Environmental DNA

eDNA Metabarcoding Applications Across Italian Marine Coastal Ecosystems: An Overview

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ABSTRACT

Climate emergency and other anthropogenic pressures urgently call for technological and methodological advances to enhance our ability to protect marine habitats and their ecosystem services. In recent years, environmental DNA (eDNA) metabarcoding has emerged as a powerful tool to achieve an integrative assessment of the environmental health status, through a broad, relatively rapid, and cost-effective taxonomic monitoring of biodiversity at different spatial scales. Here we provide a time-based overview of the applications of the eDNA metabarcoding methodology carried out across diverse Italian marine and coastal habitats, with an *in-depth* scrutiny of the commonly adopted operative procedures, from sampling to sequencing. The lack of standardization and low replicability in space/time arose as major issues of several monitoring campaigns, preventing appropriate cross-comparability of previous studies. Given the wide potential of eDNA metabarcoding surveys along the Italian coastline, this review aims to boost a wider application of eDNA metabarcoding for biodiversity inventories and to avoid major methodological weaknesses that could compromise the long-term value and broad spatial scope of future monitoring plans.

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1 | Introduction

In the last decades, marine conservation efforts increased steadily, driven by the key role of marine ecosystem functioning on a global scale, contributing to human well-being, climate regulation, mitigation of extreme events, matter cycling, and biodiversity (UNEP 2006). The European Water Framework Directive (WFD; European Parliament and European Council 2000), the Marine Strategy Framework Directive (MSFD; European Parliament and European Council 2008) and the new Nature Restoration Law (European Commission, Directorate-General for Environment 2022) are among the most important legislative instruments supporting EU member states towards the implementation of strategies and actions for the management, protection, conservation, and recovery of marine ecosystems. These regulations recommend conservation and restoration actions to promote the achievement of high-quality ecosystems flagged by an integrative measure of the "good environmental status". Therefore, a central issue is constant monitoring to generate updated knowledge and early warning on environmental disturbance and ecosystem degradation.

Biological monitoring allows detection of changes in the structure and organization of biotic communities, with sensitive species replaced by more tolerant ones, reflecting a cumulative response to multiple natural and anthropogenic (antagonistic vs. additive and synergic) factors occurring simultaneously over time. Biological monitoring serves as an integrative measure of the ecological quality of the ecosystem, overcoming the inherent quality-quantitative and spatio-temporal limitations of chemical–physical monitoring.

Traditionally, the biotic component of marine ecosystems has been studied through direct or indirect analysis (i.e., sampling, sorting, morphological identification of specimens, or video/photo monitoring) involving technical and operational challenges, requiring high levels of taxonomic expertise spanning over a wide biological diversity. A reliable, direct taxonomical assessment may be time-consuming and expensive, often requiring invasive samplings and potentially resulting in low spatial and temporal resolution, with limited power to detect larval or juvenile stages as well as cryptic, small, elusive, or rare species (Valentini et al. 2015; Danovaro et al. 2016; Pawlowski et al. 2022).

In this framework, the growing application of molecular tools in support to traditional methods has paved the way to overcome the issues mentioned above. DNA barcoding (Arnot et al. 1993; Hebert et al. 2003) opened the so called 'molecular taxonomy Era', a new way to carry out environmental surveys (Dayrat 2005). DNA metabarcoding, originally planned to explore the diversity of microbial communities (Taberlet, Coissac, Pompanon, et al. 2012), represents a further development of DNA barcoding to screen across entire biological communities at the same time. Given its promising potential, its application is effective in a variety of fields: paleo-genomics, trophic interaction analysis, plants and animals distribution, symbioses, pollution response, biodiversity monitoring and air/soil/water quality assessment (Beng and Corlett 2020; Compson et al. 2020; Pawlowski et al. 2020). Its expansion has also been possible due to two additional key achievements that led the metabarcoding to be widespread. The first one is the introduction of the concept of environmental DNA (eDNA, Ogram et al. 1987), defined by Taberlet, Coissac, Hajibabaei, et al. (2012) as "a complex mixture of genomic DNA from many different organisms found in an environmental sample" that associated with metabarcoding allow to survey an ecosystem in a quickly and effective way. Secondly, the development and continuous improvement of High-Throughput Sequencing (HTS, Shendure and Ji 2008) that allows a rapid and simultaneous identification of a huge quantities of sequences and consequently of organisms.

The combination of eDNA, metabarcoding, and HTS has become a powerful tool in environmental science (Figure 1),

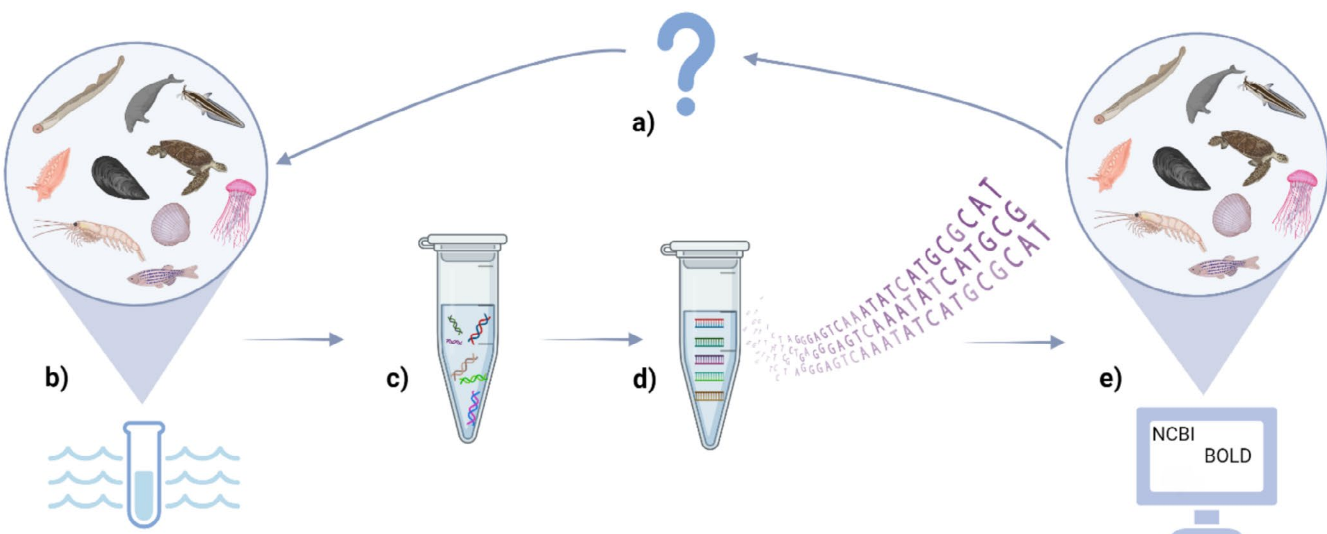

FIGURE 1 | eDNA metabarcoding workflow. (a) Initial biological question; (b) sampling of the environmental matrix; (c) extraction of eDNA from the matrix; (d) PCR amplification of the chosen molecular marker/s, attach of the unique barcode and HTS processing; (e) Bioinformatic quality filtering, taxonomic assignation, and data analysis to answer to the initial biological question. NCBI (National Center for Biotechnology Information) and BOLD (Barcode of Life Data System) are common databases for taxonomic assignment. Created in biorender.com.

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with the number of studies and the development of new protocols and instruments continuously increasing (Ruppert et al. 2019). DNA fragments, both intracellular and extracellular, are present in all environmental matrices and can be analyzed to obtain a comprehensive snapshot of habitats from a small amount of sediment, water, or other sources without stressing the environment (Pietramellara et al. 2009; Beng and Corlett 2020).

While the potential of the eDNA metabarcoding approach is clear, its application could be further improved as some limitations persist, including a low ability to identify organisms at lower taxonomic levels, such as genus or species (Ruppert et al. 2019; Schenekar et al. 2020). In this regard, traditional (i.e., descriptive, based on morphology and morphometry) taxonomy is mandatory to improve the available reference databases, on which metabarcoding is deeply linked. Indeed, to obtain a good resolution in the eDNA metabarcoding results it is required a high identity value between the query sequences and sequences from already identified species, considering that “unassigned” are the result of a non-correspondence to the reference database selected (Danovaro et al. 2016; Wangensteen et al. 2017). The second point that negatively affects the routine adoption of eDNA metabarcoding is the lack of shared approaches: different protocols and instruments are nowadays adopted. This led to difficult comparisons among different studies and sites, resulting in a heterogeneous set of biological information that does not allow reaching generalized, shared conclusions (Kumar et al. 2020). The main challenge is to couple the specific characteristics of each target ecosystem and the need for a shared and standardized experimental design to obtain accurate, repeatable, and reliable results with a robust statistical support. Furthermore, given the high sensitivity of this approach, contamination with exogenous DNA can occur, posing the issue of stringent experimental protocols (Hinlo et al. 2017). In fact, different types of contamination can occur in each step of a DNA metabarcoding workflow, starting from the sampling (i.e., no change/clean the instruments and consumables among the replicas) to the amplification step. This latter is also the most sensitive one, where three main types of contamination can occur: PCR product carryover, sample-to-sample contamination, and the one derived from the PCR environment (contaminated laboratory room) or from consumables (Taberlet et al. 2018). To avoid and monitor the contamination problem, it is mandatory to set up an adequate protocol with several control points (i.e., controls).

Taking all these issues into account, it is imperative to define a standardized and reproducible experimental design, based on the state of art of the selected area and of other similar eDNA metabarcoding studies. Such an approach becomes even more important in the case of planned periodic assessments, as happens in the monitoring of ecosystem and biodiversity changes over time.

We provide here an overview of the eDNA metabarcoding studies conducted so far throughout the Italian marine habitats, characterized by a variety of ecosystems and a high number of species and endemism rate (Danovaro et al. 2010; Barredo et al. 2016). Although the term eDNA is used to indicate the genetic material freely spread in the environment, here we review marine eDNA, including investigations dealing with stomach/gut content, feces, and bulk DNA from organisms mix or artificial substrate (Ruppert et al. 2019), as these are often carried out within monitoring framework investigations.

Our goal is to merge foundational knowledge on the eDNA metabarcoding approach to (a) inform the development of shared guidelines and standardized protocols, (b) harmonize future marine biodiversity monitoring to deliver more accurate, consistent, and comparable datasets, and eventually, and (c) advance the achievement of the Good Environmental Status of the Italian marine waters.

2 | Systematic Review of eDNA Metabarcoding Studies in Italian Marine Systems

2.1 | The Italian Marine Ecosystems in the Mediterranean Sea

Due to its paleogeographic, biogeographic, and ecological characteristics (Bianchi and Morri 2000), the *Mare nostrum* is recognized as a biodiversity hot spot (Danovaro et al. 2010; Barredo et al. 2016) hosting more than 7.5% of global biodiversity in a relatively small area compared to the rest of the other oceans (Coll et al. 2010; Danovaro et al. 2010), with a high percentage of endemic species and key habitats (i.e., *Posidonia oceanica* (Linnaeus) Delile, 1813 meadows, coralligenous bioconstructions, and deep-sea animal forests).

By its central position in the Mediterranean Sea and nearly 7600 km coastline (Caloiero et al. 2020; Mancuso et al. 2018) at high physiographic and geomorphological heterogeneity, Italy presents a large variety of marine ecosystems, such as rocky habitats, sandy beaches, caves, transitional and coastal lagoons, together with a substantial number of islands. Moreover, particularly in its southeasternmost part, it is first affected by non-indigenous (and particularly lessepsian) species, acting as a key regional hub in the monitoring of climate and ecosystem change (Occhipinti-Ambrogi et al. 2011).

The Italian marine area does not have a unique environment, but the natural communities vary according to the geographical region. To describe the heterogeneity of the Italian seas and to group areas characterized by shared biotic and abiotic factors, Bianchi (2004) divided the Italian Seas into nine main biogeographical sectors (Table 1).

2.2 | Material and Methods

This review follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al. 2009) for eDNA metabarcoding studies in Italian marine ecosystems and was performed on January 15, 2024.

The research was limited to the title, abstract, and keywords, and was not restricted by publication year or languages. Scopus, Web of Science, and Google Scholar databases were used.

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TABLE 1 | Subdivision of the Italian Seas into nine biogeographical sectors (Bianchi 2004).

Sector	Geographic area
1	Ligurian Sea and the northern part of the Tyrrhenian Sea
2	Northern Tyrrhenian Sea, including Sardinia (and Corsica)
3	Southern Tyrrhenian Sea, including Sicily and Pantelleria Islands
4	Sicily channel
5	Southern Mediterranean Sea, including Pelagie Islands
6	Ionian Sea
7	Southern Adriatic Sea
8	Central Adriatic Sea
9	Northern Adriatic Sea

The string used to retrieve the data was constructed as follows: ("*metabarcoding*" OR "*eDNA*" OR "*environmental DNA*") AND ("*Italy*" OR "*Adriatic*" OR "*Tyrrhenian*" OR "*Ionian*" OR "*Ligurian*") AND ("*marine*" OR "*sea*" OR "*lagoon*").

A step of manual curation of the data retrieved was necessary to remove duplicate results and papers that did not correspond to our query.

We investigated the sampling procedure and samples conservation, the eDNA extraction protocol, and the sequencing step. We also make consideration of the chosen sample type in relation to the target of the study.

Qgis 3.34.1 (QGIS Association 2020) was used to map the sites involved in each study and to cluster them according to the classification of the Italian seas proposed by Bianchi (2004). The European shape file was obtained from the European environmental agency (https://www.eea.europa.eu/data-and-maps/data/eea-coastline-for-analysis-1/gis-data/europe-coastline-shapefile).

3 | eDNA Metabarcoding Studies in the Marine Italian Ecosystems

From the PRISMA research and after a manual check, we obtained 49 papers (Appendix S1) about eDNA metabarcoding studies performed in the Italian marine ecosystems.

3.1 | Temporal Distribution of the Studies Retrieved

The number of studies has generally increased with the years (Figure 2), reaching a peak of 17 papers in 2023. However, 2024 (with one paper) has been excluded from this chart because only a small portion of the year can be considered.

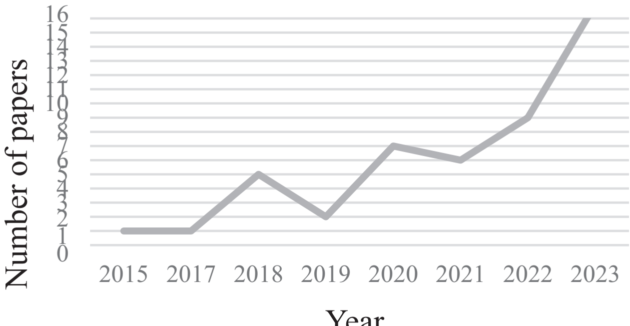

FIGURE 2 | Number of published studies on eDNA metabarcoding in the Italian marine ecosystems over the years.

3.2 | Sample Types and Targets Examined in the Studies Retrieved

Although the 49 studies focused on different sample types (Figure 3a), the most frequent is water (29 papers), with a large variety of targets (Figure 3b). The majority of water-related studies focused on plankton and phytoplankton (Penna et al. 2017; Armeli Minicante et al. 2019; Gaonkar et al. 2020; Minicante et al. 2020; Ruggiero et al. 2022; Mordret et al. 2023; Saidi et al. 2023; Turk Dermastia et al. 2023; Russo et al. 2023) to investigate the community composition and/or the genetic structure as in Ruggiero et al. (2022). Two eDNA metabarcoding studies sampled water to understand the potential of this approach to investigate the occurrence of outbreaks of pathogens. Vibrionaceae community was investigated by Banchi et al. (2022), while Reñé et al. (2022) analyzed the phytoplankton parasites.

The fish communities have also been extensively studied through water samples obtained mainly from coastal environments (Aglieri et al. 2020, 2023; Cananzi et al. 2022). The same happened for the application of this technique to find traces of marine mammals in the marine environments (Valsecchi et al. 2021, 2023; Boldrocchi et al. 2023). Lastly, water samples were also investigated to determine the entire metazoan diversity focusing, also in this case, on coastal ecosystems (López-Escardó et al. 2018; Di Capua et al. 2021).

It is interesting to notice that the method used for collecting water is quite different among the studies. For example, in Maggio et al. (2023) not only was the water column used but also bilge water to investigate NIS (Non-indigenous species) presence. NIS are also studied by Stefanni et al. (2018), and Schroeder et al. (2020, 2022, 2023) using plankton nets and by Aglieri et al. (2020) using water samples. Other examples of particular water samples came from Maiello, Talarico, Brodie, et al. (2022), Maiello, Talarico, Carpentieri, et al. (2022), Albonetti et al. (2023) and Cicala et al. (2024) that used the water dropping from the fishing net to investigate the effect of trawling.

In six papers, the studies relying on water samples integrated the analysis by investigating the sediment as a complementary eDNA source. This was the case of studies exploring the eukaryotic communities (López-Escardó et al. 2018; Cordier

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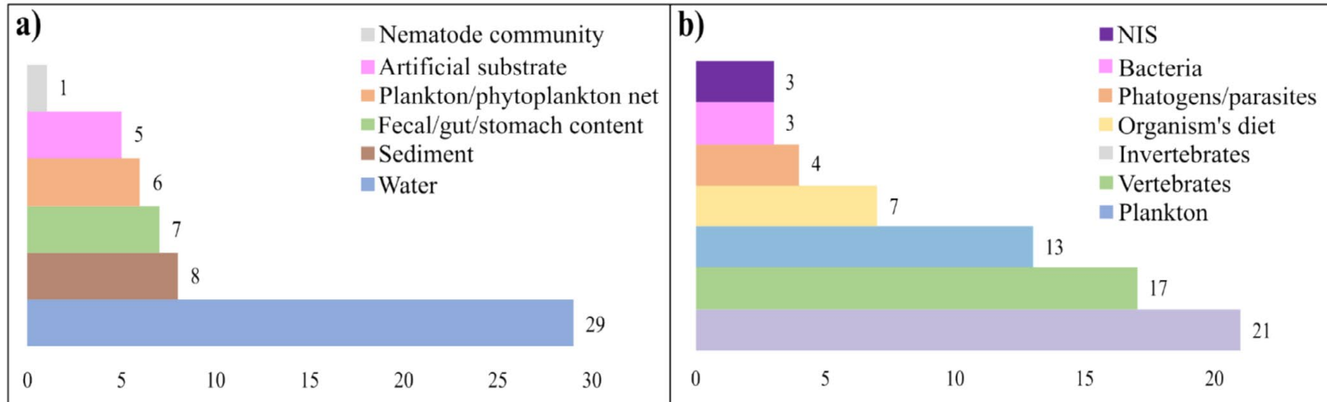

FIGURE 3 | Bar charts showing the main categories (a) sample type and (b) targets considered in the 49 studies investigated in the present review. Some studies involved more than one sample type and/or target.

et al. 2019; Tagliabue et al. 2023) or the microbial ones (Bellec et al. 2020; Basili et al. 2021). In one case (Piredda et al. 2018) the combination of the two matrices was applied to describe diatom diversity.

In four studies, sediment was exclusively collected, like in Good et al. (2022), to study meiofaunal organisms or to investigate specific communities like foraminifera (Frontalini et al. 2020; Cavaliere et al. 2021) or to do paleo-genomics (Barrenechea Angeles et al. 2023).

Even if DNA derived from artificial substrates and bulk DNA does not strictly fall into the eDNA concept, they have frequently been used to investigate the local community and have provided useful information on the biodiversity of the studied areas. ARMS (Autonomous Reef Monitoring Structures) were used to study invertebrates and benthic organisms (Pearman et al. 2020; Thomasdotter et al. 2023) like ASUs (Artificial Substrate Units; Cahill et al. 2018; Mugnai et al. 2023); while EMBs (Epilithic Microbial Biofilms) were used to investigate the microbial biofilm of macroalgal canopies (Pedicini et al. 2023).

Further, seven studies did not primarily investigate biodiversity, but used DNA metabarcoding of gut content or feces to answer some biological questions. Notably, the feces of *Pinna nobilis* Linnaeus, 1758 were scanned by a metabarcoding approach to detect bivalve pathogens (Manfrin et al. 2023). Similarly, the diet of Mediterranean seahorses was investigated through DNA metabarcoding of their feces (Lazic et al. 2021, 2023). Dietary and trophic niche investigations were carried out by DNA metabarcoding on stomach content of *Merluccius merluccius* (Linnaeus, 1758), a key resource for Mediterranean fisheries (Riccioni et al. 2018, 2022). The gut content of the non-native ctenophore *Mnemiopsis leidyi* A. Agassiz, 1865 was also investigated by DNA metabarcoding to understand its predatory impact on the mesoplankton community of the Venice Lagoon (Schroeder et al. 2021, 2022, 2023).

Lastly, Dell'Anno et al. (2015) directly targeted a deep-sea nematode community, pooling together a pre-sorted number of specimens to extract their bulk DNA and scan their taxonomic diversity using a DNA metabarcoding approach.

3.3 | Geographic Distribution of the Studies Retrieved

An overview of the localities of the studies performed in the Italian marine ecosystems (distributed over the nine ecogeographical sectors, as defined in Table 1) is mapped in Figure 4.

S3 and S9 received the most attention, with 19 and 20 papers. The highest number of studies was carried out in the Southern Tyrrhenian Sea (S3), particularly in the Gulf of Naples because of the occurrence of the Stazione Zoologica Anton Dorhn, one of the oldest marine stations in Europe. Similarly, for S9, where the Lagoon of Venice was particularly investigated because of the proximity of several historical marine stations in the North Adriatic Sea and because it is one of the main hubs for biological invasions of non-native species driven by shipping and aquaculture. These two sectors are followed by S1 with seven papers and S2 with six. A lower number of eDNA metabarcoding studies (4) was carried out in the Central Adriatic Sea (S8), mainly in the coastal waters and offshore areas of Emilia Romagna. S4, S6, and S7 are comparable (three papers each), while only one study was performed in S5.

3.4 | Protocols Adopted in the Studies Retrieved

In the 49 studies here analyzed, different protocols for sampling, eDNA extraction, library preparation, and sequencing were used (Appendix S1).

Sediment sampling is typically conducted using a box corer or a Van Veen grab sampler and usually involves collecting the top few centimeters of material. In the reviewed papers, between 0.4 and 10 g of sediment were used during the extraction step. It is well established that sediment sampling methods are closely related to factors such as depth, target characteristics, and the features of the sea floor (Pawlowski et al. 2022). While box corer and Van Veen grab sampler are the most commonly used techniques globally, alternative methods are also available (i.e., scuba diving sampling, Substrate-Independent Benthic Sampler; Cahill et al. 2018; Tagliabue et al. 2023). Moreover, cores are generally preferred over grab samplers

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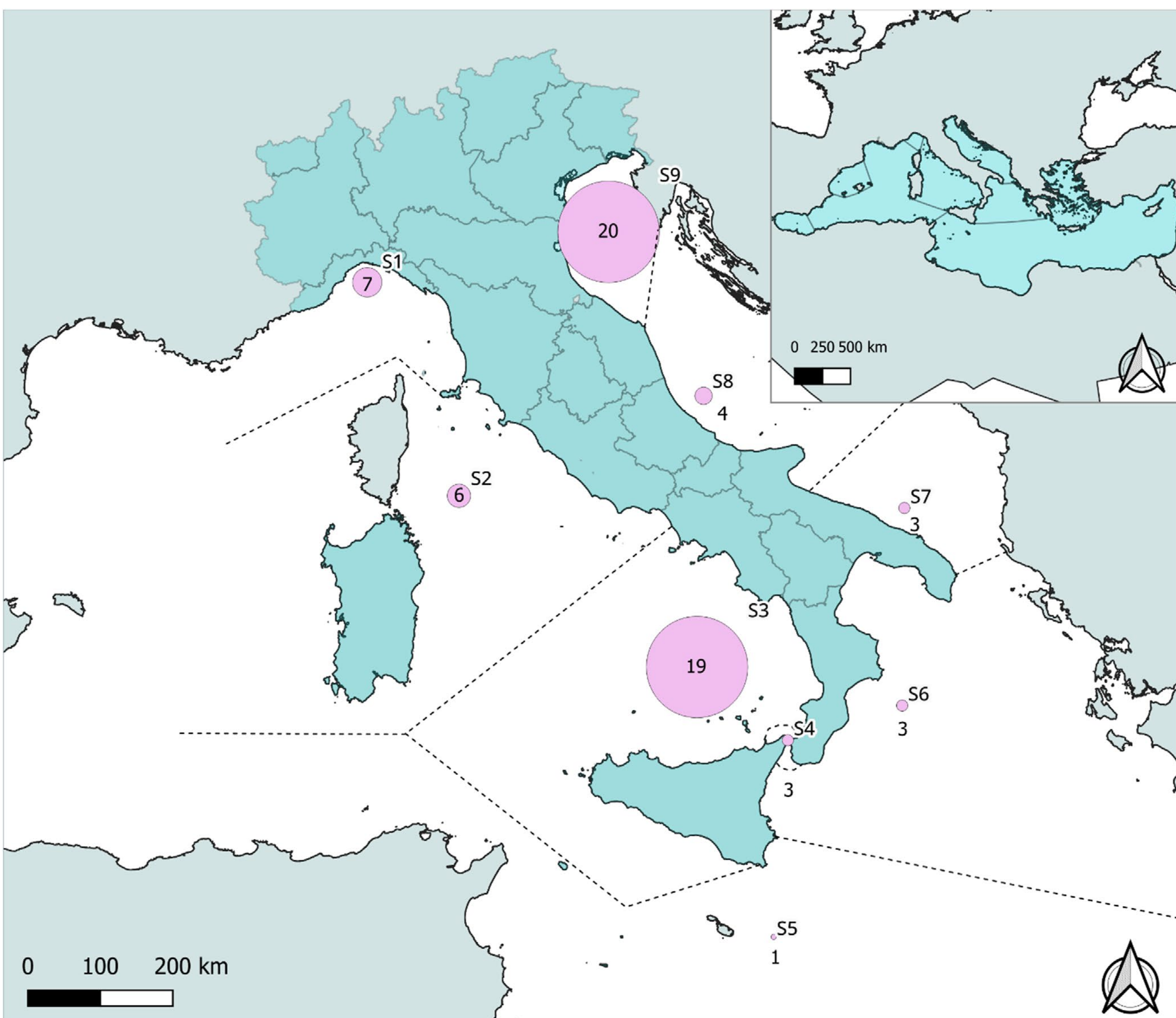

FIGURE 4 | Map showing the distribution of eDNA metabarcoding studies in the Italian marine ecosystems with the position of Italy in the Mediterranean Sea (in the top-right corner). The works are grouped using the zonation of the Italian seas proposed by Bianchi (2004) (dotted lines); if the paper involved more sites from the same area it counts as one, while if the study analyzed different areas the count is valid for each of them. The pink circles represent the number of works performed in each area, the bigger it is, the higher is the number off work. (S1) Ligurian Sea and the northern part of the Tyrrhenian Sea; (S2) northern Tyrrhenian Sea, including Sardinia (and Corsica); (S3) southern Tyrrhenian Sea, including Sicily and Pantelleria Islands; (S4) Sicily channel (S5) southern Mediterranean Sea, including Pelagie Islands; (S6) Ionian Sea; (S7) southern Adriatic Sea; (S8) central Adriatic Sea; (S9) northern Adriatic Sea. The number in the circle or near indicates the number of studies represented by the circle itself.

because they allow for better standardization and preserve the vertical sediment profile (Lins et al. 2021). Typically, the first 1–2 cm of sediment are collected; however, in some cases, the superficial layer is avoided to exclude pelagic species. It is often noted that the precise amount of sediment used during extraction is less important than the number of replicates, as sediment is a highly heterogeneous sample type (Hestetun et al. 2021; Pawlowski et al. 2022).

Regarding the collection of water samples (in 29 papers), the most used method involved the use of the Niskin bottle (60%). Volumes from one to three liters are commonly utilized, while sampling more than 10 L was rare but was specifically employed when looking for eDNA from marine mammals because of their rarity. Water was mostly collected from the surface, with a few studies that have collected it also near the bottom, based on the investigated target of organisms. Cellulose filter was the preference for the filtering step, but other options like glass microfiber filters were used. A 0.22 μm pore size is used in about 28% of the analyzed papers, followed by 0.45 μm and 1.2 μm pore sizes with about 24%; a 0.8 μm filter was used in about 14% of the papers (Figure 5a). A filter mesh of 0.22 μm was applied in the detection of small organisms' communities (i.e., microbiota) (Bellec et al. 2020; Basili et al. 2021), but also for fishes or benthic organisms (Maiello, Talarico, Brodie, et al. 2022; Maiello, Talarico, Carpentieri, et al. 2022; Tagliabue et al. 2023). A 1.2 μm filter was used to investigate plankton communities (i.e., Armeli Minicante

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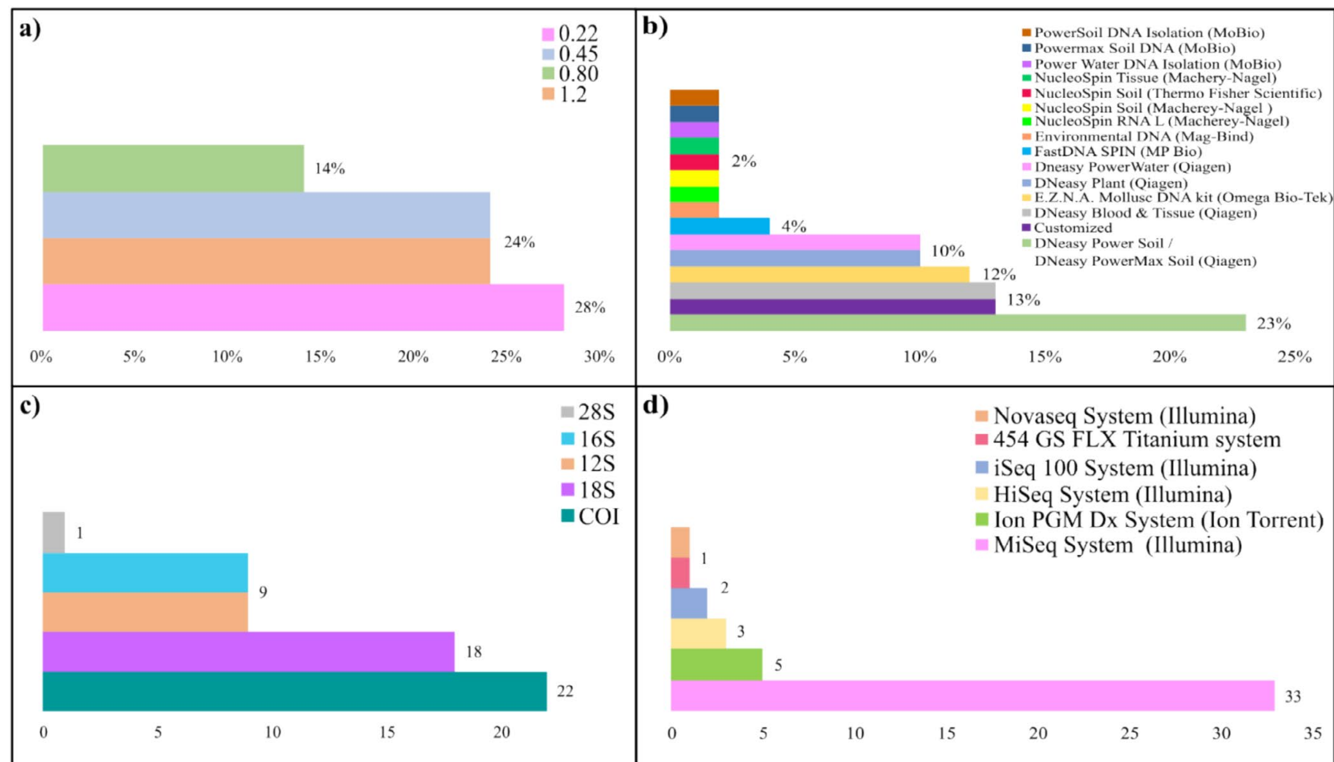

FIGURE 5 | Bar chart illustrating, across the 49 reviewed papers, the main: (a) filter pore size (μm); (b) DNA extraction commercial kits; (c) molecular markers; (d) sequencer systems. If the total number exceeds the number of papers reviewed (49), multiple approaches were used in some papers. Conversely, if the number is lower, the approach was not specified in all papers.

et al. 2019; Minicante et al. 2020; Ruggiero et al. 2022; Russo et al. 2023) and only in one case for metazoan diversity (Di Capua et al. 2021). A 0.45 μm filter was mostly used for marine mammals (Valsecchi et al. 2021, 2023; Boldrocchi et al. 2023) and for fish communities (Aglieri et al. 2020, 2023; Cananzi et al. 2022). Lastly, a 0.8 μm pore size was utilized for diatoms (Piredda et al. 2018; Turk Dermastia et al. 2023), phytoplankton parasites (Reñé et al. 2022) and metazoan communities (López-Escardó et al. 2018).

The filtration method and filtration time vary significantly between the studies, as do the storage approaches. However, in most cases, filters—after water filtration—are stored at −20°/−80°C.

DNeasy PowerSoil (Qiagen) and DNeasy PowerMax Soil (Qiagen) are the commercial kits for DNA extraction most used in the selected papers (23%, Figure 5b), followed by DNeasy Blood & Tissue Kit (Qiagen) (13%) and E.Z.N.A. Mollusk DNA kit (Omega Bio-Tek) (12%). There was also a large use of customized extraction protocols (13%). Qiagen PowerWater DNA Isolation kit and DNeasy Plant kit (Qiagen) are common kits used for DNA extraction from water samples (with 6 and 5 papers, respectively), followed by Qiagen PowerSoil DNA Isolation kit and Qiagen DNeasy Blood & Tissue kit (4 papers each).

Almost all studies used DNeasy power soil or DNeasy PowerMax Soil (Qiagen) to extract eDNA from sediment samples. However, for the study of artificial substrates or stomach/gut content and feces, there was no preference for a particular commercial kit. Lastly, the E.Z.N.A. Mollusk DNA kit (Omega Bio-Tek) was used for the extraction from material collected with a plankton net.

The choice of the molecular marker(s) is strictly target-oriented (Figure 5c). Most studies (34 out of 49) used only one molecular marker, few used two (14), while only one used five different markers (Cordier et al. 2019; this paper aimed also at a methodological comparison among the relative marker efficiencies). In 22 papers, COI (Cytochrome Oxidase subunit I) was used, followed by 18S rRNA, applied in 18 studies. 16S rRNA and 12S rRNA were used in two papers. Lastly, 28S rRNA was rarely used.

In five studies, fish communities or a specific focus on Chondrichthyes were investigated through the 12S rRNA gene in combination with the COI gene; in two papers, only 12S was used. This marker was also used (in combo with 16S) for the investigation of marine mammals; in Valsecchi et al. (2023) a special region of 16S (Mar Ver 2) was used to investigate the presence of the monk seal. 16S and 18S alone were applied to the investigation of microbial diversity. The 18S was also involved in the study of the metazoan community as COI.

Regarding the sequencing System (Figure 5d), the MiSeq Illumina System is for sure the most used (34 papers out of 49), followed by the Ion PGM Dx System (Thermo Fisher Scientific, 5 papers) used most for sequencing planktonic samples. Other Systems were HiSeq Illumina System (3 papers), iSeq 100

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Illumina System (2 papers), 454 GS FLX Titanium system (1 paper), and Novaseq Illumina System (1 paper). Only two studies used more than one HTS technology.

4 | Discussion

The necessity of this review arises from several reasons.

First, it is due to the increasing use of eDNA metabarcoding as an effective complementary tool for biodiversity investigations aimed at assessing and monitoring aquatic ecosystems (Wangensteen et al. 2017; Beng and Corlett 2020; Pawlowski et al. 2020). Second, it highlights Italy's 8000 km of coastline in the Mediterranean marine biodiversity (Danovaro et al. 2010; Barredo et al. 2016). Thus, a state-of-the-art overview and key points regarding the use of this technique in Italy have become necessary.

In total, 49 papers published between 2015 and 2023 (and one in the early 2024) dealing with the application of eDNA metabarcoding (in a broad sense) to inventory biodiversity across different Italian marine and coastal ecosystems were obtained and have been reviewed. Although no single solution exists for eDNA metabarcoding approaches, a comparison of the selected studies has been carried out here to highlight the advantages and disadvantages of the different protocols used so far, based on the sample type and biodiversity targets (Table 2).

4.1 | Temporal Distribution of the Studies Retrieved

The first use of DNA metabarcoding in the world dates back to the last century (Giovannoni et al. 1990) but the first study on marine biodiversity using eDNA metabarcoding in Italy was published only in 2015 (Dell'Anno et al. 2015), quite later compared to other areas of the world (Ruppert et al. 2019; Schenekar et al. 2020). After 2015, the number of studies increased steadily until 2023 when the largest number of papers was published (17). This is not surprising when considering the constant improvement in molecular technologies, which allowed an increasingly straightforward application of these methodologies alongside a significant reduction in relative costs (Ruppert et al. 2019; Taberlet et al. 2018). Simultaneously, there has been an increase in expert personnel as well as user-friendly instruments.

4.2 | Geographic Distribution of the Studies Retrieved

The studies—object of the present review—are not equally distributed across the Italian Seas, with most of the areas quite poorly represented, even though hosting environments of biological interest. Throughout the Italian peninsula, Sectors 3 and 9 faced particular attention due to many research groups working on eDNA metabarcoding analyses in that specific area and the presence of particularly interesting sites like the hydrothermal vents in the Naples area (Mattison et al. 1998; Rizzo et al. 2022) and the Venice lagoon (S9, Degobbis et al. 1986; Franco et al. 2009; Anelli Monti et al. 2021).

4.3 | Field of Application

Over the years and due to the increasing interest in eDNA metabarcoding studies, the field of application has considerably expanded. In the beginning, microbiology was the main field of application, but later more specific fields of research, like ethology and neurophysiology of both single and multiple species, were involved (Taberlet et al. 2018). More recently, conservation ecology (as environmental status assessment and discovery of NIS or elusive taxa) has received a particular attention, becoming one of the main fields of application. The study of invertebrate diversity revealed useful to assess the anthropic impact and establish environmental quality (Pawlowski et al. 2020). Vertebrates, mainly fishes, are mostly used to unveil the bottom trawling impact and/or the commercial implications.

4.4 | Sample Types

The choice of sample type across the 49 papers analyzed here varies according to the different research targets. Water samples are the most commonly used in Italy, even though sediment samples have historically been the most frequently used sample type in the world (Taberlet et al. 2018). The DNA extracted from Italian water samples was employed not only for detecting vagile organisms (i.e., fish and marine mammals) or planktonic specimens, as expected, but also for assessing entire eukaryotic or metazoan communities. Additionally, a novel investigation method involves collecting water dripping from fishing nets or rolls of gauze inserted in the nets (Maiello, Talarico, Brodie, et al. 2022; Maiello, Talarico, Carpentieri, et al. 2022; Albonetti et al. 2023; Cicala et al. 2024). Studies using this method aim to assess the impact of bottom trawling or detect specific groups of organisms (i.e., *Chondrichthyes*), yielding promising results.

Among the 49 papers reviewed, only three used both surface and bottom waters, but without any direct comparison between the two datasets obtained. Moreover, in two cases, the two datasets are pooled together. Comparing results from surface and bottom waters could be an interesting area of analysis, especially considering the scant information existing in the literature (Ruppert et al. 2019; Jeunen et al. 2020; de Vargas et al. 2015). Surface water is easier to collect (no need of scuba divers or specific instruments such as Niskin bottles) and could be a viable option depending on the target organisms.

In recent decades, artificial substrates, a specific type of eDNA source, such as ARMS, have been developed to investigate species recruitment in benthic and meiofaunal communities (Leray and Knowlton 2015). This method is notable for its ability to work in a standardized environment (and protocols), allowing a clearer comparison across different geographical and temporal data.

Sediment sampling was also used in Italian eDNA metabarcoding studies to investigate both eukaryotic and microbial communities. The inconsistency in the choice of a unique sample type across Italian studies mirrors the global situation. To date, there is not a clear consensus on the best sample type for eDNA metabarcoding studies in the marine environment (according to the target), and systematic analyses of the effect of the initial sample type choice are still rare (Tagliabue

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TABLE 2 | Overview of the main methods used in eDNA metabarcoding assessment of biodiversity across Italian coastal and marine habitats.

		Advantages	Disadvantages	Target
Sample type	Artificial structures	Spatial and temporal data	Not detect the entire biodiversity	Benthic communities
	Plankton and phytoplankton net	High concentration of the targeted community		Plankton and phytoplankton communities
	Fecal, gut, and stomach content	Trophic investigation	Not detect the entire biodiversity	Targeted species
	Sediment	High eDNA yield Spatial accuracy	Broad temporal range High sampling effort More inhibitors	Benthic communities Benthic fish Eukaryotic communities Fouling organism Microbial communities
	Water	Low sampling effort Temporal accuracy	Broad spatial range	Planktonic communities Vagile communities
Molecular markers	16S	Specific to communities		Archaea, Bacteria
		Complete 12S	Less efficient than 12S	Fish
	12S	High number of primer sets		Fish
	18S	Broad range of taxa	Low taxonomic resolution	Biodiversity assessment
	COI	Reference database Sensitive analysis	Less frequently used	Biodiversity assessment
Quantity[a] (g)	0.2–0.5	Lower sampling effort	Underestimate the overall biodiversity	Microorganism
	10	Detect more organisms	Higher sampling effort Only with few commercial kit	Meiofauna
Volume[b] (l)	1–3	Good compromise between yield and sampling effort	Underestimate the overall biodiversity	Biodiversity assessment
	4–5	Valsecchi et al. (2021)	Filter clog	Biodiversity assessment
	10	Detect rare trace	Higher sampling effort	Rare trace Mammals
Filter porosity[b] (μm)	200	Pre-filtration Avoid filter clog	Higher sampling effort	
	0.22	Retain small particles	Higher filtration time	Microbial community
	0.45	Lower filtration time	Not retain small particles	Metazoan

(Continues)

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TABLE 2 | (Continued)

		Advantages	Disadvantages	Target
Kit	Commercial kit	Standardization	Cost	Based on target
	Custom	Cost	Low efficiency	Based on target
Sequencer	Illumina	High quality Low cost Standardization	High quality for short reads	
	Oxford Nanopore	Long reads sequencing Quick	Higher error rate	
	PacBio	Long reads sequencing	Higher error rate Higher cost	

Note: Each method is associated with the most suitable target organisms, along with its respective advantages and disadvantages. Further details on each step are provided in the discussion section.
[a]Only for sediment samples.
[b]Only for water sample types.

et al. 2023). Based on the current literature, we know that the taxonomic composition is strictly related to the sample type (Holman et al. 2019; Koziol et al. 2019; Tagliabue et al. 2023). However, Koziol et al. (2019) recommended selecting the sample type depending on the biological taxa of interest. This latter methodology can positively affect the robustness of the results obtained both in terms of representativeness of the community investigated and the statistical significance of the results obtained. In this research, water samples are dominated by Dinophyceae, which are also well represented in sediment samples, where microeukaryotes are also abundant. Moreover, water is preferable for planktonic and vagile organisms (i.e., fishes), while sediment well represents fouling and benthic communities (Brandt et al. 2021) including benthic fishes (Turner et al. 2015). Although sediment was the most used sample type in the past, water is becoming increasingly common due to its simpler sampling procedure; collecting water is less problematic than collecting sediments from marine bottoms (Gaonkar et al. 2020; Antich et al. 2021; Basili et al. 2021; Aglieri et al. 2023). On the contrary, water eDNA represents a broad spatial range due to wave and current action that transport the material (Antich et al. 2021; Goldberg et al. 2016; Hajibabaei et al. 2019; Nichols et al. 2022; Reimer and Gösser 2023). Sediment samples, instead, have a higher yield compared to water, both in terms of the number of reads and OTUs detected (Holman et al. 2019; Koziol et al. 2019), with few exceptions (Tagliabue et al. 2023). This is because DNA tends to accumulate in sediments where the decay rate is lower than in water (Barnes et al. 2014; Sakata et al. 2020). Moreover, big fragments of benthic species could be captured during the sampling, and this could influence the final eDNA yield. Considering the available literature, sediment is often indicated as the preferable first choice for investigating metazoan communities (Brandt et al. 2021; Tagliabue et al. 2023). However, recent evidence suggests the best approach to achieve accurate estimates of overall diversity would be the integration of different sample types, usually water and sediment (Holman et al. 2019; Koziol et al. 2019; Tagliabue et al. 2023). In fact, water samples are often complemented by sediment (Atienza et al. 2020; Antich et al. 2021; Pawlowski et al. 2022) because these two sample types (water and sediment) can yield different results, being more efficient for one or another kind of community. This concept also emerged in the reviewed papers: water and sediment samples produce different results in terms of alpha/beta diversity and community composition, calling attention to the importance of targeting the appropriate sample type.

4.5 | Molecular Markers

A main point of debate is the selection of the molecular marker to achieve the best level of taxonomic assignment, as DNA metabarcoding across different taxa may require the choice of different markers. The choice of primer sets is even more specific. Across the 49 selected papers, fish diversity was mostly investigated using the 12S marker, often in conjunction with COI, typically without a direct comparison between them, sometimes with the final merging of the two datasets for an overall taxonomic assessment. As a notable exception, Valsecchi et al. (2021) compared the datasets obtained from

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DNA metabarcoding carried out using two "universal" marine vertebrate primer sets, MarVer1 and MarVer3, for 12S and 16S, respectively. The two markers yielded concordant results, though with some differences in the efficiency of detection between the teleost and cetacean species groups. MarVer3 detected more teleost signals than MarVer1, which, in contrast, performed better in detecting cetaceans, including the occurrence of the sperm whale, which, on the contrary, was not unveiled by MarVer3.

There is a consensus in using 18S (especially the V4 region) (Santoferrara 2019) for studying planktonic and microbial communities, in addition to the 16S marker, particularly for prokaryotic organisms. However, the two markers have never been directly compared in the studies analyzed.

Despite COI being globally less frequently used than 18S (van der Loos and Nijland 2021), in Italy the opposite strategy has been adopted so far. Two papers utilized these two markers together, consistently showing that COI and 18S yield comparable results and a similar trend in community composition analyses, but with some differences. According to Good et al. (2022), the overall COI dataset detected more amplicon sequence variants (ASVs), but the 18S dataset includes a higher number of Metazoan ASVs. Particularly, roundworms (Nematoda) were underrepresented in the COI dataset. Stefanni et al. (2018) investigated the zooplankton community using both the COI and 18S (V9) markers. COI detected more ASVs than 18S, with differences in terms of phyla assignment: for the phylum Chordata, COI better captured the diversity of vertebrate eDNA traces, while 18S captured more efficiently the diversity of chaetognath and tunicate eDNA in the monitored sample types. Lastly, to detect benthic and planktonic eukaryote diversity, Cordier et al. (2019) compared five molecular markers: 18S V1V2, 18S V4, 18S 37F, 16S, and COI. The five markers showed similar trends, but 18S V1V2 more consistently highlighted the community changes along a distance gradient from the impact considered in the research.

The choice of target-specific molecular markers adopted in the Italian eDNA metabarcoding studies is quite in agreement with the selection of markers in the world eDNA literature (Lear et al. 2018).

To investigate the fish community, several markers have been used over the years, with COI, 12S Cytb (Cytochrome b), and 16S (often in combination with 12S) as the most common ones. Ribosomal gene markers (12S and 16S) offer higher taxonomic coverage than the coding genes (COI and Cytb). Moreover, several studies suggest that the 12S marker is preferred for fish eDNA metabarcoding over COI, despite the latter having greater representation in reference databases (Collins et al. 2019; Xiong et al. 2022). In fact, COI shows a higher level of non-specific amplification than 12S (Collins et al. 2019). 16S is often used alongside 12S in fish community analyses, resulting in lower efficiency but complementing the detection offered by 12S (Valsecchi et al. 2021).

16S gene is used in almost all studies targeting Archaea and Bacteria because it is very specific to these communities and outperforms other markers (i.e., 18S). The V4 and V9 regions are the most commonly used (Danovaro et al. 2010; Hermans et al. 2018; Lear et al. 2018; Taberlet et al. 2018).

For biodiversity assessment of eukaryotic and/or metazoan communities (including eDNA *sensu lato*), COI and 18S genes are the most commonly used "universal" markers, often providing similar trends in biodiversity assessments (Wangensteen et al. 2018). COI is a high variable gene, well-represented in public databases (i.e., BOLD: The Barcode of Life Data System, https://www.boldsystems.org), while 18S is a well-conserved gene with lower taxonomic resolution but capable of covering a broader range of taxa (Leray and Knowlton 2016; Wangensteen et al. 2018; Braukmann et al. 2019). Even though COI is less frequently used than 18S (van der Loos and Nijland 2021), it provides more sensitive analyses (Wangensteen et al. 2018; Casey et al. 2021) because it is more specific and primer-dependent. For these reasons, COI is a good choice if the target is overall biodiversity, but the final choice should be based on the study's specific goals (e.g., Nematoda and Chaetognata are best detected using 18S).

Although it is possible to identify the best marker for each type of target community, a multi-marker approach is ideally the best solution, as it may provide a comprehensive investigation of biodiversity. This is why such studies have become increasingly common over the recent years (Clarke et al. 2017; Cordier et al. 2019; Bellec et al. 2020; Good et al. 2022).

4.6 | Protocols

When discussing eDNA metabarcoding analyses, it is essential to consider the sampling procedure.

While sediment does not need particular pre-operation before the DNA extraction, in the case of water samples, more steps must be taken into consideration. There is a general consensus that filtering water is preferred over precipitation methods (Deiner et al. 2015; Lear et al. 2018). However, the reviewed literature does not provide a clear guideline on the optimal volume of water to be collected. This is a crucial consideration given the typically low concentration of eDNA in water, which may necessitate sampling large volumes (Lear et al. 2018). Additionally, studies have shown that eDNA distribution in seawater can be patchy, and the volume of water filtered significantly impacts biodiversity estimates (Bessey et al. 2020; Miya et al. 2020). Although more water generally results in the detection of more organisms, a balance must be struck considering the sampling effort required. Notably, Valsecchi et al. (2021) recommended filtering 4–5 L of marine water per filter as an optimal approach. Expanding the consideration out of Italy, sampling volumes between 1 and 3 L are commonly used, balancing eDNA yield and filtration time and avoiding filter clogging. However, for detecting marine mammals or fish biodiversity, volumes up to 10 L may be necessary to capture even rare DNA traces (Bessey et al. 2020).

Another important aspect is filter porosity, which corresponds to the size of the targeted biological material. Only two out of the 49 reviewed papers used more than one filter type. For example, Cananzi et al. (2022) compared glass fiber filters (1.2 μm,

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Whatman) and cellulose acetate filters (0.45 μm Sartorius) to investigate the fish community in the Venice Lagoon but found no significant differences, and they are considered replicas in the study. Similarly, Valsecchi et al. (2021) tested three types of nitrocellulose filters (0.22 μm, 0.45 μm, and 0.8 μm) for marine vertebrates and found no significant differences but suggested using 0.45 μm filters to retain smaller biological particles without requiring excessive filtration time.

Lower porosity filters can capture more material with a limited volume of water before membrane saturation, while higher porosity filters allow for larger volumes but may miss smaller particles (Barnes and Turner 2016). Typically, filters with a porosity of around 0.45 μm or larger are used for detecting metazoan, fish, and marine mammals, while smaller communities, such as the microbial one, are filtered through a 0.22 μm filters (Deiner et al. 2018; Li et al. 2018). Pre-filtration using 200/300 μm filters is commonly employed to facilitate the filtration process and prevent early membrane saturation. The filter type also influences the final eDNA yield, with nitrocellulose membranes often outperforming other materials (Hinlo et al. 2017; Deiner et al. 2018; Jeunen et al. 2019; Kumar et al. 2020). Water samples should be filtered as soon as possible after collection, ideally within 24 h, to prevent DNA degradation (Hinlo et al. 2017; Lear et al. 2018). On-site filtration using a vacuum system is recommended to preserve DNA, avoid contamination during sample transport, and save time (e.g., filtering between moving to one site and another; Yamanaka et al. 2016). However, caution must be taken to properly clean, the instruments between replicas, with bleach or ethanol. Filters should be stored on ice and in dark conditions (Laramie et al. 2015) until they can be preserved at −20°C or in Longmire's lysis buffer (Renshaw et al. 2015; Hinlo et al. 2017; Spens et al. 2017).

Regarding eDNA extraction, detailed information on the kit is rare in the reviewed papers. However, there is a clear trend towards using Qiagen kits across different research groups, regardless of sample type. Nowadays, a wide variety of commercial kits have been developed for specific biological targets, making them preferable to custom protocols. The use of commercial kits also helps standardize the metabarcoding workflow. Qiagen kits are considered the best choice due to their widespread use and efficiency in multiple studies, but other companies such as Gentaur, Thermo Fisher Scientific, Macherey-Nagel, and MP Biomedicals (Mahmoudi et al. 2011; Hinlo et al. 2017; Lear et al. 2018; Jeunen et al. 2019; Pawlowski et al. 2020).

Although some studies employed sequencing platforms from Thermo Fisher Scientific (5 papers) or Roche (1 paper), Illumina platforms, particularly MiSeq (for long-read applications), are predominantly used due to their cost-effectiveness. As highlighted by Ruppert et al. (2019), Illumina sequencers outperform other NGS platforms in short reads, and for this reason, the use of Illumina sequencing platforms is overwhelming in recent years (Lear et al. 2018).

Although bioinformatics analysis pipelines and databases are often customized and depend on the specific objectives of the study as well as the expertise of the researchers, certain common patterns can still be observed across various workflows. Obitools and DADA2 are among the most widely used tools for processing sequencing data, from sequence trimming and error correction to taxonomic assignment. For taxonomic classification, researchers often rely on widely accepted reference databases such as NCBI, which provides comprehensive genomic resources. However, alternative databases like PR2, which is tailored for protists and environmental eukaryotes, and SILVA, which focuses on ribosomal RNA sequences, are also frequently employed. Additionally, custom databases are often created to accommodate specific taxonomic groups or to improve the accuracy of taxonomic assignment in particular studies. These choices in tools and databases reflect the diverse nature of sequencing studies and the need for flexibility in addressing different research questions and sequencing technologies.

5 | Conclusions

This review showed the multidisciplinary field of studies that eDNA metabarcoding can cover with its numerous advantages compared to the traditional approaches. In contrast, the heterogeneity of the protocols used so far is still problematic (Ruppert et al. 2019; Schenekar et al. 2020). Each study used different methodologies and instruments. Sometimes, it is possible to find the same general workflow, but this occurs primarily when the study focuses on the same topic and is conducted by the same research group, while more often, even if the biological question and the target are similar, the protocols adopted are different. This creates incomparable results even if retrieved from the same studied area. Furthermore, considering the small period since the introduction of this approach, only a few research groups have had the time to monitor the same site and use the same approach. To date, comparisons remain scarce and the ability of eDNA to provide information about environmental change over time has not yet been fully exploited.

In spite of the heterogeneity of various methods in each step of the eDNA metabarcoding workflow, it is possible to summarize a list of recommended methodological procedures and technological opportunities, taking into consideration the available literature, the targets of the study, and the characteristics of the investigated biotic components and their habitats. Our recommendations include:

1. Sample type: unless focusing on specific communities that may require a specific sample type (i.e., meiofauna and sediments), the integration of both water and sediment provides a more comprehensive investigation of marine biodiversity. Additionally, if the goal is to assess environmental status, sediment samples may be sufficient as they better target benthic species (which are sensible to environmental stressors). On the contrary, water samples are recommended for vagile species.
   1.1 Water samples: 1–3 L should be filtered on-site using nitrocellulose filters: 0.45 μm for macro-organisms and 0.22 μm for microbial communities. Filters should be preserved at −20°C or in Longmire's lysis buffer.
   1.2 Sediment samples: should be sampled using core instruments, and the first centimeters should be considered for eDNA extraction. Multiple replicas are recommended, while 0.20–0.5 g of sediment is sufficient for eDNA extraction.

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2. Molecular marker: the selection should be aligned with the target organism: 12S for fish communities, 16 for Archaea and Bacteria, and an integration of 18S and COI markers for overall biodiversity assessment. The choice of primers is related to the species target.
3. eDNA extraction: commercial kits are preferable to custom protocols, with Qiagen kits indicated so far as a good value for money solution.
4. Sequencing platform: Illumina platforms, particularly MiSeq, offer the best cost-effectiveness for sequencing.
5. Bioinformatics pipelines are often based on Obitools or the DADA2 algorithm, and taxonomic classification uses different databases depending on the target (e.g., NCBI, PPR2, SILVA). Custom pipelines and databases are often used.

In conclusion, the integration of both traditional (morphological, descriptive) taxonomy and eDNA metabarcoding is considered the most powerful way to investigate marine biodiversity. Only a few studies have adopted this strategy so far (i.e., Stefanni et al. 2018; Aglieri et al. 2020; Maiello, Talarico, Brodie, et al. 2022), but these always identified a positive correlation between the two methodologies.

eDNA metabarcoding can be a useful and reliable approach, but it can suffer from severe limitations related to the proper verification of the taxonomic identification of ASVS, often requiring also knowledge of the behavioral ecology of the species.

The efficiency of eDNA metabarcoding is strictly dependent on the accuracy of verified reference databases, developed by the integration of morphological analysis of voucher specimens with traditional DNA barcoding. In this scenario, there is a call for increasing integration of novel and traditional methods, also to drive the pan-European environmental policy for biodiversity monitoring and conservation towards an optimized, standardized monitoring tool. This review was planned as a starting point in search for an optimization of methodologies and techniques to detect the impact of global warming and other anthropogenic disturbances on marine biodiversity in Italy. Here, we analyzed 49 papers, but additional efforts are required for a more robust methodological standardization, along with broader analyses in terms of time and space, with the support of traditional taxonomists delivering verified collections of specimens and tissues to be used as voucher samples for DNA barcoding and compilations of verified ASVs datasets. Although EU marine ecosystems are various and populated by a heterogeneous biodiversity, their monitoring must be carried out as a single entity, so as to deliver comparative analyses and assessments at broad geographic and long temporal scales. Integrative methodological efforts will be key to the achievement of biodiversity conservation and management.

Author Contributions

Alice Tagliabue: research, writing – original draft. **Giulia Furfaro:** writing – review and editing, supervision. **Stefano Piraino:** ideation, writing – review and editing, supervision. **Andrea Galimberti:** review. **Antonia Bruno:** review. **Lorenzo Zane:** review. **Alberto Pallavicini:** review.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting this review are derived from publicly available resources cited within the article.

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