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# Exploring the Biodiversity of a European NATURA 2000 Mediterranean Lagoon through eDNA Metabarcoding

**Valeria Specchia [1,2,*], Benedetta Saccomanno [1], Francesco Zangaro [1,3], Eftychia Tzafesta [1] and Maurizio Pinna [1,2,3,*]**

[1] Department of Biological and Environmental Sciences and Technologies, DiSTeBA, University of Salento, Via Monteroni 165, 73100 Lecce, Italy
[2] NBFC, National Biodiversity Future Center, 90133 Palermo, Italy
[3] Research Centre for Fisheries and Aquaculture of Aquatina di Frigole, DiSTeBA, University of Salento, 73100 Lecce, Italy
* Correspondence: valeria.specchia@unisalento.it (V.S.); maurizio.pinna@unisalento.it (M.P.)

**Abstract:** Coastal lagoons are considered important habitats both for ecological functions and biodiversity worldwide. Thus, they provide relevant ecosystem services and valuable natural resources. However, coastal lagoons are highly susceptible to anthropogenic pressures that can cause biodiversity losses and require specific biomonitoring programs as well as management measures. In this research, we applied environmental DNA (eDNA) metabarcoding to investigate the biodiversity of a poorly known Mediterranean lagoon included in the European Natura 2000 Network. We used the cytochrome oxidase I (COI) gene marker to capture the entire biodiversity of this highly diversified aquatic coastal environment. With a low sampling effort and rapid laboratory practices, a large amount of valuable biodiversity data was generated and analyzed. Interestingly, this straightforward and broad molecular surveying of biodiversity unveiled a wide variety of taxonomic groups, such as benthic macroinvertebrates, zooplankton, phytoplankton, and macroalgae, which are frequently used as ecological indicators. We were able to detect species that were previously morphologically identified, as well as species never identified before. This research underlines the validity of eDNA metabarcoding in assessing the biodiversity in a poorly known and protected Mediterranean lagoon ecosystem, as well as in identifying the early warnings of environmental stressors. Finally, the research highlights the need to investigate multiple target genes and primers set for a larger analysis of specific species.

**Keywords:** eDNA metabarcoding; cytochrome oxidase I (COI); Aquatina Lagoon; European Union NATURA 2000 Network; biodiversity assessment

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## 1. Introduction

Biodiversity loss is increasing at alarming rates due to anthropogenic pressure, resulting in species extinctions and a reduction in genetic diversity [1]. Land and sea overexploitation, pollution, and climate change are only some of the anthropogenic factors leading to species loss and ecosystem alteration [2]. Efforts in preserving genetic diversity and ecosystem functioning are now considered a global priority to circumvent extinction events and the consequent irreversible depletion of resources and ecosystem services [3].

Apart from political and economic rerouting towards more sustainable societal growth, a key aspect of biodiversity conservation is based on ecosystem preservation. An example of this is the European initiative NATURA 2000 Network, by which several sites, stretching across 27 European Countries, are now regarded as protected areas to preserve endangered and rare species and habitats [4].

However, these conservation efforts are strictly related to the availability of biomonitoring tools and programs able to provide reliable data on biodiversity, species distribution,

and the abundance of both native and non-indigenous species (NIS) in a brief time and at affordable costs.

To date, the assessment of biodiversity has been established only through the identification of morphological traits and counting of sampled living individuals. These methods present some undeniable drawbacks, being not only low throughput in nature, but also invasive and often destructive of the same organisms and ecosystems they are trying to preserve [5,6], having an impact on both high-density and rare species. Additionally, their accuracy is undermined by difficulties in distinguishing phenotypically similar organisms and juvenile stages, estimating hard-to-detect species and maintaining the morphological features unaltered during laboratory handling [7,8]. For all these reasons, it is necessary to explore new methods for effective species identification and classification [9].

Molecular biology has been historically applied to answer ecological questions such as population genetics, species evolution, the assessment of the divergence time between species, and even mating rates [10]. The field of molecular ecology is now seeing further development thanks to the advent of environmental DNA (eDNA) metabarcoding [11]. This approach involves examining the genetic content of an environmental sample in a nondestructive way [12,13]. From terrestrial to aquatic ecosystems, eDNA metabarcoding is providing an assessment of biodiversity and ecosystem fitness [14–16], the tracking of NIS and rare species [17–19], an unravelling of dispersal behaviors [20], and even the detection of extinct ancestral species [21,22]. Moreover, this tool allows for the exploration of an unprecedented amount of data from an ecological perspective. However, a current limitation in eDNA metabarcoding application is represented by the incompleteness of DNA barcodes in international libraries [23–27]. Furthermore, the identification of appropriate gene targets and primer sets is mandatory and needs to be modulated depending on the type of ecosystems and ecological indicators considered.

In this research, we applied eDNA metabarcoding to assess the biodiversity in a poorly known Mediterranean lagoon included in the EU NATURA 2000 Network, identified with the code IT9150003—Aquatina di Frigole. To achieve this, we sequenced COI amplicons of DNA extracted from seven surface water samples of the lagoon. The peculiarity of this lagoon is in the intake of both fresh and marine water from two opposite channels, creating a gradient of salinity that allows a great variety of species to co-exist. The lagoon hosts different species of crustaceans and mollusks, aquatic plants, macro-algae, and phytoplankton; moreover, it is also a natural nursery for fish and the nesting of migratory birds [28]. However, previous morphological surveys have mainly focused on the identification of macrofauna and macrobenthos, leaving most of the microscopic biodiversity, which not only affects population dynamics, but also comprises efficient ecological indicators, uncovered. To preliminarily assess the efficiency of eDNA metabarcoding in this transitional water ecosystem, we also provide a comparison between the results generated in this study and previous morphological identifications. Transitional water ecosystems provide important ecosystem services as well as a diversified area for conservation. Yet, they are often undervalued and understudied when compared to marine, terrestrial, and freshwater environments [29–32]. Therefore, it is important to test the application of high-throughput molecular methods for biodiversity assessment on largely understudied Mediterranean transitional water ecosystems for improving conservation management strategies and sustainable development.

## 2. Materials and Methods

### *2.1. Sampling Protocol and eDNA Extraction*

On October 2020, seven surface water samples were collected from the "Aquatina di Frigole" lagoon, which is part of the European NATURA 2000 Site IT9150003 (Adriatic Sea, South-East Italy). Each sample consisted of 1 L of surface water which was immediately processed at the Research Centre for Fisheries and Aquaculture—University of Salento—located in the NATURA 2000 site. To avoid contamination, all the equipment was carefully autoclaved or rinsed with sterile bi-distilled water. To avoid filter clogging and reduce

polymerase chain reaction (PCR) inhibitors such as particulate and humic substances, prefiltration was applied before filtration. First, each sample of 1 L was divided into two subsamples of 0.5 L. Each 0.5 L subsample was prefiltered through a 1.6 µm glass filter 42.5 mm in diameter (Whatman® glass microfiber filters, Grade GF/A). Secondly, the two resulting filtrates were combined and filtered again through a 0.45 µm cellulose filter 47 mm in diameter (Advantec Mixed Cellulose Ester filters). The 0.45 µm filters were used for DNA extraction through the DNeasy PowerWater kit following the manufacturer's protocol.

### *2.2. DNA Amplification and High-Throughput Sequencing*

The PCR amplification of a fragment of approximately 350 bp of the mitochondrial gene cytochrome oxidase subunit I (COI) was performed using the degenerated primers mCOIintF 5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′ [33] and dgHCO-2198 5′-TAAACTTCAGGGTGACCAAARAAYCA-3′ [34] linked to indexes. The reaction was performed in a volume of 50 µL composed of 5 µL of 10× reaction buffer; 1 µL of $MgCl_2$ (50 mM); 1 µL of dNTP mix (10 mM); 1 µL of each primer (10 mM); 10 ng DNA; 0.5 µL of Life Technologies Platinum Taq (5 U/µL); 39.5 µL of sterile bidistilled water. The amplification process included the following phases: denaturation (95 °C for 30″), annealing (45 °C for 30″), extension (72 °C for 30″) repeated for 30 cycles, preceded by an initial denaturation step at 95 °C for 5 min, and followed by a final extension at 72 °C for 5 min. All PCR products were purified with a PureLink PCR purification kit (Invitrogen, Carlsbad, CA, USA).

High-throughput, paired-end amplicon sequencing was carried out on the Illumina Miseq platform (BMR Genomics©, Padua, Italy), generating an average of 114.297 reads among samples. The bioinformatic analyses were performed using the QIIME2 platform. After the adapters' removal, raw reads were processed using DADA2 (with default parameters except trunc_len_f = 265 and trunc_len_r = 230) and clustered into amplicon sequence variants (ASVs). The ASVs with a frequency of 0.05% were discarded. The remaining ASVs were divided into annotated and not annotated groups. The assigned sequences were then manually annotated through NCBI BLASTn to confirm taxonomy and filtered to a minimum of 75% for query coverage and identity. The number of reads and corresponding percentages obtained after each quality filtering step and taxonomic assignment are summarized in Table S1. The FASTQ files are deposited in GenBank (SRA) of NCBI with identification number PRJNA847192.

## 3. Results

### *3.1. Species Biodiversity Assessment by eDNA Metabarcoding*

The DNA extracted from seven water samples was amplified with COI primers and sequenced with next-generation sequencing technology (NGS). The quality filtered sequences obtained by high-throughput sequencing were annotated taxonomically, and the total percentage of each phylum, based on the number of reads, was estimated (Table 1). The obtained results reveal that 40% of the total reads belong to the phylum Arthropoda, followed by Rhodophyta and Ochrophyta, with 22% and 13% of reads, respectively. Both Cnidaria and Annelida represent about 10% of reads, while only 1.8% of reads were assigned to Mollusca. Although in a small percentage, NGS also revealed the presence of Chordata, Porifera, Echinodermata, and Nemertea (0.4–0.2%). In addition, we also found the trace of three phyla of algae, Chlorophyta (0.1%), Charophyta (0.06%), and Chrysophyta (0.01%).

Interestingly, the experiment unveiled a very large spectrum of taxa of the main macrotaxonomic guilds, including benthic macroinvertebrates, zooplankton, phytoplankton, and macroalgae (Figure 1; Table S2) Following this taxa classification into macro guilds corresponding to ecological indicators, we evaluated both the percentage of reads (Figure 1) and the number of molecular taxonomic units (MOTUs) (Figure 2). The group phytoplankton/macroalgae and benthic macroinvertebrates displayed similar percentages of reads; however, the former presented more than double the amount of MOTUs ($n$ = 90)

compared to the latter ($n$ = 42). Zooplankton was the least represented, with 13% of reads and 17 MOTUs.

**Table 1.** Percentage of reads for each Phylum. Percentage was calculated as the sum of reads of each phylum against the total number of annotated reads.

| Phylum | Percentage of Reads |
|---|---|
| Arthropoda | 40.38% |
| Rhodophyta | 22.38% |
| Ochrophyta | 13.27% |
| Cnidaria | 10.42% |
| Annelida | 10.39% |
| Mollusca | 1.77% |
| Chordata | 0.44% |
| Porifera | 0.34% |
| Echinodermata | 0.20% |
| Nemertea | 0.19% |
| Chlorophyta | 0.13% |
| Charophyta | 0.06% |
| Chrisophyta | 0.01% |

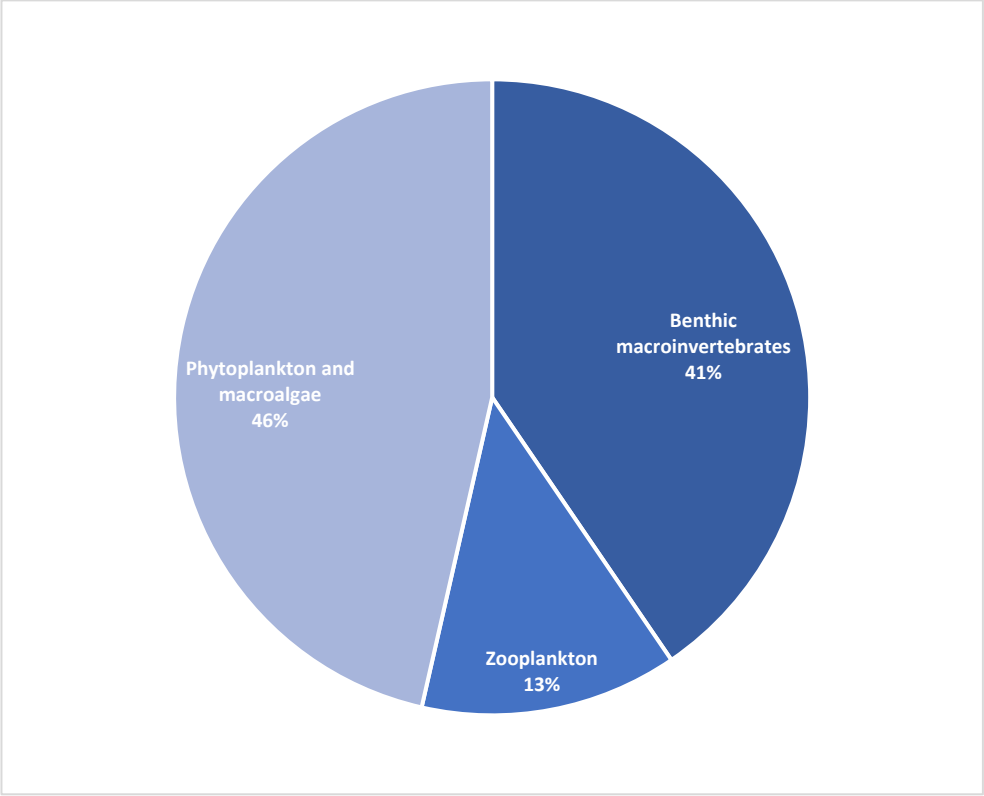


**Figure 1.** Percentage of reads for each macro-taxonomic guild. Percentage was calculated as the sum of reads of each guild against the total number of annotated reads.

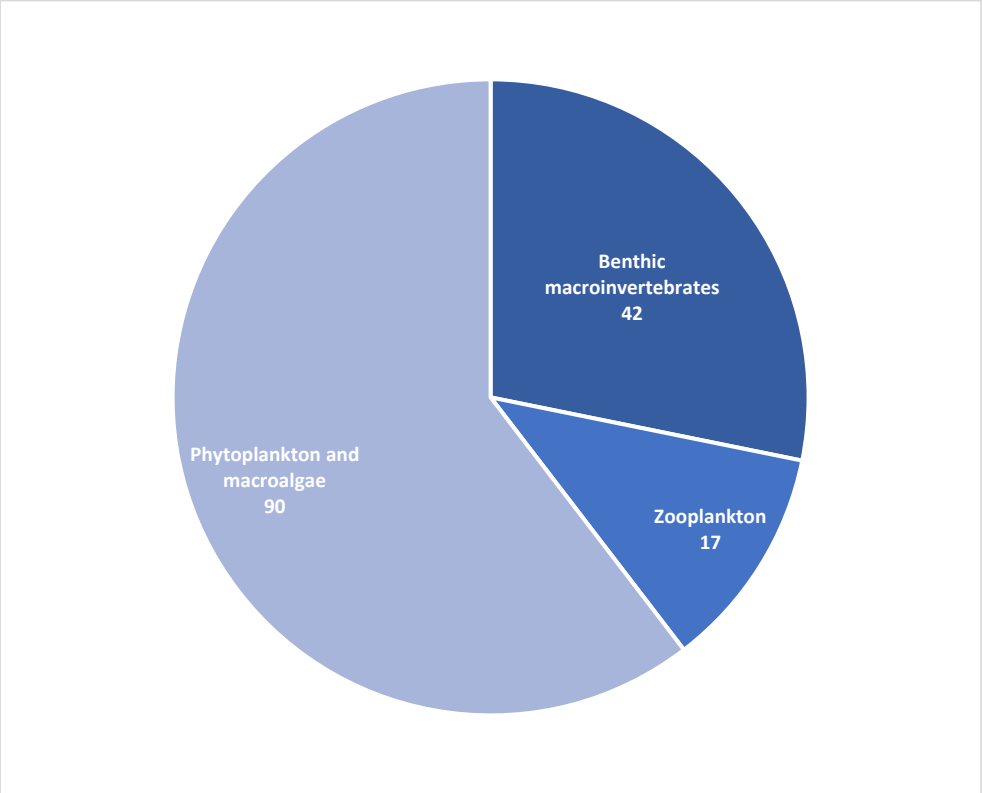


**Figure 2.** Number of MOTUs for each macro-taxonomic guild.

### *3.2. Molecular and Morphological Species' Identification Are Congruent and Complementary*

Records of morphological surveys in the Aquatina Lagoon are scarce and scattered throughout different years and seasons, and a complete list of species is still missing. Nevertheless, we obtained a list of species generated through the European-funded project IMPRECO [28], which attempted to define the main biotic indices in the lagoon through the phenotypic evaluation of organisms found in sediment samples. Comparing it with our results, eDNA metabarcoding unveiled 61 different genera among phytoplanktonic algae versus 26 genera in the morphological analysis. We were able to detect among the phytoplankton *Chaetoceros* sp. (88% identity), *Pseudo-Nitzschia* sp. (88% identity), *Cylindrotheca closterium* (93% identity), and *Navicula veneta* (95% identity), which were previously identified morphologically. Moreover, it unveiled 15 genera of Cnidaria versus 1 genus in the morphological analysis; however, it did not reveal many genera of benthic macro-invertebrates that were identified by morphological studies using the traditional sampling of sediments. Additionally, eDNA metabarcoding unveiled the presence of *Ercolania viridis* (98% identity), known indigenous swimming nudibranchs.

## 4. Discussion

In this study, we applied eDNA metabarcoding for a preliminary biodiversity assessment in a coastal lagoon protected under the Natura 2000 Network. Intending to capture a large variety of taxa, we analyzed amplicons derived from degenerated COI primers that we previously used to amplify single species sampled in the Aquatina Lagoon [26]. We selected the cytochrome oxidase subunit I (COI) gene as a marker because it is considered the main barcode gene and shows a high interspecific variation and a low intraspecific variation [35,36]. Although its use is mainly recommended for the animal kingdom, this study shows that COI barcodes can be also effectively applied for the identification of phytoplankton species as well. This is not surprising seeing that COI is a conventional reference gene with more than 4.7 million deposited sequences and growing literature evidence [37]. Nonetheless, about 90% of the total reads in each sample had to be discarded

after filtering, solely due to a lack of annotation in reference databases. This is currently the main shortcoming of molecular metabarcoding, wherein the detection of most species is hindered by the incompleteness of reference barcode libraries, especially in benthic biomonitoring [24,26,38]. For instance, the Aquatina Lagoon is known to host a high density of *Cymodocea nodosa* which was not identified in this study due to a lack of records of its COI sequence in the reference libraries. It is therefore of paramount importance to fill in the gap by sequencing a selection of representative taxa to improve sequencing resolution, especially in less studied benthic environments.

However, using generally low-effort sampling and rapid laboratory procedures, a large amount of valuable data was generated. We detected 13 different phyla, spanning three main ecological indicators with different body sizes and evolutionary adaptations, which would not have been recovered all using a traditional sampling technique. These results show that, in a lagoon environment characterized by shallow water, eDNA extracted from surface water and the use of the COI marker gene allows for the identification of both planktonic and benthic organisms. Specifically, phytoplankton organisms are abundant in an environment in which decomposition processes in sediment influence nutrient availability in the water column.

We were also able to identify up to genus level, including some of the phytoplankton, such as *Chaetoceros* sp., *Navicula* sp., *Pseudo-Nitzschia* sp., which were morphologically identified by Caroppo in 2009 [39]. Phytoplankton is well known for its ability to produce toxic metabolites with adverse effects on animals and even human health [40–42], and/or for their ability to degrade the overall water quality due to red tides causing seawater discolorations, mucilage, and anoxia [43]. It is therefore important to monitor them and make sure their biomass remains within safe levels. This is where molecular identification plays a crucial role. In fact, it has often been reported that several potentially dangerous species were morphologically misidentified [44–48]. Additionally, phytoplankton is considered a major ecological indicator because it can rapidly flag ecological imbalance thanks to its high plasticity to abiotic stresses. Therefore, implementing the methods for the routine detection of phytoplankton will also aid in the assessment of water quality and shift in population dynamics due to climate change.

Considering the comparison between our data and the IMPRECO database [28], we also demonstrated how eDNA seems more useful to identify planktonic species compared to a morpho-taxonomic approach which, in turn, better estimates multicellular eukaryotes. Strikingly, almost no Chordata and, in particular, no fishes were detected with eDNA, even if those who were morphologically identified have publicly available COI barcodes. Despite the sampling being carried out in October, when fish move towards the sea, we were still expecting to find some DNA lingering in the water. However, a study by Collins et al. [49] demonstrated that COI universal primers applied to a heterogeneous environmental sample lead to an underestimation of the fish population by preferentially amplifying prokaryotes and other metazoans. Therefore, ribosomal markers should be preferred over mtDNA markers to detect fish and large crustaceans more accurately [50], as also demonstrated by the use of 12S as the main metabarcoding gene for detecting and identifying ichthyofauna and crabs in deep waters [51–53]. Consequently, further studies need to be performed with other gene markers to improve the efficacy of molecular surveys in this highly heterogeneous environment.

In conclusion, the analysis of water samples was proven to be efficient in monitoring the global biodiversity of this NATURA 2000 site; however, for specific ecological indicator classes, this study indicates the need to use multiple primer sets and/or multiple gene targets.

This study shows that molecular surveys can be applied to accelerate and improve biodiversity assessment and biomonitoring programs aimed at the conservation of Mediterranean coastal lagoon ecosystems and early warning responses to stress and climate change.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14110991/s1, Table S1: Total number of reads for each sample, percentages of reads without hit in NCBI and percentages of reads of annotated sequences are reported; Table S2: Taxonomy of annotated sequences.

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