divided into shallow, deep, and mid-water) (APPENDIX 6 -

Table S6.), obtained through eDNA collections. Of these, 85 were detected only in one of these layers: 38 in shallow layers, 42 in deep layers, and 5 in mid-water layers. These results confirm the need for sampling at different depths to provide a more complete picture of the species present at a site. For example, even in a dynamic coastal environment, where Dukan et al. (2024) found no significant differences between species detection in surface and bottom samples — as 73.58% of species were shared between these depths — at least eight species (or 15.09%) would not have been detected if sampling were limited to the surface.

In other cases, when the goal of the research is not to recover the highest number of species detected but rather to detect or quantify the eDNA of a target species, sampling at multiple depths may be unnecessary. However, this does not mean that depth is no longer a relevant factor; on the contrary, the accuracy and quality of eDNA detection can be significantly increased by considering the depth at which the sample will be collected, based on prior knowledge of the target organism's ecology and behavior (HARPER *et al.*, 2019; JANOSIK *et al.*, 2021; LITTLEFAIR *et al.*, 2021).

Although surface water sampling is, in some cases, effective for detecting eDNA from some benthic species (CARIM *et al.*, 2016; CURRIER *et al.*, 2018; LIU *et al.*, 2022; SHAW *et al.*, 2016), in certain situations, sampling from depth can be much more effective. For example, Janosik et al. (2021) detected eDNA of *Scaphirhynchus suttkusi* in benthic samples from the Tombigbee River during winter, whereas surface samples did not yield any detectable eDNA. Additionally, Carim et al. (2016) demonstrated that the eDNA of *Mysis diluviana*, which inhabits deeper waters during the day, had a concentration on average 173 times higher in benthic samples than in surface and thermocline samples.

Knowledge of species ecology can also be crucial for interpreting data obtained through eDNA. As far as is known, the eDNA technique alone is not capable of providing information about a species' ontogeny (YANG *et al.*, 2017). However, through eDNA analysis combined with sampling at different depths and prior knowledge of the natural history of *Larimichthys polyactis* and *Larimichthys crocea*, Wang, Lu, Zhao, Yang et al. (2021) and Wang, Lu, Zhao, Du et al. (2021) were able to infer that in certain sites, marked by the detection of eDNA near the surface, were associated with pelagic eggs, larvae, and juveniles of both species. On the other hand, detection at other points, with high eDNA concentrations in

intermediate and deep layers, was linked to adult individuals. Studies like these suggest the potential to use eDNA data in vertical and horizontal analyses for detecting spawning and breeding sites.

Finally, the association between vertical sampling at different times, such as day and night, may indicate species' vertical migration behaviors. Canals et al. (2021) showed that the eDNA patterns of fish collected during the day and night were aligned with the daily vertical migration of species from the continental slope of the Bay of Biscay. Govindarajan et al. (2023) also observed this vertical migration pattern in fish through eDNA, and similar patterns were studied for Copepoda (DOWELL *et al.*, 2024; FENG *et al.*, 2022) and Dinoflagellata (DOWELL *et al.*, 2024).

1.3.5 Guidelines and recommendations for eDNA sampling

In this section, we present sampling recommendations considering the vertical perspective of the water column, based on the suggestions from the studies evaluated and the conclusions of this review.

Regarding the need for sampling at different depths, not all studies support this approach. For example, Dukan et al. (2024) argue that sampling at a single depth may be sufficient, and if the goal of the research is to capture the full spectrum of biodiversity at a location, increasing the number of replicates at the same depth may be more efficient than sampling at multiple depths. In contrast, Robinson et al. (2023) advocate that sampling different depths may be more meaningful than increasing the number of sampling locations. It is important to note that, while Dukan et al. (2024) conducted sampling in a dynamic coastal environment with high water mixing, Robinson et al. (2023) worked in a fjord with strong halocline stratification, which substantially influences their results and, consequently, their recommendations.

Samples collected only at the surface were significant for some studies and recommended for specific cases. Boldrocchi et al. (2024) demonstrate that surface water sampling was sufficient for detecting the deep-diving cetacean *Ziphius cavirostris* through qPCR. Although this species inhabits depths of up to 3000 m, it must come to the surface to breathe, which makes it possible to detect

its eDNA even in the shallower layers. Paine et al. (2021), Currier et al. (2018), and Katano et al. (2017) recommend surface water sampling in small streams, highlighting the ease of this approach, especially since there is no need to enter the water, thus avoiding disturbance to the animals and habitat. The choice of surface samples, due to the ease of collection process, was also emphasized by Uthicke et al. (2018) for coral reefs and by Zhou et al. (2023) for Poyang Lake in warmer climates. Kamoroff and Goldberg (2018), on the other hand, recommend sampling near the surface to avoid false positives from possible dead individuals on the waterbody's bed. However, depending on the source of "non-living" eDNA, concentrations may be higher regardless of depth, as observed by Yamamoto et al. (2016), who noted an increase in eDNA concentration of *Trachurus japonicus* at different depth layers near a fish market. These authors emphasize the importance of investigating exogenous sources of eDNA in the environment before establishing sampling points.

Sampling near the bottom has been recommended to improve the detection of benthic species (BURGOA CARDÁS *et al.*, 2020; CARIM *et al.*, 2016; WU *et al.*, 2019) and sessile benthic organisms (LOR *et al.*, 2020; MARQUES *et al.*, 2024). Antich et al. (2021) highlighted that water collections, even at depth, are limited for analyzing benthic communities. On the other hand, Shaw et al. (2016) argued that sediment samples are less effective at detecting fish communities compared to water samples. To overcome these limitations, Clarke et al. (2021), Picard et al. (2023), and Wu et al. (2019) suggest paired sampling of water and sediments to detect benthic communities, fish, and bottom-dwelling species such as *Palaemon paucidens*.

Guri et al. (2024) and Roblet et al. (2024) recommend sampling at two layers: one closer to the surface and another closer to the bottom, which are sufficient for detecting and differentiating fish communities. However, studies in deeper marine waters, such as Murray et al. (2024), emphasize the importance of including more collection depths to achieve better taxonomic resolution for each depth layer. Other studies, such as those by Wang, Lu, Zhao, Yang et al. (2021) and Wang, Lu, Zhao, Du et al. (2021), and Zhang et al. (2022), highlight the need to maximize sampling points, both vertically and horizontally.

Dowell et al. (2024) and Adams et al. (2023) emphasize the relevance of sampling at different times to capture the daily vertical migration patterns of

certain species. The mobility of these species can cause the eDNA to be present or absent depending on the timing of the sample collection. Additionally, Sevellec et al. (2024) not only suggest considering different periods and depths but also recommend that samples be collected during different tidal phases.

Regarding factors that can cause stratification in the water column, such as the halocline (JEUNEN *et al.*, 2020) and thermocline (FUKUMORI *et al.*, 2024; KLOBUCAR, RODGERS and BUDY, 2017; LITTLEFAIR *et al.*, 2021), sampling above and below the stratification point is likely essential for a comprehensive view of the environment. In environments with a seasonal thermocline, planning samples for turnover periods, when the waters are more mixed, may make sampling from only one depth sufficient (KLOBUCAR, RODGERS and BUDY, 2017; LITTLEFAIR *et al.*, 2021).

This comprehensive review elucidates the primary factors governing the vertical distribution of eDNA in aquatic systems. The findings presented herein serve as a foundational framework for optimizing sampling protocols in eDNA-based studies and advance our understanding of environmental DNA ecology.

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2. CHAPTER 2 - VERTICAL DISTRIBUTION OF FREE AND MULTI-FRACTION ENVIRONMENTAL DNA IN A CONTROLLED AQUATIC SYSTEM: INSIGHTS INTO DISPERSAL DYNAMICS AND SAMPLING IMPLICATIONS‡

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ABSTRACT

The study of environmental DNA (eDNA) is becoming an increasingly valuable tool for investigating and monitoring aquatic communities. However, significant gaps remain in understanding its dynamics and distribution in aquatic environments. We performed controlled experiments to investigate the vertical distribution of eDNA in a simulated lentic water column, specifically comparing the dispersion of free extracellular DNA (f-exDNA) and multi-fraction DNA. Aliquots containing f-exDNA or multi-fraction DNA from two target species (*Limnoperna fortunei* and *Oreochromis niloticus*) were injected at the bottom and/or surface of a 5-meter experimental column. Water samples were collected at different depths over 8 h, and target eDNA concentration was determined by qPCR, allowing the assessment of the temporal scale of eDNA diffusion while minimizing the influence of water turbulence. f-exDNA rapidly disperses throughout the water column, becoming homogenous within 30 min. Multi-fraction DNA exhibited slower and more complex distribution dynamics, sinking rapidly when released at the surface and rising gradually when introduced at the bottom, achieving homogenization after 8 hours, likely due to the presence of larger particles. These findings highlight the influence of particle size on DNA dispersion and provide key insights for designing field sampling strategies that account for DNA fractions and vertical dynamics.

Keywords: aquatic environment; DNA distribution; DNA particles; eDNA; real-time PCR; water sampling.

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2.1 INTRODUCTION

Environmental DNA (eDNA) refers to the genetic material shed by organisms into their environment (BARNES and TURNER, 2016). eDNA samples consist of various biological particles, including free DNA, organelles, cells, tissue fragments, and metabolic waste (TURNER *et al.*, 2014; WILCOX *et al.*, 2015). When suspended in an aquatic environment, these biological particles can be sampled along with the water, extracted, and detected through molecular biology techniques (FICETOLA *et al.*, 2008). The application of eDNA for aquatic species surveillance and monitoring is widely adopted and offers distinct advantages over traditional methods. For instance, this approach can detect single or multiple species in a single sample (HARPER *et al.*, 2019) and can be used to estimate relative biomass (PILLIOD *et al.*, 2014; TAKAHARA *et al.*, 2012). The main advantages of this approach are the shorter time requirements, increased cost-effectiveness, increased taxonomic resolution, and non-invasive sampling (EILER *et al.*, 2018; HUNTER *et al.*, 2015; THOMSEN *et al.*, 2012). Recently, several studies have applied this method to detect aquatic organisms, such as fish (DAL PONT *et al.*, 2021; WANG *et al.*, 2022; XIN *et al.*, 2024), mussels (EGETER *et al.*, 2022; MARQUES *et al.*, 2024; XIA *et al.*, 2018), oysters (DUGAL *et al.*, 2024), jellyfish (MINAMOTO *et al.*, 2017; PENG *et al.*, 2023; TAKASU *et al.*, 2019; YE *et al.*, 2024), elasmobranchs (DUNN *et al.*, 2022; LEURS *et al.*, 2023), and amphibians (BRAMMELL *et al.*, 2023; MOSS *et al.*, 2022; O'DONNELL, FOX and INGRALDI, 2023).

Although eDNA is a powerful tool for investigating and monitoring aquatic communities, its field sample collection and analysis methods lack standardization (HINLO *et al.*, 2017). Most methods typically collect water samples at the surface or bottom of the water column (BUXTON *et al.*, 2017; KATANO *et al.*, 2017), whereas sampling at multiple depths is rare (ANDRUSZKIEWICZ *et al.*, 2017; YAMAMOTO *et al.*, 2016). Horizontal dispersion of eDNA is well-studied in rivers (DEINER and ALTERMATT, 2014; JERDE *et al.*, 2016; JO and YAMANAKA, 2022; PONT, 2024; SANSOM and SASSOUBRE, 2017), but the vertical distribution is still scarcely explored from a

functional perspective. Vertical zoning, which describes the structuring of species and communities across depths, is closely linked to vertical eDNA dynamics. This zoning can exhibit dramatic changes within a few meters (CHAPPUIS *et al.*, 2014), leading to variations in eDNA concentration, composition, and spatial distribution as biological communities shift with depth. While some studies report negligible impacts of vertical distribution on eDNA detection and composition (ANDRUSZKIEWICZ, SASSOUBRE and BOEHM, 2017; CURRIER *et al.*, 2018; EICHMILLER, BAJER and SORENSEN, 2014; HERVÉ *et al.*, 2022; MOYER *et al.*, 2014), others detected significant differences (AMBERG *et al.*, 2019; ANDRUSZKIEWICZ *et al.*, 2017; BURGOA CARDÁS *et al.*, 2020; CARIM *et al.*, 2016; HÄNFLING *et al.*, 2016; HARPER *et al.*, 2020; JEUNEN *et al.*, 2020; KLOBUCAR, RODGERS and BUDY, 2017; LACOURSIÈRE-ROUSSEL *et al.*, 2018; LITTLEFAIR *et al.*, 2021; MINAMOTO *et al.*, 2017; MURAKAMI *et al.*, 2019; ROBINSON *et al.*, 2023). These studies, however, vary significantly regarding characteristics of the water body architecture, water composition, depth sampled, sampling strategy, target organism, detecting technique, extraction protocol, and molecular marker. This leads to an inconsistent pattern of results, which can be misinterpreted as being solely due to the biological characteristics of the organism, rather than a combination of these characteristics and the environmental depth at which the sample was collected (MINAMOTO *et al.*, 2017). However, these studies tend do not consider how eDNA moves through the column as (a) the sources of biological material are still in the water, releasing particles, while the particles that are still in the water are being degraded and moved horizontally, and (b) they do not consider time as one of their variables, only depth.

Another key factor in eDNA vertical dispersion is the form in which DNA fragments exist. The term eDNA comprises the various fractions in which DNA may be present in the environment, such as intracellular DNA (iDNA), free extracellular DNA (f-exDNA), weakly bound extracellular DNA (wb-exDNA), and tightly bound extracellular DNA (tb-exDNA) (NAGLER *et al.*, 2022). The different forms of DNA in the aquatic environment exhibit different dispersion behaviors, mainly due to the strong tendency for DNA bound to larger particles (>1 μm) to sink (NEVERS *et al.*, 2021; TURNER *et al.*, 2014; TURNER, UY and EVERHART, 2015).

Water bodies are complex systems with varied hydraulic dynamics. Studying the vertical aspects of eDNA in a natural system is difficult due to many factors acting in the water column at once (JANE *et al.*, 2015). Flow, hyporheic exchanges, streambeds, surface-subsurface exchange, sediment, and colloidal interactions are some of the factors that contribute to this complexity (SHOGREN *et al.*, 2016; SHOGREN *et al.*, 2019). Controlling these variables in a field experiment to understand how they affect the vertical dynamics of eDNA is not logistically viable, so they must be studied individually in a controlled environment.

In this study, we investigated the behavior of eDNA particles in a simulated lentic freshwater column. To this end, we built an experimental apparatus in the form of a long vertical tube, injected DNA at different depths (bottom and surface), and monitored how it moves vertically through the water column over time. Understanding eDNA behavior in the water column is crucial for interpreting species distribution and improving sampling strategies. A controlled environment is ideal for this purpose, as it allows the introduction of variables as our understanding of these dynamics evolves.

2.2 MATERIALS AND METHODS

2.2.1 Experimental setup

We built three experimental apparatus to emulate a water collumn of a lentic freshwater aquatic environment using 5-meter-high polyvinyl chloride (PVC) tubes (20 cm diameter, total volume: 160 L). Chromatographic septa were placed at six depths (0, 1, 2, 3, 4, and 5 m) to allow water sampling using sterile 1-mL medical syringes from the cylinder's external side (**Figure 3**). This design minimized turbulence within the water column.

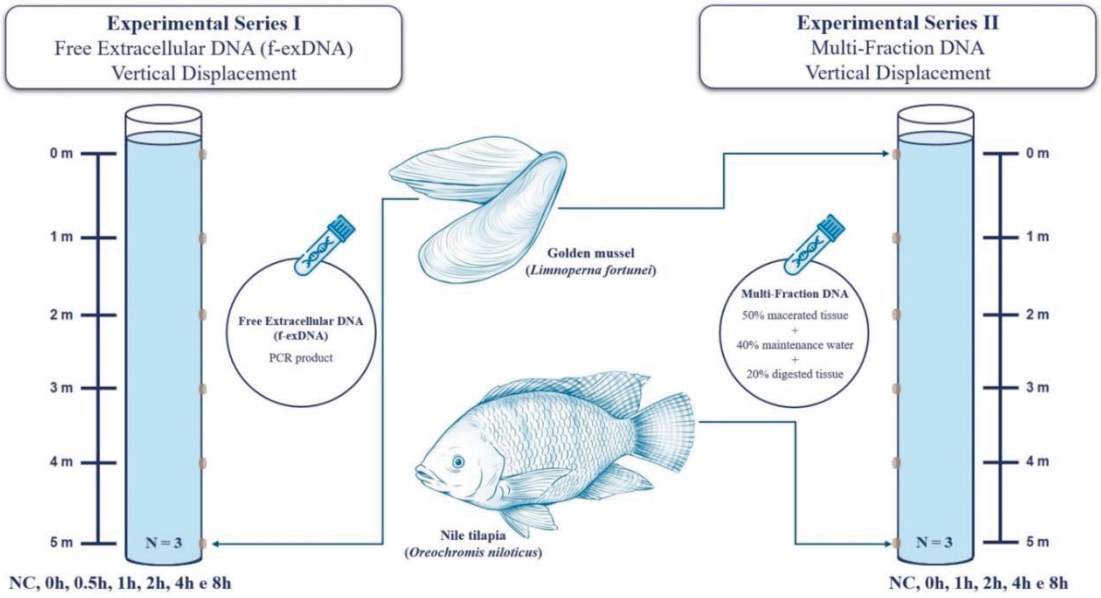

Figure 3. Schematic representation of the experiments evaluating the vertical displacement of free extracellular DNA (f-exDNA) and multi-fraction DNA from *Limnoperna fortunei* (golden mussel) and *Oreochromis niloticus* (Nile tilapia) during an 8-h period.

Before each experiment, the apparatus was decontaminated using a two-step process. First, we washed the tubes thoroughly with a 6% sodium hypochlorite solution to remove DNA and biological particles. Next, residual chlorine was neutralized by rinsing three times with DNA-free water, prepared by treating Curitiba tap water with 10% sodium hypochlorite (0.2 mL/L), followed by 50% sodium thiosulfate solution (0.1 mL/L). Residual chloride was monitored using colorimetric tests (ZALL, FISHER and GARNER, 1956).

2.2.2 Preparation of the DNA fractions

Two target species were used for DNA preparation: *Limnoperna fortunei* (Dunker, 1857) (golden mussel) and *Oreochromis niloticus* (Linnaeus, 1758) (Nile tilapia), selected due to their invasive potential (BOLTOVSKOY and CORREA, 2015; CASSEMIRO *et al.*, 2018), leading them to be used as models in studies involving eDNA (ANDRADE, RAZZOLINI and BAGGIO, 2021; PIE *et al.*, 2017).

Two independent experiments were conducted using DNA aliquots of different compositions (**Figure 3**). In the first experiment, we injected a DNA aliquot containing only copies of free extracellular DNA (f-exDNA) from *L.*

fortunei. In the second experiment, we used DNA aliquots theoretically composed of various DNA fractions (iDNA, f-exDNA, wb-exDNA, tb-exDNA) (Nagler et al. 2022) from both target species.

To obtain an aliquot of free extracellular DNA, we amplified fragments of approximately 100 bp of the *COI* gene from a genomic sample of *L. fortunei* using PCR. The set of primers used in the reaction was designed by Pie et al. (2017) and is described in Table 2. Each assay was run in a 25 µL final volume reaction, with concentrations: 100 µM each primer, 0.25 mM dNTP mix, 1 U Platinum Taq DNA Polymerase, 1 X Platinum Taq buffer and 2 mM $MgCl_2$. Thermocycling conditions were performed as follows: 1 min at 95 °C for initial denaturation, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 30 sec and at 70 °C for 30 sec. To obtain a high DNA concentration for the stock test solution, we carried several independent PCRs, and the resulting products were pooled and later quantified using Qubit 4 fluorometer.

The multi-fraction DNA for each species was obtained by mixing three components in different proportions: macerated tissue (50%), animal maintenance water (40%), and digested tissue (10%). The macerated tissue component consisted of the supernatant obtained by the mechanical maceration of 10 g of tissue from the target species, followed by homogenization in 40 mL of ultrapure water and centrifugation at 6000 RPM (2 min – 4 °C). The maintenance water was taken from a container where individuals of the target species were kept for 24 hours, under constant aeration (O2 saturation - 75-80%), controlled temperature (22-23 °C), and density (20 g/L). The digested tissue was prepared to ensure the presence of extracellular DNA (exDNA) and was obtained by digesting muscle tissue from the target species (0.05 g) in 200 µL of digestion buffer (1% SDS, 30 mM Tris, and 10 mM EDTA) and 20 µL of proteinase K. the digestion process was performed in a dry bath incubator (56 °C) for 24 h.

The inoculation samples were stored at -80 °C and thawed before use. qPCR was used to quantify target DNA concentrations: ~2.0 x 10^6 ng/L for *L. fortunei* as f-ex-DNA, ~3 ng/L (*O. niloticus*), and ~86 ng/L (*L. fortunei*) for the multi-fraction DNA.

2.2.3 Experimental procedures

Two experimental series were conducted independently. Each experiment started by filling the entire apparatus with DNA-free water, keeping the surface point (0 m) 10 cm from the waterline. It was followed by a 24-hour period to allow water movement to decrease. The water temperature was measured immediately before the start of the experiments, with an average of 22.0 ± 0.8 °C. Both experiments were conducted in triplicate.

2.2.4 Experimental Series I: f-exDNA vertical displacement

The goal of this experiment was to establish the time for the vertical displacement of extracellular DNA particles in the water column (from the bottom to the surface). For this purpose, aliquots (1 mL – 2,0 X 10^6 ng/L of DNA) of free extracellular DNA (PCR product) of *L. fortunei* were inoculated into the experimental apparatus (n=3) at the 5 m depth. At 0, 0.5, 1, 2, 4, and 8 h following the inoculation, 2 mL water samples were collected at each sampling depth (0, 1, 2, 3, 4, and 5 m). The samples were stored in sterile Eppendorf tubes (2.0 mL) and preserved at -20 °C until processing. For the control samples, a 2 mL sample was collected at each depth (0, 1, 2, 3, 4, and 5 m) immediately before the inoculation of the DNA aliquot into the apparatus. The control samples underwent the same storage and preservation procedures as the other samples.

2.2.5 Experimental Series II: Multi-fraction DNA vertical displacement

The objective of this experiment was to evaluate the period of vertical DNA displacement (from the bottom to the surface and from the surface to the bottom) of a sample containing DNA particles of various sizes – simulating an eDNA source. For this purpose, we used multi-fraction DNA aliquots (20 mL) containing DNA molecules from two species of aquatic organisms (*L. fortunei* [86.23 ng/L of DNA] and *O. niloticus* [3.29 ng/L of DNA]). The eDNA aliquot of *L. fortunei* was inoculated 0 m (the surface end of the apparatus) and the eDNA

aliquot of *O. niloticus* was injected at the 5 m (at the bottom of the apparatus). Both injections were performed simultaneously. At 0, 1, 2, 4, and 8 h following the inoculation 2 mL water samples were collected at each depth (0, 1, 2, 3, 4, and 5 m). Control samples were also collected before the eDNA injection. Sample storage and preservation was performed as described for the experimental series I.

2.2.6 DNA amplification and quantification

The samples were thawed at room temperature for processing. Free DNA samples from experimental series I did not require digestion. Samples from experimental series II (containing multi-fraction DNA) underwent a digestion process (900 µL of sample, 200 µL of digestion buffer [1% SDS, 30 mM Tris, and 10 mM EDTA], 45 µL of proteinase K followed by incubation at 56 °C for 24 hours. Then, all samples underwent DNA extraction using the Solid Phase Reversible Immobilization (SPRI) protocol (DEANGELIS, WANG and HAWKINS, 1995), where 1 mL of the sample was incubated in a solution with a final concentration of 12.5% weight/volume PEG-8000, 0.7 M NaCl, and 0.02 mg/mL carboxylated magnetic beads, at room temperature for 10 minutes, to condense DNA and adhere to the magnetic beads. The samples were then magnetized using neodymium magnetic racks (NEB) and the supernatant was removed. The samples were dried at room temperature and eluted in 100 µL of TE buffer (10 mM Tris-HCl and 1 mM EDTA) and gently mixed. After separating DNA from the magnetic beads, the samples were magnetized again, and the supernatant containing DNA was collected and stored.

After extraction, the samples were quantified by qPCR using a hydrolysis probe (TaqMan) to determine the initial concentration of COI gene fragments for each target species. For *L. fortunei*, the same primers and probe previously used in the process of obtaining the extracellular DNA aliquot were employed, as described by Pie et al. (2017). For *O. niloticus*, a new set of primers and probe was designed, tested and validated prior experimentation. The primer and probe process of design and validation is detailed in the Supplementary Information file

(APPENDIX 7 - Supplementary Information). The sequences of the primers and probes for both species are presented in Table 2.

Table 2. Sequences of primer and probes for *Limnoperna fortunei* (Pie et al. 2017) and *Oreochromis niloticus* used for free-extracellular DNA synthesis and qPCR analysis

Target species	Type	Gene/ probe	Sequence	Position	Size (pb)
Limnoperna fortunei	Primer	COI	GGGACTGGTTGGACAGTTTAT	Forward	21
			ACGCACCAGCTAAATGAAGA	Reverse	
	Probe	FAM	CCCAGCAGTTGACATAGCTGCTTT	-	24
Oreochromis niloticus	Primer	COI	ACATGAAACCCCCTGCCATCTC	Forward	21
			CCTCCGGCAGGGTCAAAGAAG	Reverse	
	Probe	FAM	TGCCCGTTCTTGCCGCCGGCATCACAA	-	27

The qPCR assays for each target species were performed with a final volume of 10 µL, with the following concentrations: 0.75 µM each primer, 0.25 µM probe, 0.06 M betaine, 0.05 µg/µL BSA, 0.3 µL vegetable glycerin P.A., and 1X QuantiNova Probe PCR Kit (Qiagen). Each sample was analyzed in triplicate, with 3 µL of extract used in each reaction. Cycling conditions were 2 min at 95 °C for enzyme activation, followed by 50 cycles of denaturation at 95 °C for 5 seconds and annealing and extension combined at 60 °C for 5 seconds. The qPCR assays were run in a RotorGeneQ 5plex+HRM (Qiagen).

For quantification, a standard curve was built for each primer by running a five-order serial dilution of the stock solution previously quantified using Qubit, also performed in triplicate. Each run was analyzed using RotorGeneQ Series Software (Qiagen), with quantification analysis. Threshold was calculated with automatic option, with a 0.35 upper bound limit, and quantification of DNA concentration (ng/L) was done in the “dynamic tube” mode.

2.2.7 Statistical Analyses

All data were analyzed using Python programming in the Spyder 6.02 environment. Data cleaning and loading were performed using the Pandas

library. The datasets were grouped into three experimental series: free extracellular DNA of *L. fortunei*, multi-fraction DNA of *L. fortunei*, and multi-fraction DNA of *O. niloticus*.

To ensure the validity of the statistical analyses, we verified the data's normality using the *Shapiro-Wilk, Kolmogorov-Smirnov*, and *Anderson-Darling* tests and homoscedasticity using the Breusch-Pagan test. Exploratory data analysis was performed using the Seaborn and Matplotlib libraries. Heatmaps were generated to identify trends in eDNA concentration across depths and time, facilitating the interpretation of vertical displacement patterns.

2.3 RESULTS

2.3.1 Experimental Series I: f-exDNA vertical displacement

Immediately after the inoculation of free extracellular DNA (0 h), the highest concentration was observed at the bottom of the water column (5 m). Within 30 minutes, DNA concentrations increased at intermediate depths (3–4 m), indicating the initial stages of vertical dispersion. After 1 h, DNA concentrations became evenly distributed across the water column, demonstrating rapid homogenization of small DNA particles in lentic conditions. This distribution persisted for up to 8 hours, with slight variations observed at the surface (0 m) and bottom (5 m) (**Figure 4**).

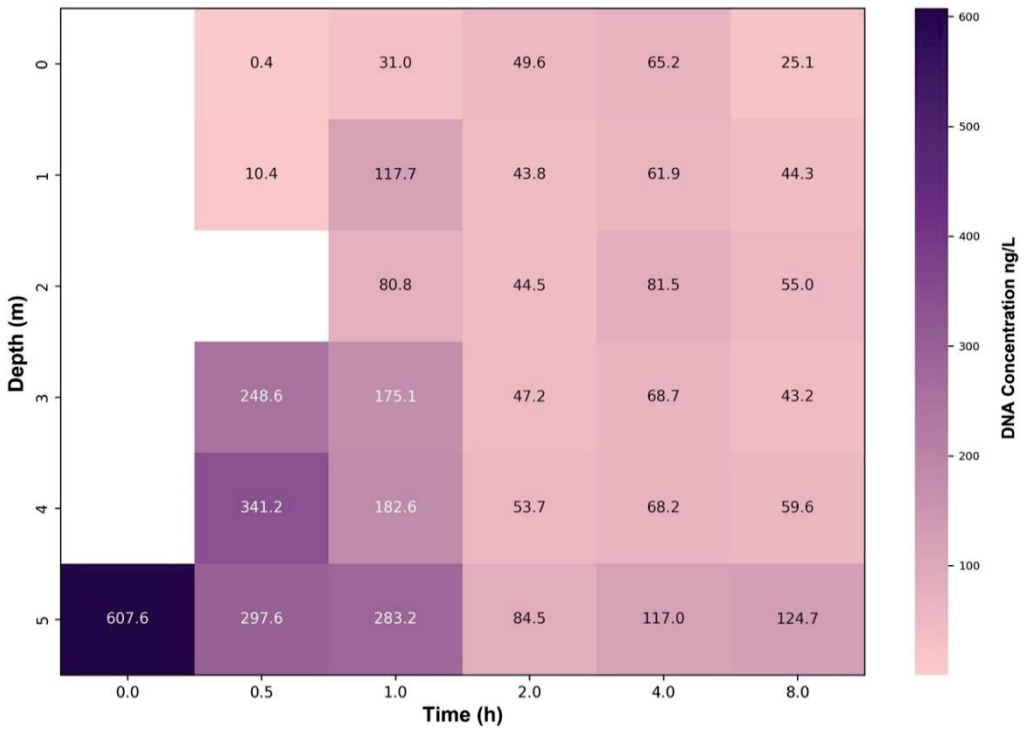

Figure 4. Heatmap of the mean concentration of free extracellular DNA (ng/L) in a lentic freshwater column (0–5 m depth) over time (0–8 h), showing vertical displacement. The numbers represent the DNA concentration (ng/L) at specific depths and times. The aliquot, derived from *Limnoperna fortunei*, was injected at a depth of 5 m at time zero.

2.3.2 Experimental Series II: Multi-fraction DNA vertical displacement

The multi-fraction DNA exhibited a slower and more complex displacement pattern compared to f-ex-DNA. When inoculated at the bottom (5 m), multi-fraction DNA remained concentrated near the injection site for the first hour, with minimal movement toward the upper layers (**Figure 5**). After 2 hours, DNA was detected at intermediate depths (3–4 m), indicating the beginning of an upward movement. By the 8th hour, multi-fraction DNA was present throughout the water column; however, concentrations remained highest at the bottom and diminished toward the surface.

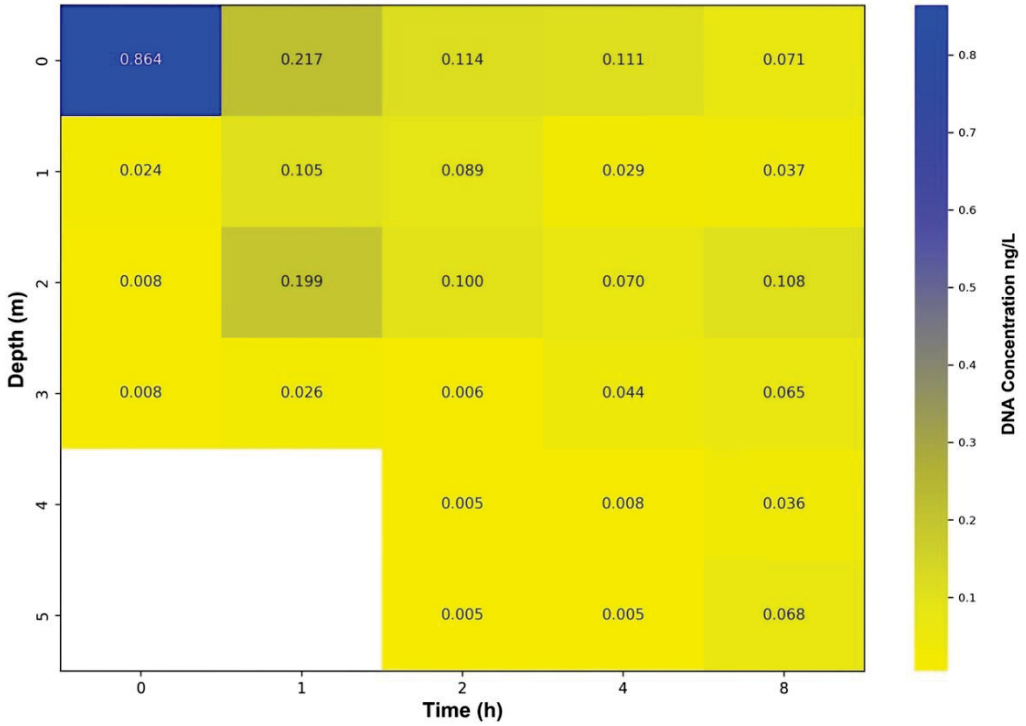

Figure 5. Heatmap of the mean concentration of multi-fraction DNA (ng/L) in a lentic freshwater column (0–5 m depth) over time (0–8 h), showing vertical displacement. The numbers represent the DNA concentration (ng/L) at specific depths and times. The aliquot, derived from *Oreochromis niloticus*, was injected at a depth of 5 m at time zero.

When multi-fraction DNA was inoculated at the surface (0 m), downward displacement occurred more rapidly. At 30 minutes, DNA was detected at 3 m depth. By 2 hours, DNA reached the bottom (5 m) but showed lower concentrations in intermediate layers (4–5 m). Homogenization occurred by the 8th hour, but with residual DNA accumulation near the surface and bottom (**Figure 6**).

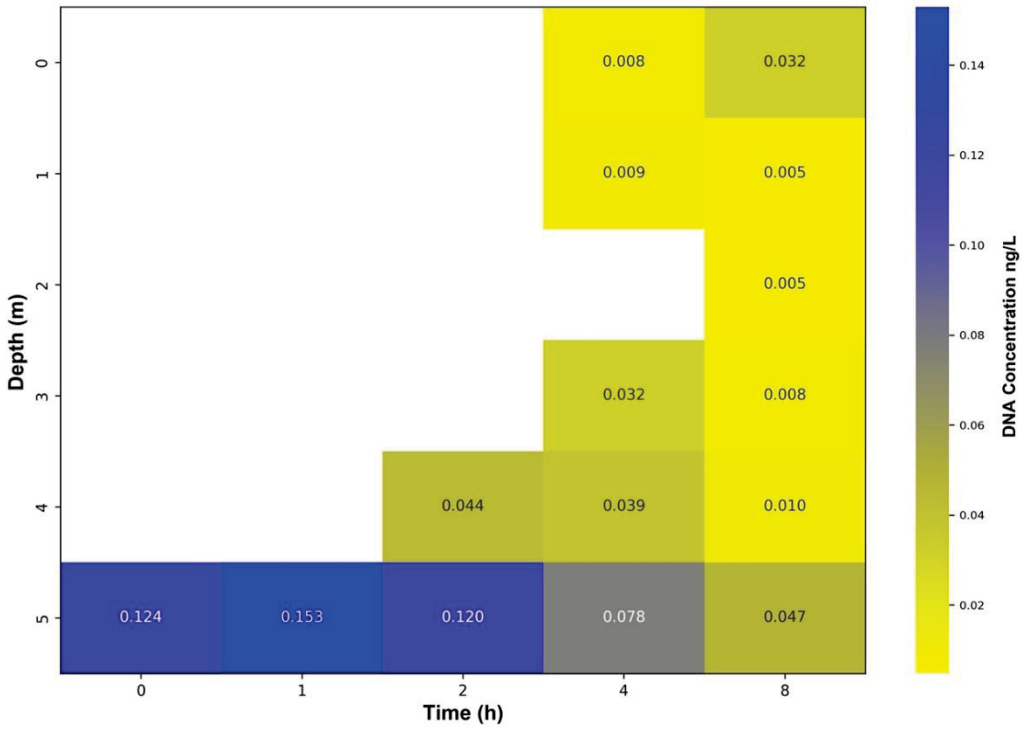

Figure 6. Heatmap of the mean concentration of multi-fraction DNA (ng/L) in a lentic freshwater column (0–5 m depth) over time (0–8 h), showing vertical displacement. The numbers represent the DNA concentration (ng/L) at specific depths and times. The aliquot, derived from *Limnoperna fortunei*, was injected at the surface (0 m) at time zero.

2.3.3 Comparative analysis between f-exDNA and multi-fraction DNA

The free extracellular DNA demonstrated rapid and uniform homogenization across the water column within 1 hour, highlighting its high dispersal capacity. In contrast, multi-fraction DNA showed delayed and heterogeneous dispersion, influenced by particle size and density. Larger particles tended to sink, concentrating near the bottom, while smaller particles contributed to gradual vertical movement.

2.4 DISCUSSION

Our results were successful in investigating the vertical displacement patterns of f-exDNA and multi-fraction DNA in the water column under controlled conditions. As the application of eDNA becomes increasingly used for monitoring diverse aquatic biota, understanding the factors affecting its distribution becomes

essential for improving detection accuracy. We observed that f-exDNA disperses rapidly, within minutes, even without turbulence, a finding of particular importance since natural water currents would likely further enhance homogenization. However, it is essential to recognize that eDNA in natural environments exists as a polydisperse mixture (TURNER *et al.*, 2014; WILCOX *et al.*, 2015), where particle size strongly affects vertical behavior (NAGLER *et al.*, 2022). To further investigate this phenomenon, we conducted a second experiment using DNA from two aquatic species to evaluate the vertical movement of multi-fraction eDNA.

The multi-fraction DNA of *O. niloticus* exhibited higher resistance to vertical dispersion, with the highest concentrations consistently observed at the deepest part (5 m) across all sampling times. Compared to f-exDNA, the multi-fraction eDNA ascended from the bottom to mid and upper levels of the water column at a rate four times slower, reflecting the impact of particle size on dispersal dynamics. This disparity in distribution speed aligns with the expected influence of particle size, as multi-fraction eDNA includes larger particles that settle more readily due to gravitational forces. eDNA particles in the water column vary in size, ranging between 1 and 10 µm (TURNER *et al.*, 2014; WILCOX *et al.*, 2015), which limits their capacity to remain suspended in the water column, resulting in their accumulation near the bottom of lentic systems. Gravitational sedimentation plays a dominant role in shaping the vertical distribution and concentration of multi-fraction eDNA. We observed initial upward dispersion of eDNA particles at 2 hours, intermittent dispersion at 4 hours, and near-homogeneous distribution at 8 hours. This gradual dispersion may be driven by lighter particles within the f-exDNA fraction or by the progressive breakdown of larger particles into smaller, more mobile fragments. Our hypothesis is supported by findings from the *L. fortunei* f-exDNA experiment, where only small, lightweight DNA fragments were present, leading to rapid homogenization.

Although the dissipation of multi-fraction eDNA was slower than that of free extracellular DNA, our experiment confirmed that eDNA molecules can move from the bottom to the surface in a 5-meter-deep aquatic environment, even in undisturbed conditions. In contrast, a study using dead goldfish (*Carassius auratus*) placed at the bottom of 2-liter containers found no DNA in the surface or middle layers (0 and 21 cm depth) but detected it only near the dead animal at

the bottom (41.9 cm depth) (KAMOROFF and GOLDBERG, 2018). This delay may reflect the equilibrium between DNA release from the decomposing body and its degradation, possibly accelerated by microbial colonization promoted by the presence of the organism itself (KEENAN, EMMONS and DEBRUYN, 2023). In our experiment, DNA injection was designed to mimic the immediate release of DNA from a living organism, providing insight into the dynamics of newly released eDNA. Over time, it is likely that DNA in the water column would degrade due to the action of environmental factors such as microbial activity, UV radiation, and enzymatic processes (NEVERS *et al.*, 2021; ZHAO, VAN BODEGOM and TRIMBOS, 2021).

Still addressing the dispersion from the bottom to the surface, our results revealed an intermittent pattern, characterized by the absence of DNA at a depth of 2 meters during the 0.5-hour sampling time in the free extracellular DNA experiment and at 4 hours in the multi-fraction DNA experiment. Although DNA was detected at shallower depths (0 and 1 meter) during these times, its concentration remained low, accounting for only 1.2% and 11.4% of the total DNA measured, respectively. This suggests that super-light fractions, likely consisting of free extracellular DNA or DNA bound to buoyant particles, ascended more rapidly to the surface, while denser particles required more time to distribute throughout the water column. Intermittent eDNA has also been documented in horizontal distribution studies. Jerde et al. (2016) and Shogren et al. (2017) observed similar patterns when analyzing fish DNA in semi-natural experimental flows. Their findings suggest that eDNA does not behave deterministically as a conservative tracer but exhibits stochastic dynamics influenced by environmental and physical factors.

As expected, distinct dispersion patterns emerged for DNA injected at the surface and the bottom of the experimental water column. By the final sampling time (8 hours), DNA from the target species was detected at all depths; however, surface-to-bottom dispersion occurred more rapidly. For instance, multi-fraction eDNA from *L. fortunei* was detected at a depth of 3 meters shortly after being injected at the surface and was present at all depths within 2 hours. Previous studies investigating eDNA concentrations following source removal reported a rapid decline in suspended eDNA levels (NEVERS *et al.*, 2021; ZHAO, VAN BODEGOM and TRIMBOS, 2021), primarily attributed to natural degradation

processes (JOSEPH *et al.*, 2022). However, it is plausible that this decline is strongly influenced by the gravitational sedimentation of particles containing eDNA, as highlighted by Harrison et al. (2019).

Environmental DNA is not a monodisperse phase in nature but instead comprises particles ranging from single DNA molecules to tissue fragments, typically between 0.2 and 180 μm, with most particles falling in the 1–10 μm range (TURNER *et al.*, 2014). The size of DNA particles plays a critical role in studies comparing different depths, as particle interaction with filter pore size directly influences the captured eDNA profile. In our experiments, we bypassed the filtration step because the tested eDNA fractions were already concentrated, and the experimental samples were relatively small. Although the particle size distribution of eDNA is consistent among closely related taxa, as documented in fishes (BARNES *et al.*, 2021), it varies significantly across broader taxonomic groups, such as water fleas (MOUSHOMI *et al.*, 2019). Moreover, this distribution changes over time, as larger particles tend to fragment into smaller ones (MURAKAMI *et al.*, 2019). The lack of standardization in sampling and processing methods — such as sample volume, filtration techniques, and the time between collection and measurement — further complicates comparisons across studies, amplifying the influence of particle size distribution on the results. On the other hand, the behavior of different particle sizes in the water column remains poorly understood (ALLAN *et al.*, 2021). This lack of understanding introduces potential bias in eDNA sampling, as the captured eDNA may not accurately reflect the true concentration of eDNA at a given sampling point. Filter pore size and sample volume configurations can introduce errors that vary significantly between sampling locations.

We also hypothesize that the solubility of eDNA particles influences their behavior in the water column. Although most eDNA particles exhibit hydrophilic properties, some are hydrophobic and may interact differently with their environment. Environmental DNA can bind to colloidal particles, altering its natural suspension dynamics primarily due to changes in weight. This interaction may lead to localized accumulation within the water column or variations in diffusion speed (CAI, HUANG and ZHANG, 2006; CAI *et al.*, 2006). When bound particles become too dense, they may sink, resulting in the deposition and accumulation of eDNA on the substrate (ZHAI, WANG and PUTNIS, 2019). The

size of suspended particles also plays a crucial role, as finer substrates capture more eDNA due to their smaller pore size (SHOGREN *et al.*, 2016). This lighter eDNA-substrate complex can be easily resuspended into the water column, potentially introducing sampling biases or, for example, sampling near the bottom may unintentionally resuspend substrate-bound eDNA, leading to the overrepresentation of trapped eDNA in water sample (TURNER *et al.*, 2014). Hydrogeomorphic features of the studied system must also be considered, as slope variations and adsorption sites can significantly influence eDNA distribution by providing sequestration points (FREMIER *et al.*, 2019). While our results elucidate the behavior of free extracellular DNA and multi-fraction DNA particles in a relatively small-scale water column, they provide a controlled baseline to understand how these dynamics occur in the absence of external interference.

This concept is closely tied to a fundamental aspect of understanding eDNA dynamics in the water column: its relationship with the particle size distribution (PSD) of suspended materials. Studies by Turner et al. (2014) and Wilcox et al. (2015), conducted with fish species such as *Cyprinus carpio* and *Salvelinus fontinalis*, demonstrate that although eDNA occurs in particles of various sizes in aquatic environments, most detections are associated with PSDs between 1 and 10 µm. This pattern suggests that detected eDNA may be primarily contained within organelles, such as mitochondria (oDNA), or small intact cells (iDNA), as their sizes correspond to this range (NAGLER *et al.*, 2022; TURNER *et al.*, 2014; WILCOX *et al.*, 2015). Alternatively, and not exclusively, this DNA may be of extracellular origin, adsorbed onto larger mineral or organic particles through divalent cations (tb-exDNA) or cationic bridges (wb-exDNA) (BRANDÃO-DIAS *et al.*, 2023; NAGLER *et al.*, 2022).

The high proportion of larger particles (> 1 µm) likely plays a significant role in species detection, as these particles tend to settle on the substrate rather than remain suspended in the water column, especially in lentic systems with limited resuspension factors (TURNER, UY and EVERHART, 2015). In this study, we did not directly measure the PSD associated with eDNA, but it is plausible to assume that particle size proportions resemble those described in previous research, particularly in samples containing multi-fraction DNA. The sedimentation tendency of these larger particles may not only explain the faster movement of DNA from the surface to the bottom but also account for the

observed decline in DNA concentrations over time across all experimental assays.

It is important to highlight that our assays may not fully represent DNA dispersion patterns in environments deeper than 5 meters. Deeper natural environments are often more influenced by water column stratification processes, such as haloclines and thermoclines (BOEHRER and SCHULTZE, 2008). In such stratified systems, eDNA tends to remain confined within specific layers, such as those established by haloclines (JEUNEN *et al.*, 2020; KAWAKAMI *et al.*, 2023) or thermoclines in freshwater (FUKUMORI *et al.*, 2024; KLOBUCAR, RODGERS and BUDY, 2017; LITTLEFAIR *et al.*, 2021) and saltwater environments (ANDRUSZKIEWICZ *et al.*, 2017; KAWAKAMI *et al.*, 2023; ZHANG *et al.*, 2020). As a result, sampling at a single depth in these environments may fail to adequately capture the diversity of organisms across the entire water column. Depending on the study objectives, sampling strategies should consider the natural history of the target organisms, prioritizing layers most relevant to their ecology (CARIM *et al.*, 2016; FUKUMORI *et al.*, 2024; LITTLEFAIR *et al.*, 2021). Alternatively, multi-depth sampling may be necessary for multi-species surveys, such as those conducted with metabarcoding approaches (ANDRUSZKIEWICZ *et al.*, 2017; JEUNEN *et al.*, 2020; LITTLEFAIR *et al.*, 2021; ZHANG *et al.*, 2020).

Our results indicate that sampling at any depth may be sufficient to detect the presence of a species via environmental DNA 8 hours after its release in a lentic aquatic environment up to 5 meters deep. This time frame could be shorter in natural environments, where even lentic systems experience disturbances caused by factors such as wind (REARDON *et al.*, 2014) or bioturbation (ADÁMEK and MARŠÁLEK, 2013), which enhance water mixing between the surface and the bottom. These findings align with previous studies that detected target species' DNA at all depths in shallow environments (< 5 meters) (CURRIER *et al.*, 2018; EICHMILLER, BAJER and SORENSEN, 2014; MOYER *et al.*, 2014). Given the numerous factors acting simultaneously in complex aquatic systems, it is essential to analyze these components separately to understand their individual behaviors. This approach enables the development of robust models applicable to realistic field conditions. With so many factors acting simultaneously in complex aquatic systems, it is important to analyze their

components separately and understand how they behave individually so that we can build a more robust model that can be applied to realistic field conditions.

While this study has contributed to advancing the understanding of eDNA dynamics in controlled environments, caution must be exercised when extrapolating these findings to natural and more dynamic systems due to the diverse characteristics of aquatic ecosystems, including depth, hydrological patterns, and biotic and abiotic factors. Future research should focus on the interactions between eDNA, colloidal particles, sedimentation, and water mixing processes, such as turbulence and bioturbation, across a broader range of natural environments. Integrating molecular tools with innovative experimental designs and comprehensive field studies will be essential for developing robust models to predict eDNA dynamics in real-world scenarios. Such advancements will enhance the application of eDNA in conservation, ecological monitoring, and environmental management, making it a more reliable tool for addressing pressing environmental challenges.

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GENERAL CONCLUSIONS

This study investigated the vertical dispersion of environmental DNA (eDNA) in the water column, exploring both the available scientific literature and controlled experiments to understand the factors influencing its distribution. The results indicate that the vertical dynamics of eDNA are influenced by multiple physical, chemical, and biological factors, which can affect both the composition of detected communities and the concentration of eDNA at different depths.

The systematic review revealed that most eDNA studies still focus on collecting samples from the surface, potentially underestimating the biodiversity present in deeper layers. Data analysis showed that 37 studies indicated differences in the composition of detected communities across depths, while 28 studies identified variations in eDNA concentration. These differences were particularly evident in environments with thermal and halocline stratification, where species segregation along the water column directly influences eDNA dispersion patterns.

Controlled experiments demonstrated that the free eDNA fraction disperses rapidly, becoming homogeneous within a few hours, while eDNA associated with larger particles exhibits distinct behavior, tending to settle more quickly. This finding highlights the need to consider the form of eDNA when interpreting its distribution in the water column. The differential dispersion between eDNA fractions has direct implications for sampling strategy design, indicating that collecting samples at multiple depths may be essential for a more representative assessment of biodiversity in aquatic environments.

Given these findings, it is evident that improving eDNA sampling and analysis methodologies is crucial, including approaches that consider not only spatial distribution but also the dynamic processes affecting the persistence and mobility of genetic material in water. Future studies should focus on testing the influence of different environmental variables on eDNA dispersion in natural environments, as well as developing predictive models to assist in planning more efficient sampling strategies.

In summary, this study contributes to advancing knowledge on eDNA ecology, providing insights to enhance its use in environmental monitoring and

biodiversity conservation. Considering the vertical behavioral dynamics of eDNA is a fundamental aspect of ensuring the reliability of data generated by this tool, reinforcing the need for sampling protocols that more accurately reflect the diversity and distribution of organisms in aquatic systems.

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APPENDIX 1 - TABLE S1.

Table S1. Terms used in each category that comprise the search strings. Terms from the 1st category were searched exclusively in the titles of the articles, whereas terms from the 2nd and 3rd categories were searched exclusively in the abstracts of the articles.

1st category[1]	2nd category[2]	3rd category[2]
enviromental DNA	surface water	resuspension
eDNA	lake	stratification
	aquatic	water column
	sea	vertical
	marine	depths
	waterbodies	surface
	pond	site occupancy
	lotic system	spatial distribution
	river	downstream
	freshwater	eDNA movement
		upstream
		hydrological processes
		longitudinal
		community structure
		spatial structure

[1] terms searched in the titles of the articles.

[2] terms searched in the abstracts of the articles.

APPENDIX 2 - TABLE S2.

Table S2. Search strings used for retrieving articles from Scopus, Web of Science, and PubMed databases. Each query was tailored to the syntax and filtering capabilities of the respective database, targeting specific terms in titles and abstracts to ensure relevant literature retrieval within the defined time frame and language constraints.

Databases	Serach String
Scopus	(TITLE ("environmental DNA" OR "eDNA")) AND (ABS ("surface water" OR "lake" OR "aquatic" OR "sea" OR "marine" OR "waterbodies" OR "pond" OR "lotic system" OR "river" OR "freshwater")) AND (ABS ("resuspension" OR "stratification" OR "water column" OR "vertical" OR "depths" OR "surface" OR "site occupancy" OR "spatial distribution" OR "spatial structure" OR "downstream" OR "eDNA movement" OR "upstream" OR "hydrological processes" OR "longitudinal" OR "community structure")) AND PUBYEAR > 2007 AND PUBYEAR < 2025 AND (LIMIT-TO (DOCTYPE , "ar") OR LIMIT-TO (DOCTYPE , "re") OR LIMIT-TO (DOCTYPE , "ip") OR LIMIT-TO (DOCTYPE , "dp")) AND (LIMIT-TO (LANGUAGE , "English"))
Web of Science	((TI=("environmental DNA" OR "eDNA")) AND (AB=("surface water" OR "lake" OR "aquatic" OR "sea" OR "marine" OR "waterbodies" OR "pond" OR "lotic system" OR "river" OR "freshwater")) AND (AB=("resuspension" OR "stratification" OR "water column" OR "vertical" OR "depths" OR "surface" OR "site occupancy" OR "spatial distribution" OR "spatial structure" OR "downstream" OR "eDNA movement" OR "upstream" OR "hydrological processes" OR "longitudinal" OR "community structure")) AND PY=(2008-2024) AND LA=(English)) AND DT=(Article OR Early Access OR Data Paper OR Review)
PubMed	(("environmental DNA"[Title] OR "eDNA"[Title]) AND (("surface water"[Abstract] OR "lake"[Abstract] OR "aquatic"[Abstract] OR "sea"[Abstract] OR "marine"[Abstract] OR "waterbodies"[Abstract] OR "pond"[Abstract] OR "lotic system"[Abstract] OR "river"[Abstract] OR "freshwater"[Abstract]) AND ("resuspension"[Abstract] OR "stratification"[Abstract] OR "water column"[Abstract] OR "vertical"[Abstract] OR "depths"[Abstract] OR "surface"[Abstract] OR "site occupancy"[Abstract] OR "spatial distribution"[Abstract] OR "spatial structure"[Abstract] OR "downstream"[Abstract] OR "eDNA movement"[Abstract] OR "upstream"[Abstract] OR "hydrological processes"[Abstract] OR "longitudinal"[Abstract] OR "community structure"[Abstract]))) AND ("2008/01/01"[PDAT] : "2024/12/31"[PDAT]) AND (English[Language]) AND (Journal Article[ptyp] OR Review[ptyp] OR "Article in Press"[ptyp])

Table S3. List of metadata fields, descriptions, and possible answers for each field, related to data extraction from the selected works.

Item Order	Metadata fields	Description	Possible answers
1	Study ID	Refers to the 4-digit number found in the filename of the article being evaluated	####
2	DOI	Specifies the DOI (Digital Object Identifier) of the article	Article DOI
3	Study title	Title of the study	Study title
4	Authors	Name(s) of the study's authors	Names of the authors in the bibliographic format
5	Year of publication	Year the study was published	Year of Publication
6	Keywords	Keywords representing the study	List of Article Keywords
7	Source/journal	Name of the journal or source of publication	Journal/Source Name
8	Citation	Study citation in the "Author-Date" format	List of Authors in Bibliographic Format (APA)
9	Study location	Defines the general type of location where the research was conducted	Continental; Coastal; Open Sea; Estuary/Fjord; N.A.; N.P.
10	Continent/ocean	Continent or ocean associated with the study area	Continent/ocean name(s)
11	Country/Region	Country or broad region within a continent or ocean where the research was conducted	Country/Region name(s)
12	Region/State	Region, state, or area within a country or ocean where the research was conducted	Region/State name(s)
13	City/Specific Location	City or precise location within a region, state, sea, or ocean where the research was conducted	City/Specific Location name(s)
14	Water Type	Type of water associated with the research	Freshwater; Marine; Brackish Water
15	Type of Experimental Environment	Type of environment where the research was conducted	Microcosms; Mesocosms; Constructed Semi-Natural Environments; Natural Waters
16	Aquatic Environment Group	General group of aquatic environments associated with the research	Freshwater Fluvial Systems; Freshwater Lentic Water Body; Micro and Mesocosms; Shallow Marine or Estuarine Environments (< 50m); Deep Marine Environments (> 50m)
17	Aquatic Environment	Specific aquatic environments associated with the research	Coastal Regions (Marine Coastal Area); Island Coast (Marine Coastal Area); Bays (Marine Coastal Area); Creeks (Fluvial System); Streams (Fluvial System); Lakes (Lentic Water Body); Open Sea; Ponds (Lentic Water Body); Tributary (Fluvial System); Rivers (Fluvial System); Estuaries; Coral Reefs; Atoll; Wetlands; Mangroves; Fjords; Tidal Pools; Reservoirs; Aquariums; Fish Farms; Artificial Lakes; Urban Ponds; Lagoon; Agricultural Channel; Marine Channel, among others, as appropriate
18	Number of water bodies evaluated in this study	Total number of water bodies evaluated in this study	Total number of waterbodies assessed in the study
19	Waterbody Name	Name(s) of the waterbody(ies) where the research was conducted	Proper name of the waterbody(ies)
20	Maximum depth (m)	Maximum depth of the waterbody	Maximum depth in meters (m)
21	Water Surface Area (ha) (Lentic Environments	Area of the water surface in lentic environments	Area in hectares (ha)
22	Average Width (m) (Lotic Environments)	Average width in lotic environments	Width in meters (m)
23	Discharge (m^3/s) (Lotic Environments)	Flow rate in lotic environments	Discharge in cubic meters per second (m^3/s)
24	Current Velocity (m/s) (Lotic Environments)	Velocity of the current in lotic environments	Current velocity in meters per second (m/s)
25	Total Volume (m^3) (Aquariums and Mesocosms	Total volume of water in aquariums and mesocosms	Total volume in cubic meters (m^3)

Table S3. (Continued...)

Item Order	Metadata fields	Description	Possible answers
26	eDNA Analysis Methods	eDNA analysis methods employed	Conventional PCR; qPCR; ddPCR; PCR Multiplex; Barcoding; Metabarcoding; Other method
27	Research/Experiment Period(s)	Research period	Date or period as provided by the article
28	Taxa Studied	Kingdom, Phylum, Class, Order, Family, Genus, and/or Species focused on in the study	List of taxa
29	Perspective Addressed by the Study	Specifies the perspective(s) of eDNA dispersion or sampling addressed in the study	Latitudinal; Longitudinal; Vertical
30	Type of Data Present in the Study	Describes the type of data generated, associated with the perspective	Vertical Analysis of Communities; Vertical Analysis of eDNA Concentration; Longitudinal Analysis of Communities; Longitudinal Analysis of eDNA Concentration; Latitudinal Analysis of Communities; Latitudinal Analysis of eDNA Concentration; Vertical Dispersion of eDNA from Known Source; Longitudinal Dispersion of eDNA from Known Source; Latitudinal Dispersion of eDNA from Known Source; Water Column/Substrate Analysis; [Margin/Coast]/[Central Area/Offshore] Analysis; Sedimentation/Resuspension
31	Sampled Depths (m)	Sampling depths in the study	Sampling depths in meters and/or with the term used by the article
32	Q1	Do the communities/species/taxa identified by eDNA differ between vertical layers?	Yes; No; N.A.
33	Q2	In which vertical layer is there greater diversity of communities/species/taxa?	Surface; Bottom; Intermediate; N.A.; (Other)
34	Q3	Does the eDNA concentration of communities/species/taxa differ between vertical layers?	Yes; No; N.A.
35	Q4	In which vertical layer is the eDNA of communities/species/taxa most concentrated?	Surface; Bottom; Intermediate; N.A.; (Other)
36	Q5	Does the stratification of the water column influence the vertical distribution of eDNA?	Yes; No; N.A.
37	Q6	What type of water column stratification influences and is described in the article?	Halocline Stratification; Pycnocline Stratification; Hydrological Stratification; Temperature Stratification; Light Stratification; Oxygen Stratification; N.A.
38	Q7	Does eDNA in the water column reflect the species' preferred depth layer?	Yes; No; N.A.
39	Q8	Does the vertical layer where eDNA is detected tend to reflect its release location?	Yes; No; N.A.
40	Q9	Is the eDNA of less complex organisms (e.g., protozoa, metazoans) more restricted to water layers compared to the eDNA of more complex organisms (e.g., macrofauna, fish, mammals)?	Yes; No; N.A.
41	Q10	Is surface water sampling effective for detecting benthic and/or deep-water organisms?	Yes; No; N.A.
42	Q11	Does sampling near the bottom favor the detection of benthic species?	Yes; No; N.A.
43	Q12	Can eDNA be used to detect vertical, daily, or seasonal migrations of aquatic organisms?	Yes; No; N.A.
44	Q13	Does the horizontal transport of eDNA differ between surface and deep water layers?	Yes; No; N.A.
45	Q14	Does an increase in discharge promote acceleration in the homogenization and dilution process of eDNA?	Yes; No; N.A.
46	Q15	Does a change in flow cause resuspension of eDNA from sediments?	Yes; No; N.A.
47	Q16	Does eDNA sampling reveal greater differences in biological communities from a vertical perspective than from a horizontal perspective in aquatic ecosystems?	Yes; No; N.A.
48	Q17	Can eDNA transported by freshwater increase detected diversity in estuaries?	Yes; No; N.A.
49	Q18	Do tides and currents impact the detection of eDNA?	Yes; No; N.A.
50	Q19	Do small lentic environments exhibit greater homogenization of eDNA in the water column?	Yes; No; N.A.

Table S3. (Continued...)

Item Order		Metadata fields	Description	Possible answers
51	Q20		Is sedimentation a relevant factor for the removal of eDNA from the water column in lotic environments?	Yes; No; N.A.
52	Q21		Does the size of the eDNA fragment (free extracellular DNA, intracellular DNA, particle-bound DNA) influence its persistence and transport in the environment, potentially indicating spatiotemporal proximity to the source organism?	Yes; No; N.A.
53	Q22		Is eDNA degradation lower in sediments than in the water column?	Yes; No; N.A.
54	Q23		Is eDNA more concentrated in sediments than in the water column?	Yes; No; N.A.
55	Q24		Does sediment sampling offer a higher probability of species detection compared to water sampling?	Yes; No; N.A.
56	Q25		Does eDNA detection in water indicate recent presence of the organism, while detection in sediment is associated with long-term persistence?	Yes; No; N.A.
57	Q26		Are eDNA signals more homogeneous in the water column than in sediments?	Yes; No; N.A.
58	Q27		Does the type of substrate influence the persistence of eDNA?	Yes; No; N.A.
59	Q28		Can eDNA accumulated in the substrate and/or the presence of carcasses/dead animals lead to false positive detections?	Yes; No; N.A.
60	Q29		Does eDNA sampled from the water column represent only a local and transient signal of species presence?	Yes; No; N.A.
61	Q30		Do species in the water column release their DNA inconsistently over time, with moments of high and low eDNA release?	Yes; No; N.A.
62	Q31		Are eDNA results equivalent to research and/or historical data obtained through conventional methods?	Yes; No; N.A.
63	Q32		Should the ecology of the species be considered in sampling planning to increase the probability of detection via eDNA?	Yes; No; N.A.
64	Q33		Do eDNA concentrations increase significantly in specific areas during the breeding season?	Yes; No; N.A.
65	Q34		Do eDNA data reveal any detections outside the expected habitat?	Yes; No; N.A.
66	Q35		Does the article make recommendations for sampling?	Yes; No; N.A.
67	Sampling recomendations		Sampling recommendations from the article	Sampling recommendations
68	Exclusively - Surface		List of taxa present exclusively at the surface	List of taxa
69	Preferentially - Surface		List of taxa preferentially at the surface	List of taxa
70	Exclusively - Bottom		List of taxa present exclusively at the bottom	List of taxa
71	Preferentially - Bottom		List of taxa preferentially at the bottom	List of taxa
72	Exclusively - Midwater		List of taxa present exclusively in the midwater	List of taxa
73	Preferentially - Midwater		List of taxa preferentially in the midwater	List of taxa
74	Exclusively - Coast/Edge		List of taxa present exclusively at the coast/edge	List of taxa
75	Preferentially - Coast/Edge		List of taxa preferentially at the coast/edge	List of taxa
76	Exclusively - Central/Offshore		List of taxa present exclusively in the central/offshore	List of taxa
77	Preferentially - Central/Offshore		List of taxa preferentially in the central/offshore	List of taxa
78	Additional information		Additional or important information for understanding the data in the article	Additional information

APPENDIX 4 - TABLE S4.

Table S4. Distribution of datasets across different depth thresholds and the number of sampled depths for three water environments: Freshwater, Marine, and Mixed/Brackish. The table shows the number of datasets categorized by depth ranges (Very Shallow, Shallow, Medium, Deep, Very Deep) and the number of depths sampled (ranging from 2 to more than 10 depths) for each environment.

				Number of sampled depths									
	Depth thresholds (m	Datasets	2 depths	3 depths	4 depths	5 depths	6 depths	7 depths	8 depths	9 depths	≥ 10 depths	TOTAL
Freshwater	Very Shallow	≤ 1	5	3	1	1	-	-	-	-	-	-	5
	Shallow	> 1 and ≤ 3	3	2	1	-	-	-	-	-	-	-	3
	Medium	> 3 and ≤ 20	30	18	7	3	-	2	-	-	-	-	30
	Deep	> 20 and ≤ 50	24	13	2	4	2	3	-	-	-	-	24
	Very Deep	> 50	22	20	-	1	-	-	1	-	-	-	22
	TOTAL	-	**84**	**56**	**11**	**9**	**2**	**5**	**1**	**0**	**0**	**0**	**84**
Marine	Very Shallow	≤ 10	39	37	1	-	1	-	-	-	-	-	39
	Shallow	> 10 and ≤ 50	130	91	37	-	2	-	-	-	-	-	130
	Medium	> 50 and ≤ 200	213	22	36	96	56	2	1	-	-	-	213
	Deep	> 200 and ≤ 1000	25	10	2	5	-	3	-	4	1	-	25
	Very Deep	> 1000	49	12	11	17	-	3	5	-	-	1	49
	TOTAL	-	**456**	**172**	**87**	**118**	**59**	**8**	**6**	**4**	**1**	**1**	**456**
Mixed/Brackish	Very Shallow	≤ 1	-	-	-	-	-	-	-	-	-	-	0
	Shallow	> 1 and ≤ 5	1	1	-	-	-	-	-	-	-	-	1
	Medium	> 5 and ≤ 20	3	2	1	-	-	-	-	-	-	-	3
	Deep	> 20	10	7	3	-	-	-	-	-	-	-	10
	TOTAL	-	**14**	**10**	**4**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**14**
	TOTAL	-	**554**	**238**	**102**	**127**	**61**	**13**	**7**	**4**	**1**	**1**	**554**
	%	-	-	**42.96%**	**18.41%**	**22.92%**	**11.01%**	**2.35%**	**1.26%**	**0.72%**	**0.18%**	**0.18%**	-

Table S5. List of studies reporting the water layer (Shallow, Deep, or Intermediate) where the highest taxonomic diversity was detected through environmental DNA (eDNA) sampling, according to the studied taxon.

Research	Local	Taxa	Layer(s)
Liu et al. 2019	Sansha Yongle Blue Hole	Eukaryotes	Intermediate
McClenaghan et al. 2020	Labrador Sea	Aquatic Metazoans	Intermediate
Monuki et al. 2021	Leo Carrillo State Beach	Actinopterygii and Chondrichthyes	Deep
Feng et al. 2022	Western Tropical Pacific Ocean	Mesozooplankton	Intermediate
Govindarajan et al. 2022	Gulf of Mexico	Invertebrates	Shallow
Alexander et al. 2023	Gulf of Thailand	Eukaryotes	Shallow
Diao et al. 2023	Formosa Ridge	Phytoplankton and Invertebrates	Shallow
Muff et al. 2023	North-Western Mediterranean Sea and Western Indian Ocean	Actinopterygii and Chondrichthyes	Shallow and Intermediate
Robinson et al. 2023	Knight Inlet	Actinopterygii, Invertebrates and Algae	Intermediate
Fukumori et al. 2024	Lake Yunoko	Actinopterygii	Shallow
Kim et al. 2024	Western Pacific Ocean	Prokaryotes and Eukaryotes	Intermediate
Murray et al. 2024	Fram Strait	Gelatinous Zooplankton	Deep and Intermediate
Sevellec et al. 2024	Milne Inlet	Aquatic Metazoans	Deep
Sevellec et al. 2024	Churchill	Aquatic Metazoans	Shallow

Table S6. Species detected exclusively or preferentially in different layers of the water column (shallow, deep, and midwater) based on studies that sampled two or more depths. The categorization considers whether the species was found exclusively in a specific layer or if its detection was preferential in that layer, even if it was also recorded in others. The references indicate the studies that reported these observations.

Species	Exclusively Shallow	Exclusively Deep	Exclusively Midwater	Preferentially Shallow	Preferentially Deep	Preferentially Midwater
Acanthopagrus latus					Zhang et al. 2024	
Acanthopagrus sp.					Zhang et al. 2024	
Alepes kleinii					Zhang et al. 2024	
Alepocephalus agassizii		McClenaghan et al. 2020				
Ammodytes hexapterus	McClenaghan et al. 2020					
Anarhichas dendiculatus			McClenaghan et al. 2020			
Anguilla anguilla	Roblet et al. 2024	Dukan et al. 2024			Burgoa Cardás et al. 2020; Hänfling et al. 2016	
Anguilla sp.	Jeunen et al. 2020					
Anthias anthias		Roblet et al. 2024				
Antipathes curvata					Alexander et al. 2023	
Apogon imberbis		Roblet et al. 2024				
Artherina presbyter		Dukan et al. 2024				
Ascidia ahodori					Alexander et al. 2023	
Ascidia sp.					Jeunen et al. 2020	
Astatotilapia calliptera				Liu et al. 2024[1]	Liu et al. 2024[2]	
Atherinomorus lacunosus				Alexander et al. 2023		
Atherinops affinis				Monuki et al. 2021		
Atolla tenella		Murray et al. 2024				
Aurelia coerulea				Ye et al. 2024	Peng et al. 2023	
Austrominius sp.				Jeunen et al. 2020		
Austroperla sp.	Jeunen et al. 2020					
Bathykorus bouilloni		Murray et al. 2024				
Botrynemma brucei		Murray et al. 2024				
Buenia affinis		Roblet et al. 2024				
Buglossidium luteum		Dukan et al. 2024				
Caesioperca sp.					Jeunen et al. 2020	

Table S6. (Continued...)

Species	Exclusively Shallow	Exclusively Deep	Exclusively Midwater	Preferentially Shallow	Preferentially Deep	Preferentially Midwater
Calocalanus pavo				Alexander et al. 2023		
Calocalanus plumulosus				Alexander et al. 2023		
Carassius auratus		Kamoroff & Goldberg 2018[3]				
Ceratoscopelus maderensis		Rozanski et al. 2024				
Chamaesipho sp.				Jeunen et al. 2020		
Chauliodus sloani		Rozanski et al. 2024				
Chelon auratus	Roblet et al. 2024					
Chelonia mydas					Harper et al. 2020	
Chloropicon laureae					Alexander et al. 2023	
Chloropicon roscoffensis					Alexander et al. 2023	
Chrosomus neogaeus				Littlefair et al. 2021		
Chrysaora pacifica					Minamoto et al. 2017	
Citharichthys stigmaeus					Monuki et al. 2021	
Clausocalanus minor					Alexander et al. 2023	
Clinatrichus argentatus		Roblet et al. 2024				
Clupea pallasii					Robinson et al. 2023	Robinson et al. 2023
Coryphaenoides rupestris			McClenaghan et al. 2020			
Coscinasterias sp.					Jeunen et al. 2020	
Cottus cognatus					Littlefair et al. 2021	
Crystallogobius linearis		Roblet et al. 2024				
Cyanea nozakii						Ye et al. 2024
Cyclopterus lumpus	Dukan et al. 2024					
Dasyatis tortonesei		Roblet et al. 2024				
Dictyocoryne truncatum					Alexander et al. 2023	Alexander et al. 2023
Dinobryon sp.	Jeunen et al. 2020					
Diplecogaster bimaculata	Dukan et al. 2024					
Elopichthys bambusa		Zhou et al. 2023				
Epinephelus marginatus		Roblet et al. 2024				

Table S6. (Continued...)

Species	Exclusively Shallow	Exclusively Deep	Exclusively Midwater	Preferentially Shallow	Preferentially Deep	Preferentially Midwater
Euphausia sp.					Jeunen et al. 2020	
Euthynnus affinis				Zhang et al. 2024		Alexander et al. 2023
Evechinus sp.					Jeunen et al. 2020	
Farranula gibbula						Alexander et al. 2023
Galaxias sp.	Jeunen et al. 2020					
Gasterosteus aculeatus	Dukan et al. 2024					
Gnathopogon elongatus	Fukumori et al. 2024					
Gobiomorphus sp.	Jeunen et al. 2020					
Gobius cruentatus		Roblet et al. 2024				
Gobius vittatus		Roblet et al. 2024				
Gobius xanthocephalus		Roblet et al. 2024				
Gymnammodytes cicerelus	Roblet et al. 2024					
Hemibarbus maculatus	Zhou et al. 2023					
Hemigrapsus sp.				Jeunen et al. 2020		
Lagenorhynchus acutus	Cai et al. 2024					
Lamna nasus	Cai et al. 2024					
Larimichthys crocea				Wang et al. 2021[5]	Wang et al. 2021[4]	Wang et al. 2021[4]
Larimichthys polyactis				Wang et al. 2020[6]	Zhang et al. 2024	Wang et al. 2020[4]
Lepidodermella sp.	Jeunen et al. 2020					
Lepidogaster purpurea		Roblet et al. 2024				
Leuresthes tenuis				Monuki et al. 2021		
Lophius Budegassa		Rozanski et al. 2024				
Lophius piscatorius	Dukan et al. 2024					
Margariscus margarita				Littlefair et al. 2021		
Margaritifera monodonta					Lor et al. 2020	
Maurolicus muelleri	Roblet et al. 2024					
Menticirrhus undulatus					Monuki et al. 2021	
Meridiastra sp.					Jeunen et al. 2020	

Table S6. (Continued...)

Species	Exclusively Shallow	Exclusively Deep	Exclusively Midwater	Preferentially Shallow	Preferentially Deep	Preferentially Midwater
Merluccius merluccius		Rozanski et al. 2024				
Microlipophrys dalmatinus		Roblet et al. 2024				
Micromonas commoda					Alexander et al. 2023	
Mugil cephalus				Zhang et al. 2024		
Mustelus californicus		Monuki et al. 2021				
Myctophum punctatum	Roblet et al. 2024					
Mysis diluviana					Carim et al. 2016	
Nausthoe punctata					Alexander et al. 2023	
Nemopilema nomurai					Ye et al. 2024	
Neogobius melanostomus		Dukan et al. 2024				
Neophocaena asiaeorientalis asiaeorientalis					Stewart et al. 2017	
Neosalanx taihuensis	Zhou et al. 2023					
Notoscopelus elongatus	Roblet et al. 2024					
Odontamblyopus rubicundus					Zhang et al. 2024	
Oncorhynchus nerka					Fukumori et al. 2024	
Oncorhynchus spp.				Robinson et al. 2023		
Ophiactis sp.					Jeunen et al. 2020	
Oxyporhamphus microterus				Alexander et al. 2023		
Pagellus erythrinus	Roblet et al. 2024					
Palaemon paucidens					Wu et al. 2019	
Pampus echinogaster				Zhang et al. 2022		
Parablennius pilicornis	Roblet et al. 2024					
Parablennius zvonimir	Roblet et al. 2024					
Paracalanus sp.					Jeunen et al. 2020	
Paralabrax clathratus					Monuki et al. 2021	
Paralabrax nebulifer					Monuki et al. 2021	
Paralepis coregonoides			McClenaghan et al. 2020			
Paralichthys olivaceus					Suzuki et al. 2024	

Table S6. (Continued...)

Species	Exclusively Shallow	Exclusively Deep	Exclusively Midwater	Preferentially Shallow	Preferentially Deep	Preferentially Midwater
Pelagomonas calceolata					Alexander et al. 2023	
Phaeocystis globosa					Alexander et al. 2023	
Phaeocystis sp.					Jeunen et al. 2020	
Phocoena phocoena	Cai et al. 2024					
Pholis gunnellus		Van Driessche et al. 2024				
Phoxinus phoxinus					Hänfling et al. 2016	
Phycis phycis		Roblet et al. 2024				
Pimephales promelas				Littlefair et al. 2021		
Pinna nobilis					Marques et al. 2024	
Pomatoschistus pictus	Dukan et al. 2024					
Potamopyrgus sp.	Jeunen et al. 2020					
Psenopsis anomaia					Zhang et al. 2024	
Pseudaspius hakonensis	Fukumori et al. 2024					
Pseudocaranx dentex				Murakami et al. 2019		
Pseudochattonella sp.					Jeunen et al. 2020	
Pseudo-nitzschia cuspidata					Alexander et al. 2023	
Pseudopleuronectes yokohamae					Suzuki et al. 2024	
Pseudoscourfieldia marina					Alexander et al. 2023	
Ptychobranchus fasciolaris					Currier et al. 2018	
Rajella bigelowi		McClenaghan et al. 2020				
Reinharditus hippoglossoides			McClenaghan et al. 2020			
Salmo salar		Dukan et al. 2024			Hänfling et al. 2016	
Salvelinus alpinus					Hänfling et al. 2016	
Salvelinus namaycush					Littlefair et al. 2021	
Sarda sarda	Roblet et al. 2024					
Sardinops sagax				Monuki et al. 2021		
Saurogobio gymnocheilus	Zhou et al. 2023					
Scaphirhynchus suttkusi					Janosik et al. 2021	

Table S6. (Continued...)

Species	Exclusively Shallow	Exclusively Deep	Exclusively Midwater	Preferentially Shallow	Preferentially Deep	Preferentially Midwater
Scophthalmus rhombus		Dukan et al. 2024				
Scorpaena scrofa		Roblet et al. 2024				
Sebastes mentella			McClenaghan et al. 2020			
Sebastes spp.					Robinson et al. 2023	Robinson et al. 2023
Selar crumenophthalmus				Alexander et al. 2023		
Semicossyphus pulcher					Monuki et al. 2021	
Seriola dumerili				Suzuki et al. 2024		
Seriphus politus					Monuki et al. 2021	
Serranus cabrilla		Roblet et al. 2024				
Silurus meridionalis	Zhou et al. 2023					
Sponchylosoma cantharus		Roblet et al. 2024				
Squatina californica					Monuki et al. 2021	
Stolephorus insularis					Zhang et al. 2024	
Symphodus cinereus		Roblet et al. 2024				
Symphodus doderleini		Roblet et al. 2024				
Symphodus rostratus		Roblet et al. 2024				
Syngnathus typhle		Roblet et al. 2024				
Tethya seychellensis				Alexander et al. 2023		
Thalassoma pavo	Roblet et al. 2024					
Thryssa dussumieri				Zhang et al. 2024		
Torpedo marmorata		Roblet et al. 2024				
Trachimus draco		Dukan et al. 2024				
Trachurus mediterraneus	Roblet et al. 2024					
Trachurus trachurus	Roblet et al. 2024					
Tripterygiom melanurum	Roblet et al. 2024					
Trisopterus minutus		Dukan et al. 2024				

Table S6. (Continued...)

Species	Exclusively Shallow	Exclusively Deep	Exclusively Midwater	Preferentially Shallow	Preferentially Deep	Preferentially Midwater
Trypauchen vagina					Zhang et al. 2024	
Umbrina roncador					Monuki et al. 2021	
Upeneus guttatus				Alexander et al. 2023		
Vibrio fluvialis					Alexander et al. 2023	
Xiphias gladius	Roblet et al. 2024					
Zeugepterus regius	Dukan et al. 2024					
170	**38**	**42**	**5**	**27**	**63**	**8**

[1] littoral ecomorph
[2] deep ecomorph
[3] dead
[4] adults
[5] eggs and larvae
[6] larvae and juveniles

APPENDIX 7 - SUPPLEMENTARY INFORMATION

Development of a Molecular Marker for Nile Tilapia (*Oreochromis niloticus*)

Design of the Molecular Marker

For the development of the primer and probe set, the mitochondrial gene sequences for *COI*, *12S*, and *16S* available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) were evaluated for *O. niloticus* and related species. The *COI* gene presented the highest number of sequences, totaling 27 species distributed across five genera of the Cichlidae family, including nine species from the genus *Oreochromis* (Table SS1.).

Table SS1. List of species, including family and genus identification, whose mitochondrial gene sequences (COI, 12S, and 16S) were retrieved from the GenBank database for alignment.

Family	Genus	Species	GenBank Sequence Code		
			COI	12S	16S
Cichlidae	Cichla	*Cichla kelberi*	JN988797.1	-	DQ779580.1
Cichlidae	Crenicichla	*Crenicichla sp.*	-	-	-
Cichlidae	Crenicichla	*Crenicichla vitatta*	KU288979.1	-	-
Cichlidae	Crenicichla	*Crenicichla acutirostris*	GU817292.1	-	GU817248.1
Cichlidae	Crenicichla	*Crenicichla alta*	AY263860.1	-	AY263837.1
Cichlidae	Crenicichla	*Crenicichla percna*	MH931612.1	-	-
Cichlidae	Crenicichla	*Crenicichla jegui*	MH931615.1	-	-
Cichlidae	Crenicichla	*Crenicichla caetana*	MH931627.1	-	JF520126.1
Cichlidae	Crenicichla	*Crenicichla regani*	MH931655.1	KR233977.1	AF049003.1
Cichlidae	Crenicichla	*Crenicichla wallacii*	MH931656.1	-	-
Cichlidae	Crenicichla	*Crenicichla lacustres*	GU702157.1	-	JF520153.1
Cichlidae	Crenicichla	*Crenicichla britskii*	GU701937.1	-	-
Cichlidae	Crenicichla	*Crenicichla lepidota*	HM405098.1	AF285917.1	JF520163.1
Cichlidae	Crenicichla	*Crenicichla menezesi*	KF938552.1	-	-
Cichlidae	Crenicichla	*Crenicichla haroldoi*	JN988829.1	-	-

Family	Genus	Species	GenBank Sequence Code		
			COI	12S	16S
Cichlidae	Crenicichla	*Crenicichla niederleinii*	-	-	-
Cichlidae	Geophagus	*Geophagus brasiliensis*	MG825020.1		
Cichlidae	Geophagus	*Geophagus proximus*	GU701786.1		
Cichlidae	Geophagus	*Geophagus sveni*	MH780911.1		
Cichlidae	Satanoperca	*Satanoperca pappaterra*	JN989214.1		
Cichlidae	Oreochromis	*Oreochromis niloticus*	KU565863.1	MN255618.1	MK788803.1
Cichlidae	Oreochromis	*Oreochromis mossambicus*	KU565826.1	KU821711.1	DQ426661.1
Cichlidae	Oreochromis	*Oreochromis urolepis*	KM438540.1	-	MK788839.1
Cichlidae	Oreochromis	*Oreochromis aureus*	KU565852.1	EU709731.1	DQ426663.1
Cichlidae	Oreochromis	*Oreochromis schwebischi*	MK074536.1	-	MK788821.1
Cichlidae	Oreochromis	*Oreochromis karongae*	KM438532.1	-	MK788777.1
Cichlidae	Oreochromis	*Oreochromis leucostictus*	KT193495.1		
Cichlidae	Oreochromis	*Oreochromis tanganicae*	KU194060.1		
Cichlidae	Oreochromis	*Oreochromis lepidurus*	KP027397.1		

The *COI* gene was selected as the target, and species sequences were aligned to identify a primer sequence specific to *O. niloticus*. Based on this analysis, a set of primers (PrimerQuest Tool), each consisting of 22 base pairs, was developed to amplify a 166-base-pair amplicon. The sequences of the primers (forward and reverse) and the probe are presented in Table SS2.

Table SS2. Sequences of primers (mitochondrial gene Cytochrome Oxidase Subunit I – COI) and probe designed to detect Oreochromis niloticus by qPCR, including their melting temperatures.

Primer/Probe	Sequence	Melting Temperature (°C)
COI U-F	ACATGAAACCCCCTGCCATCTC	62.61
COI U-R	CCTCCGGCAGGGTCAAAGAAG	62.99
Probe	TGCCCGTTCTTGCCGCCGGCATCACAA	68.63

Marker Validation

The molecular marker validation was conducted using High-Resolution Melting (HRM) analysis to assess the specificity of the primers and probe designed for *O. niloticus* and qPCR to assess the efficiency through the limit of detection (LoD). For HRM, reactions were performed in a final volume of 25 µL containing 1X SYBR Green Mastermix (Qiagen), 1 µM of each primer (forward and reverse), 1 µL of DNA extracted from the target species or comparative samples, and ultrapure water. Amplification was carried out with an initial HotStart activation at 95 °C for 5 minutes, followed by 45 cycles of denaturation at 95 °C for 5 seconds and annealing/extension at 60 °C for 10 seconds.

The melting curve was generated with a temperature gradient from 40 °C to 90 °C and analyzed using Rotor-Gene Q software to determine the melting temperature (Tm) of the amplified products. DNA samples from *O. niloticus*, other fish species (*Astyanax altiparanae* and *Pseudoplatystoma corruscans*), and a negative control containing only ultrapure water were used.

The analysis revealed a single melting peak for *O. niloticus* samples, indicating high specificity of the primer and probe set for the target species. No peaks were observed in samples from other species nor in the negative control. This marker was deemed suitable for detecting and quantifying *O. niloticus* in environmental DNA samples.

For the LoD test, standard curves were generated through serial dilutions of the amplicon produced by the qPCR primers in an endpoint PCR reaction. This reaction was conducted in a final volume of 25 µL, containing 25 µM of each primer, 0.32 mM dNTP mix, 1 U of Platinum Taq DNA Polymerase, 1X Platinum Taq buffer, and 5 mM MgCl2. Thermal cycling conditions included an initial denaturation at 95 ºC for 1 minute, followed by 40 cycles of 95 ºC for 10 s, annealing at 60 ºC for 10 s, and extension at 72 ºC for 30 s.

The PCR product was purified using the SPRI method (DeAngelis et al. 1995). The product was quantified using the Qubit® 4.0 (Thermo Fisher Scientific, Brazil) with the high sensitivity (HS) kit. Seven dilution points were used, comprising DNA concentrations ranging from $2x10^{-8}$ to $2x10^{-3}$ ng/µL. qPCR reactions were performed in a final volume of 10 µL containing 1X Quantinova qPCR Mastermix (Qiagen), 0,75 µM of each primer (forward and reverse), 0,25 µM of the probe and 3 µL of DNA extracted from the target species. All reactions were performed in triplicate. Detection was successful when two out of three replicates generated a positive signal above the threshold.

REFERENCES

DeAngelis, M. M., D. G. Wang & T. L. Hawkins, 1995. Solid-phase reversible immobilization for the isolation of PCR products. Nucleic acids research 23(22):4742 doi: https://doi.org/10.1093/nar/23.22.4742