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Environmental DNA Metabarcoding of Cephalopod Diversity in the Tyrrhenian Deep Sea

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Abstract: The deep sea, the largest biome on Earth, is the least explored and understood. This lack of knowledge hampers our ability to understand and protect this important environment. In this study, water and sediment samples were collected at different depths in the central Mediterranean (224–780 m), specifically, within the Dohrn Canyon and the Palinuro Seamount, to investigate the diversity of cephalopods and establish a baseline knowledge of their distribution in these sites to preserve their habitats and estimate the impacts of human-driven environmental changes. Key taxa identified included *Heteroteuthis* sp., *Loligo* sp., and *Histioteuthis* sp., which were the most abundant across all sampling stations. A low overlap in species detection was observed between water and sediment samples, confirming previous findings that the typology of environmental matrices used in eDNA metabarcoding has a significant impact on the organisms detected and, therefore, the integrated use of different matrices to better represent local biodiversity is recommended. Furthermore, this study highlights the limitations posed by gaps in reference databases, particularly for deep-sea organisms, and addresses these by emphasising the need for improved multi-marker approaches and expanded reference databases to enhance the accuracy of eDNA-based biodiversity assessment.

Keywords: eDNA metabarcoding; cephalopod assemblages; deep-sea biodiversity; Dohrn canyon

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1. Introduction

Deep-sea ecosystems (below 200 m depth) represent the largest biome on Earth, yet only a small fraction of the seafloor has been explored [1]. Recent studies show that global changes are affecting the deep sea by causing higher water temperature, lower oxygenation, and altered organic matter fluxes, impacting biodiversity and ecosystem functioning [2–4]. Moreover, technological innovations are driving the expansion of human activities and natural resources exploitation, including fisheries, oil and gas extraction, and deep-sea mining, into deeper environments, while regulatory frameworks that apply to the management and monitoring of the marine environment do not specifically address the Mediterranean deep sea [5]. Developing effective management plans relies on our understanding of what we aim to manage; thus, establishing a comprehensive baseline

is crucial, as it provides the foundation for measuring changes, identifying threats, and implementing appropriate conservation measures [6–8].

Many deep-sea ecosystems, including submarine canyons and seamounts, have been described as "keystone structures" because of their unique geomorphological and ecological characteristics, significantly contributing to marine biodiversity [9]. They provide valuable ecosystem services, such as fisheries, carbon sequestration, and nutrient cycling, as well as genetic and chemical resources for potential exploitation [9,10]. Owing to their geomorphological heterogeneity, canyons represent biodiversity hotspots, often hosting unique assemblages [11,12]. Seamounts have recently gained worldwide recognition as deep-sea biological hotspots, primarily due to the combined effects of turbulent hydrodynamic conditions and high planktonic productivity around their summits [13–15].

Information on deep-sea biodiversity remains limited, also, for the Mediterranean Sea [7], despite it being one of the most studied seas in the world [16]. The Mediterranean, where deep-sea covers approximately 80% of the Basin and waters exceed 200 m in depth, also contains one of the highest concentrations of submarine canyons in the world (approximately 500) [17]. The Dohrn Canyon and the Palinuro Seamount in the Tyrrhenian Sea are among the best studied deep-sea structures.

The Dohrn Canyon is located approximately 12 nautical miles from Naples (Italy; Figure 1). It is a bifurcate structure consisting of a western branch and an eastern branch. The shallowest part begins at approximately 250 m of depth on the continental shelf and reaches 1300 m in the Tyrrhenian plain [18]. The main water masses of the Gulf of Naples are the Modified Atlantic Water (MAW) in the upper 50–100 m, and the Levantine Intermediate Water below 200–300 m. This oceanographic structuring highly influences the spatial distribution of the dwelling fauna, vagile and sessile, which can occupy different niches and enrich the area with biodiversity. Moreover, Mediterranean canyons host numerous endemic or rare species and the Dohrn Canyon, as well, is recognised as a biodiversity hotspot and hosts high abundances of benthic taxa and charismatic species, like cold-water corals, bivalves, sponges, and other invertebrate [10,18]. Unfortunately, considerable anthropogenic impacts have also been documented: Over the years, this area has been subjected to bottom trawling and illegal dumping, which have resulted in large amounts of lost nets, longlines, and marine litter [19]. As a matter of fact, the Canyon does not meet the good environmental status required by the EU Marine Strategy Framework Directive and several potential research projects aiming at the restoration of this area have been proposed [20]. Despite the Gulf of Naples being one of the most studied areas in the world for environmental issues, knowledge about the Dohrn Canyon is still poor and mostly related to its geological setting [21]. Similarly, the Palinuro Seamount in the south-eastern Tyrrhenian Sea, a 70 km long, 25 km wide volcano complex, with its top at 80 m of depth, to date, has only been described from a geological point of view [22,23], although it is largely exploited for fishery purposes [24].

In the Mediterranean, bathymetry is one of the key drivers of diversity, linked to food limitation, freshwater inputs, and almost constant temperatures at depth [25–27], which is particularly evident in cephalopod communities, as they follow a clear depth and not a latitude or longitude gradient [28,29]. Cephalopods are an important component of deep-sea biomass and have a key role in marine food webs. They feed mainly on crustaceans, fish, mollusks, and gelatinous fauna, but some species also exhibit detritivore or cannibalistic habits [30–32]. Additionally, cephalopods are a significant dietary component for many species of commercial and conservation interest [33,34] and they are themselves of great commercial value (e.g., *Eledone cirrhosa*, *Loligo forbesii*, *L. vulgaris*). Changes in abiotic conditions (e.g., temperature, salinity) can affect cephalopod species and influence their distribution, which can shift rapidly in response to environmental stressors [35,36].

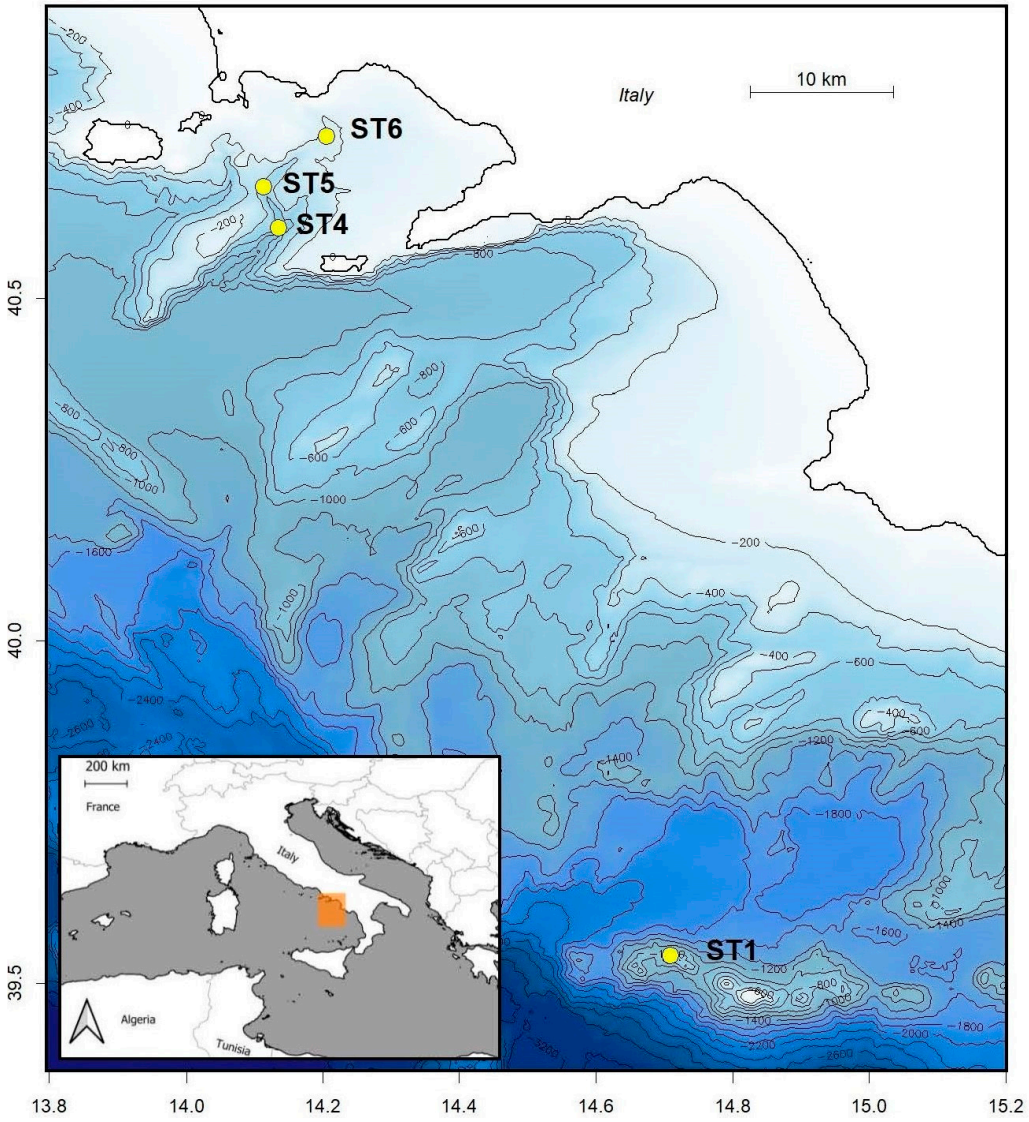

Figure 1. Sampling sites of sediment and water collected in the Central Mediterranean Sea. Details of the three stations in the Canyon Dorn, Gulf of Naples, Tyrrhenian Sea (ST4, ST5, ST6) and the one in Palinuro seamount, the South Tyrrhenian Sea (ST1) are listed in Table 1.

Table 1. Table reporting site, station, depth, and geographical coordinates of the collected samples.

Site	Station	Replicate	Depth (m)	Latitude	Longitude
Palinuro	ST1	R1 wat–R1 sed R2 wat–R2 sed	616 623	39°32.43′ N 39°32.42′ N	14°42.54′ E 14°42.53′ E
Dohrn	ST4	R1 wat–R1 sed R2 wat–R2 sed	780 767	40°36.18′ N 40°36.19′ N	14°08.15′ E 14°08.16′ E
Dohrn	ST5	R1 wat–R1 sed R2 wat–R2 sed	565 570	40°39.77′ N 40°39.75′ N	14°06.80′ E 14°06.84′ E
Dohrn	ST6	R1 wat–R1 sed R2 wat–R2 sef	225 224	40°44.23′ N 40°44.29′ N	14°12.37′ E 14°12.24′ E

Despite their key role in marine ecosystems, poor information is available on cephalopods [37]. As a matter of fact, cephalopods are often overlooked and their assessment excluded from the European Marine Strategy Framework Directive, which has been justified with a general lack of data [38]. Therefore, it is crucial to establish a base-

line knowledge of their distribution to preserve their habitats and assess the impacts of environmental changes. Several studies have described the cephalopod community inside the Mediterranean Sea and its sub-basins. In particular, the study conducted by Fanelli et al. [39] described the megafaunal species inhabiting submarine canyons and the adjacent slopes considering their geomorphological features. Using different sampling methods (e.g., baited cameras, traps, commercial trawls), 28 different species of cephalopods were identified. In the central Mediterranean abyssal plain, at depths between 200–700 m, the cephalopod species identified were *Abralia verany*, *Ancistroteuthis lichtensteninii*, *Bathypolipus sponsalis*, *Brachioteuthis riisei*, *Chenopteryx sicula*, *Chiroteuthis veranyi*, *Eledone elongata*, *E. cirrhosa*, *Galiteuthis armata*, *Heteroteuthis dispar*, *Histioteuthis bonnelli*, *H. reversa*, *Illex coindeti*, *Loligo forbesii*, *Neorossia caroli*, *Octopus salutii*, *Ommastrephes bartramii*, *Onychoteuthis banksii*, *Opisthoteuthis calypso*, *Pteroctopus tetracirrhus*, *Rondeletiola minor*, *Rossia macrosoma*, *Scaeurgus unicirrhus*, *Sepia elengans*, *S. orbignyana*, *Sepietta oweniana*, *Todarodes sagittatus*, and *Todaropsis eblanae*, with *Histioteuthis reversa* being the only species identified at depths between 700–900 m. However, scattered information on deep-sea cephalopods distribution is available and no data could be found on the presence and distribution of cephalopods in the Dohrn Canyon and in the Palinuro Seamount. Conventional biodiversity assessment methods, such as trawling and video surveys, are often biased and selective for certain taxa, as more agile organisms tend to avoid sampling gear [40]. Additionally, in such a vast and remote biome like the deep sea, sample collection may be expensive, time-consuming, and limited by logistical constraints.

Environmental DNA (eDNA), which refers to genetic material released by organisms found in the environment, represents a valid tool to provide a snapshot of the species diversity of a certain location [41,42]. It is a rapid and cost-effective technique that is quickly advancing and has found many applications in the marine environment such as monitoring species distribution, population dynamics, biodiversity assessment, prey–predator interactions, and diet estimation [43]. It can also represent a useful tool to detect species range shifts in response to changes in environmental factors [44]. The deep-sea floor acts as a DNA repository, which preserves genetic information about organisms living in the sediment, but also in the water column above it. This information can be used to assess biodiversity as well as for the monitoring of past and present environmental changes [45]. Previous studies have shown that the nature of the sample significantly influences the types of organisms detected by eDNA metabarcoding and they agree on the use of different matrices to obtain a better representation of the local biodiversity [46,47]. Most of the taxa were found to be unique to one of the matrices, with planktonic species predominating in water samples and benthic infauna in the sediment samples. However, some pelagic taxa were also detected in the sediment matrix, likely due to the deposition of genetic material on the seafloor [46]. Sedimentary eDNA has a slower decay rate compared to eDNA in the water column, allowing for the reconstruction of biodiversity over different timescales depending on the matrix analysed [48]. More recently, filter-feeding organisms have also proved to be efficient natural samplers and a valid alternative to sampling environmental matrices [49]. While studies using eDNA to investigate deep-sea biodiversity have been increasing lately, only a few have specifically focused on cephalopod diversity and never in the Mediterranean [50–52]. In the basin, a few studies have focused on deep-sea megafauna but not in the area of interest of the current work [53–56]. De Jonge et al. [57] have developed a primer targeting the V2 region of the 18S rRNA gene specific to cephalopods, facilitating the identification of a cephalopod hotspot in the Atlantic [50,52,58]. Recent findings have indicated that pelagic taxa, including cephalopods, can be retrieved from both sediment and aboveground water samples, supporting the idea that dead material, detritus, or faecal pellets can settle on the deep seafloor [46]. For these reasons, by using two sampling matrices, seawater and sediment, we aimed to retrieve as much information as possible about cephalopods' diversity.

Here, we present a first attempt to apply the eDNA metabarcoding technique to unveil cephalopods diversity in the Canyon Dohrn and Palinuro Seamount (Tyrrhenian Sea) using

two different sampling matrices. We aim to establish a baseline knowledge of the area and to assess the feasibility and effectiveness of eDNA in describing cephalopods diversity in a deep-sea environment.

2. Materials and Methods

2.1. Sampling

Sampling was conducted during the R/V OGS-Explora scientific survey in February 2018 as a part of EARTH CRUISERS (EARTH's CRUst Imagery for investigating SEismicity, volcanism and marine natural Resources in the Sicilian offshore) project funded by the Italian Ministry of University and Research (MIUR's call "Progetti Premiali 2015"), coordinated by the National Institute of Oceanography and Applied Geophysics in collaboration with the Stazione Zoologica Anton Dohrn and National Institute of Geophysics and Volcanology. Samples were collected at three different stations in the Dohrn Canyon (ST4–5–6), from depths of 224 m to 780 m. One sampling station located on top of the Palinuro seamount (ST1) was included for comparative purposes, as it is characterised by comparable depths but situated outside the Dohrn Canyon. Details about the location and depth of each station are reported in Figure 1 and Table 1. The sampling was carried out with a box corer, that was thoroughly cleaned before every deployment using a jet of surface seawater to wash away any residual and followed by a jet of freshwater. In every deployment, as final rinsing, the box corer was in contact with the sampling water from the surface to the bottom until its closure. Once the box corer was on board, water (2–5 L) at the sediment interface was collected and vacuum-filtered onto mixed cellulose esters 1.2 µm filters (MF-MILLIPORE MEMBRANE, MIXED CELLULOSE ESTERS, HYDROPHILIC, 1.2 µm, 47 mm). Meanwhile, the sediment was collected with small sterile tube corers, fractionated into three layers (0–1 cm, 1–3 cm, and 3–5 cm) and stored in different sterile falcon tubes at −20 °C until the end of the cruise. All the tools used for water and sediment collection were sterilised with 20% bleach and rinsed with MilliQ water to reduce the risk of contamination after every use. A final rinse was performed using water from the sample before collection started. For the purpose of this work, we chose to analyse only samples from the top layer of sediment (0–1 cm), because it is the most biologically active layer where recent genetic material is concentrated, and the bottom water samples. This approach allows us to focus on contemporary biodiversity, avoiding the inclusion of older or historical DNA that may be present in deeper sediment layers, which could confound the interpretation of current community composition. For each station, two independent replicates were collected, for a total of 16 samples (Table 1). On land, samples were stored at −80 °C until further analysis.

2.2. Total eDNA Extraction, Library Preparation and Sequencing

eDNA was extracted from filters with the E.Z.N.A. Mollusc DNA kit (Omega Bio-tek, Norcross, GA, USA) and from the sediment with the E.Z.N.A. Soil DNA kit (Omega Bio-tek, Norcross, GA, USA), following the manufacturer's protocol. A negative control, constituted by PCR-grade water, was processed in parallel to all eDNA extractions, following the same protocol. Then, DNA quality was checked with electrophoresis on 1% agarose gel stained with GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) and quantified using a Qubit fluorometer and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc., Monza, Italy).

To detect cephalopods, we used the Ceph18S primer pair (forward: 5′-CGC GGC GCT ACA TAT TAG AC-3′; reverse: 5′-GCA CTT AAC CGA CCG TCG AC-3′) [57], which amplifies the V2 variable region of the 18S rRNA gene, shown to be effective for taxonomic assignment [57]. PCRs were performed in a total volume of 15 µL and the reaction mixture consisted of 7.5 µL of AccuStart II PCR ToughMix 2X (Qiagen, Milan, Italy), 0.4 µL of each forward and reverse Ceph18S primer (10 mM), 5.7 µL of PCR grade water, and 1 µL of the template. The PCR thermal profile started with an initial denaturation of 3 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 56–64 °C, and 30 s at 72 °C and a final extension of 5 min at 72 °C. Given the complexity of amplifying such difficult samples, in

order to maximise the yield of the reaction, gradient PCRs were used to identify which annealing temperature worked best for our samples.

Each sample was amplified in triplicate. All reactions included a non-template control (NTC) to verify that the reaction had no external contaminations and the DNA extraction negative control (NC). NCs constituted by non-target organisms' DNA were also included: fish (*Sardina* sp. and elasmobranch) and non-cephalopod invertebrates. All PCR products were visualised on 2% agarose gel. PCR replicates were pooled together, and the band of interest corresponding to the expected amplicon length (140–190 bp) was extracted and purified from a 2% agarose gel using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Dueren, Germany). Cleaned PCR products were then prepared to be sequenced with IonTorrent PGM on a 316 chip.

2.3. Bioinformatic Analysis

Bioinformatic analyses were performed on QIIME2 [59]. The sequencing platform's output was already demultiplexed and trimmed. The DADA2 algorithm [60] was used to denoise the sequences, join paired-end sequences, and remove any chimeric sequences to obtain Amplicon Sequence Variants (ASVs).

The taxonomic assignment was performed using the qiime *feature-classifier classify-sklearn* command with the *fit-classifier-sklearn* method [61,62]. The classifiers were trained on Metazoa sequences downloaded from NCBI. The reference reads and taxonomy were processed, filtered, and evaluated with the package RESCRIPt [63]. When this method was not able to reach lower taxonomic levels, or if it retrieved taxa that did not match their known distributions, the uncertain ASVs were manually curated by careful assessment of blast search against the NCBI nr database [64].

3. Results

The sequencing process produced a total of 278,657 raw reads. After the filtering processes 18,941 reads from the water and 3975 from the sediment were retained. In the ST6R2 water sample and ST5R2 sediment sample, no cephalopod DNA was identified; hence, they were excluded from the dataset.

Eight expected taxa were retrieved, six from the filters and four from the sediment. Three taxa obtained with the bioinformatic pipeline were corrected after manual curation according to blast results; the full details are shown in the Appendix A (Table A1). Most of the reads (92%) could be assigned at least to the genus level: *Heteroteuthis* J. E. Gray, 1849; *Histioteuthis* A. d'Orbigny, 1841; *Loligo forbesii* Steenstrup, 1856; *Loligo vulgaris* Lamarck, 1798; *Eledone cirrhosa* (Lamarck, 1798); and *Onychoteuthis* Lichtenstein, 1818 (Figures 2 and 3). The most frequent taxon was *Heteroteuthis* sp., which was retrieved in all the samples except the water ones from ST5R1 and ST6R1.

In the Dohrn Canyon, *Heteroteuthis* sp. ASVs were identified in both matrices and replicates of ST4, but in ST5 and ST6, they were retrieved only in sediment samples. *Histioteuthis* sp. ASVs and *E. cirrhosa* were identified only in sediment samples, from ST4–ST5 and from ST5–ST6, respectively. The family Sepiidae was only found in water samples of ST4 and ST6. *Loligo* spp. and the family Loliginidae were found in every station only in the water samples.

Samples from the Palinuro station (ST1) showed a similar composition, with *Heteroteuthis* sp. ASVs and Sepiidae being present on both replicates and matrices. *Eledone cirrhosa* was only found in the ST1R1 sediment sample of ST1R1, while *Onychoteuthis* was exclusive of the two replicated water samples of the same station.

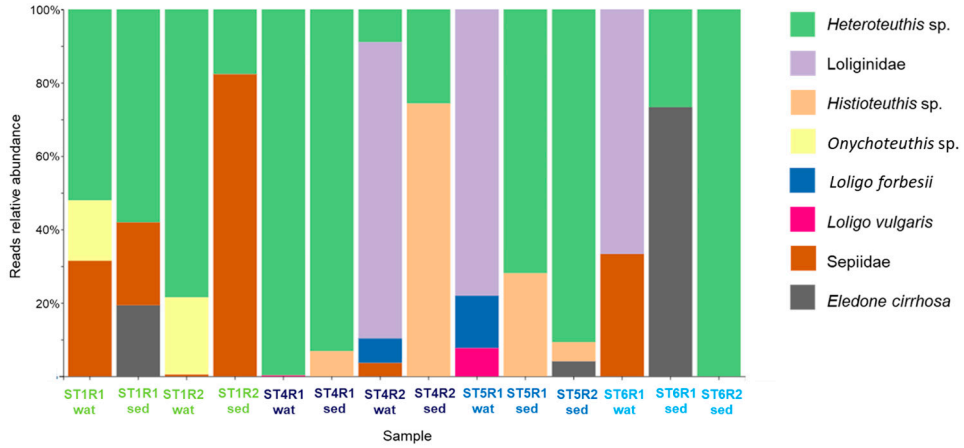

Figure 2. Relative abundance of reads of the retrieved taxa in all the water (wat) and sediment (sed) samples. In the ST6R2 water sample and ST5R2 sediment sample, no cephalopod DNA was identified; hence, they were excluded from the dataset. For sample codings, refer to Table 1.

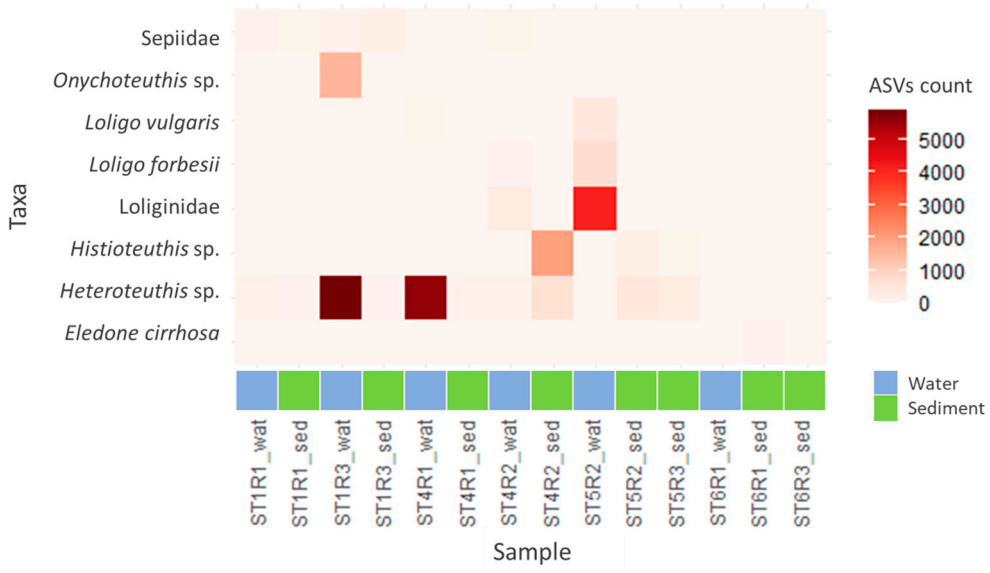

Figure 3. Shadeplot representing ASVs counts of every retrieved taxa for each sample.

4. Discussion

In this study, we successfully identified eight cephalopod taxa from deep-sea environmental DNA samples in the Mediterranean Sea. We observed a low rate of overlap between the two environmental matrices: only two taxa, *Heteroteuthis* sp. and Sepiidae, were retrieved both in sediment and water samples. Four taxa (Loliginidae, *Onychoteuthis* sp., *Loligo forbesii*, and *L. vulgaris*) were found exclusively in water samples, while two others (*Histioteuthis* sp. and *Eledone cirrhosa*) were unique of the sediment samples. Different studies [46,65] similarly found significant differences between communities detected in aboveground water samples and those in sediment, particularly for benthic organisms. In our study, a higher number of cephalopod taxa were detected in the water samples, than in the sediment ones, as reported in previous studies [46,65]. This could be related to the lower total number of reads obtained from the sediment samples in this study, which may ultimately be due to the presence of by-products of microbial activity, e.g., humic acids, which act as PCR inhibitors [66]. Moreover, Pawloski et al. [45] describe how, when the biological target is metazoans, a larger volume of soil is necessary to recover the community structure accurately. However, the low overlap between the two environmental matrices may also result from the fact that they provide insights into community composition on different time scales. Sakata et al. [48] showed that the decay rate of sedimentary eDNA

is considerably lower than the aqueous matrix, but we do not know to what extent the persistence and degradation dynamics of eDNA are influenced by deep-sea environmental conditions [67]. Sediment eDNA studies have been used to reconstruct different time scales, ranging from decadal to century-long changes in fish communities [66,68] to the reconstruction of Pleistocene diatom communities [69], depending on the depth of sediment cores/samples. In any case, we expect that the uppermost centimetre should correspond to recent decades, as deep-sea sediments typically have sedimentation rates of less than 3 cm per thousand years [70]. As a general concept, sediment is a better matrix to reconstruct biodiversity over a longer period of time, but the processing may be hampered by the higher content of PCR inhibitors. On the other hand, water may have a lower density of eDNA but may represent a better matrix to capture a snapshot of local biodiversity while dealing with fewer inhibitors. In the context of monitoring, we would recommend integrating both matrices to obtain complementary results. Our findings highlighted the predominance of *Heteroteuthis* sp. at nearly all stations in both water and sediment, suggesting that the genus may be among the most common in the area

In the manual review of uncertain taxa, some ASVs matched several different species with an equal score; thus, they were kept at the genus level to be more conservative. However, *Heteroteuthis* sp. is likely to be *Heteroteuthis dispar* (Rüppell, 1844) as it is the only Mediterranean species [71]. The persistence of this species in both sediment and water matrices suggests a stable presence and important ecological role in the studied area. *Heteroteuthis dispar* is widely distributed throughout the water column, with studies identifying it as the most abundant cephalopod in pelagic waters down to 650 m [72]. It exhibits vertical migrations, moving to the surface at night and spawning on the seabed at depths of 500–1000 m [40]. Juveniles are typically found in shallower waters, while adults inhabit deeper, near-bottom environments [40]. This broad vertical distribution and ecological adaptability highlight its significance in both pelagic and benthic food webs. *Histioteuthis* sp., the umbrella squid *Histioteuthis bonnellii* (Férussac, 1835), and the reverse jewel squid *Histioteuthis reversa* (Verrill, 1880) are the only species of this genus inhabiting the Mediterranean Sea [73] and are usually found at depths between 500 and 1500 m [74]. Similarly, caution was used with *Loligo* sp., which was reclassified as Loliginidae, as the obtained matches did not have sufficiently high scores to be confidently assigned to lower taxonomic levels. This can probably be attributed to the lack of a closer reference in the database, since the other Mediterranean *Loligo* species (*L. vulgaris* and *L. forbesii*) are present in the database and have also been retrieved in this experiment. It remains unclear whether this represents a third, unidentified species or if it is a sequence error resulting in a low score. The order Oegopsida was modified into *Onychoteuthis* sp., and likely corresponds to *Onychoteuthis banksii* (Leach, 1817), a cosmopolitan meso- and bathy-pelagic genus, which has only been captured a few times in the Mediterranean Sea [75,76]. This highlights the usefulness of eDNA metabarcoding as a tool to study elusive species that could be implemented in monitoring programs to improve knowledge on cephalopods distribution [77]. Together with *Heteroteuthis* sp. and *Histioteuthis* sp., species of this order are a significant component of the diet of many species of conservational and commercial interest, such as dolphins, whales, turtles, sharks, and fishes like swordfish and tuna [34,78–81]. *Loligo vulgaris* and *L. forbesii* are species of great commercial value in the Mediterranean, but very little information is available about their distribution in the Tyrrhenian Sea [82]. *Eledone cirrhosa* is another extremely important species in commercial landings and there is evidence of stock fluctuations in the southern Tyrrhenian Sea [83,84].

The Ceph18S primer pair is used to amplify the V2 variable region of the small 18S rRNA subunit [57]. De Jonge et al. [57] describe the suitability of this region for taxonomic assignment due to the presence of sufficient variation to allow the identification of a wide range of taxa. However, it is important to note that both in silico and empirical tests, described in the same study, demonstrated that the Ceph18S primer pair is not an optimal choice for octopod detection, as it may yield inconclusive results for sepiids, myopsids, octopotheuthids, and gonatids [57]. This result may be attributed to the incompleteness of

the reference database. Fewer than 300 out of the approximately 700 known cephalopod species have a complete or partial 18S rRNA sequence in GenBank [58]. Most of the reference sequences for cephalopods are currently mitochondrial sequences, whereas the 18S rRNA gene is nuclear.

To enhance the comprehensive coverage and precision of eDNA analysis in cephalopod biodiversity studies, a lineage specific multi-marker approach is recommended as the optimal methodology [85]. For example, the S-Cephalopoda [86] and CephMLS [87] primer sets targeting the mitochondrial 16S have proved to be complementary to the Ceph18S as they helped identify additional taxa that were not captured by the Ceph18S primer set [57]. This approach may improve the reliability of species detection when the same species is identified in multiple samples and through various markers, thereby overcoming the limitation of inadequate reference sequence data. Indeed, through the utilisation of multiple markers, the reference database can encompass species that may lack a reference sequence for a particular region but possess one for another [85]. In addition, when deep-sea benthopelagic animals are the target taxa, the use of different sample types (water and sediment) is recommended to describe completely the community structure [46].

Finally, we would suggest the combination of metabarcoding with an observational approach and include imagery data (e.g., obtained with ROVs or AUVs) to have a more complete representation of the local biodiversity [88]. As demonstrated in previous studies, video surveys can be coupled with eDNA sampling to provide complementary results and, thus, improve the detection of target species [52].

5. Conclusions

This study stands as one of the first attempts to employ an innovative molecular technique in characterising deep-sea nekton biodiversity within the Mediterranean Sea. While the initial findings are promising, the approach requires methodological refinement. Future efforts should focus on optimising sampling and processing protocols. It would be beneficial to increase the spatial coverage of the study areas and incorporate temporal series in the sampling strategy. DNA extractions should be performed onboard to minimise eDNA degradation before lab processing, following protocols that account for the removal of PCR inhibitors. Finally, exploring alternative sequencing protocols to obtain longer reads or, potentially, skip PCR amplification altogether may be worthwhile.

However, a significant challenge arises from the gaps in reference databases, which are especially pronounced for deep sea organisms considering the limited availability of sequences for multiple markers. Addressing these gaps in reference databases is critical for improving the reliability of eDNA metabarcoding, enabling more precise biodiversity assessments in understudied deep-sea environments.

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Appendix A

Table A1. Taxa identified with the bioinformatic pipelines and the corresponding manually curated corrected taxa, after identified by blast in NCBI.

Pipeline	Blast
Loligo sp.	Loliginidae
Oegopsida	*Onychoteuthis* sp.
Heteroteuthis hawaiiensis	*Heteroteuthis* sp.

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