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Review

Advancements in DNA Metabarcoding Protocols for Monitoring Zooplankton in Marine and Brackish Environments

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Abstract: Over the past century, numerous studies have proposed various organisms for the biomonitoring of aquatic systems, but only recently has zooplankton emerged as a promising indicator of water quality. The traditional identification methods, however, can be inefficient in the context of monitoring efforts, as they are often time consuming and costly. DNA metabarcoding offers a powerful alternative, providing a more efficient and reliable approach to monitor zooplankton communities. In this review, we assess the current state-of-the-art methodologies used to evaluate marine and brackish zooplankton communities through the DNA metabarcoding workflow. While several emerging approaches have been reported, no standardization has been achieved so far. The DNA extraction step has gained the most consensus, with the widespread use of commercial kits (DNeasy Blood & Tissue kit employed in ca. 25% of the studies), though there is still a significant variation in kit selection. Additionally, 18S and COI were the main molecular markers employed (ca. 61% and 54%, respectively) though the target region varied in the former. Moreover, many methodologies, particularly those used for processing zooplankton samples, lack practical validation. Some studies also fail to provide sufficient detail in their methodology descriptions hindering reproducibility. Overall, DNA metabarcoding shows great potential for the efficient monitoring of zooplankton communities, but further effort is needed to establish standardized practices and optimize the current approaches across the entire methodological pipeline.

Keywords: DNA metabarcoding; zooplankton communities; marine; brackish; monitoring; state-of-the-art methodology

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1. Introduction

Coastal regions are characterized by their high biological productivity and harbor higher biodiversity compared to the open sea, owing to their dynamic nature and continuous nutrient input from the continents [1,2]. Presently, coastal ecosystems are pivotal in driving various economic activities, including commercial and recreational fisheries, with approximately 80% of these activities centered in coastal areas [3]. Other economic ventures such as sports, oil extraction, mining, tourism, aquaculture, and biotechnological research also rely heavily on coastal resources [3–5]. However, in response to the escalating resource demands, many coastal ecosystems have endured significant degradation over the past two centuries, largely due to the neglected and inadequate management practices [6] (see, e.g., Zhao and Shen [7]).

In recent years, zooplankton has emerged as a promising indicator of water quality, owing to its ability to respond to the subtle changes in the aquatic conditions [8–10]. Zooplanktonic communities serve as a crucial intermediary between primary production and higher trophic levels, encompassing various life stages such as larvae, eggs, and other propagules of species of both economic and biomonitoring significance. Yet, the morphological identification of zooplankton poses challenges, characterized by a low-throughput process demanding a

substantial sample size, thus slowing down the developmental results. Additionally, distinguishing early developmental stages (eggs or larvae) to the species level can prove arduous due to the morphological similarities between taxa. The traditional identification methods are particularly inefficient in detecting species unless the population densities are high [11,12]. Rare species, inherently elusive, often need a high volume of samples for detection [13,14]. For example, a study on zooplankton communities in the Great Lakes estimated that over 750 samples would be required to detect 95% of the zooplankton species present [15].

To address these challenges, the integration of molecular tools with the traditional zooplankton monitoring methods has become increasingly common [16]. Over the last two decades, the advancement and refinement of DNA-based techniques, such as DNA barcoding [17], along with concurrent metabarcoding approaches, have shown significant potential for organism identification and have demonstrated high efficiency in assessing zooplankton communities [18–20]. These molecular tools have proven effective at overcoming several inherent limitations of the morphology-based, low-throughput methods. DNA fragment sequencing, for specimen identification, offers an approach as follows:

i. It is morphology-independent, thus circumventing the issues of morphology ambiguity and developmental stages prevalent in zooplankton communities;
ii. It requires minimal sampling and processing effort;
iii. It requires minimal to no expertise in morphology-based identification;
iv. It is highly sensitive, exhibiting greater efficiency in detecting rare and newly introduced species);
v. Overall, it is more time- and cost-efficient. In fact, the costs associated with DNA-based tools are comparable to or slightly cheaper than those of the traditional identification methods [21].

Indeed, the standardization of the DNA metabarcoding approach is crucial for ensuring the efficient and reliable monitoring of coastal ecosystems. Metabarcoding offers a means to standardize the identification process of highly complex community biodiversity, thereby reducing the ambiguity [22]. Given the wealth of existing information, there is an urgent need for a thorough evaluation of the efficacy of DNA metabarcoding in zooplankton communities' assessments in marine and coastal ecosystems. Consequently, we undertook a review to provide a comprehensive overview of the methodologies employed throughout the analytical chain of the DNA metabarcoding approach—from sampling to sequencing (and some relevant inputs to the bioinformatics pipelines used), thereby contributing to the advancements in this field (Figure 1). Moreover, we scrutinized the potential gaps and biases, identifying the most pressing challenges awaiting resolution and offering recommendations for future advancements within the realm of biological conservation.

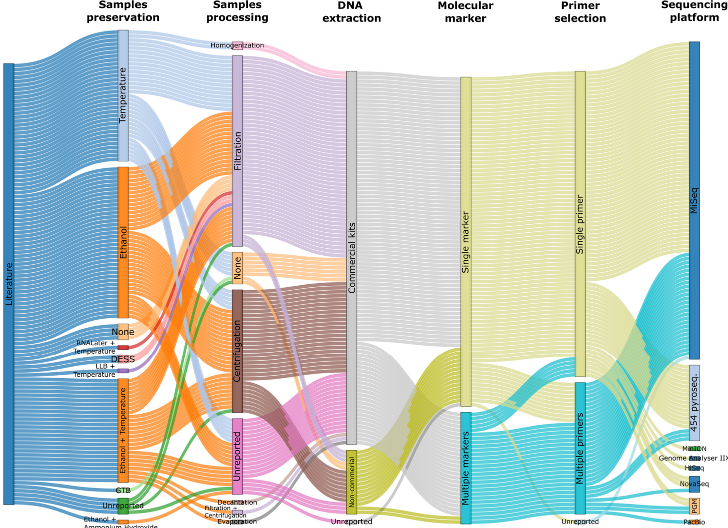

Figure 1. Overall methodological summary of this review, detailing the workflow from sampling to sequencing in metabarcoding-based studies on zooplankton communities' characterization.

2. Materials and Methods

We conducted a comprehensive literature search using the Web of Science (WoS) database (11 May 2023) to identify articles using DNA metabarcoding characterizing naturally occurring zooplankton communities in marine and coastal ecosystems. We employed various combinations of search terms related to marine and coastal environments (e.g., estuary, transition, marine, lagoon, sea, coast) and DNA metabarcoding (e.g., metabarcoding, high-throughput sequencing, HTS, next-generation sequencing, NGS, eDNA, environmental DNA), along with the term "zooplankton", searching within article titles, abstracts, and keywords. This search aimed to compile publications focused on the bulk sampling of naturally occurring zooplankton communities, typically defined as net-captured biomass. While bulk sampling involves directly filtering biomass from the water column during sample collection, we also considered additional studies involving water sampling methods (commonly associated with eDNA), when their primary focus was on zooplankton and aligned with the general objectives of this research.

In addition to the initial search results, we manually added ten relevant publications from our personal collection that were not captured by the search query, resulting in a compilation of 236 publications for review. Subsequently, we refined this list by excluding studies outside the scope of the present review: targeting non-zooplankton communities (i.e., prokaryotes or phytoplankton; n = 29), involving solely morphological analysis (n = 6), freshwater ecosystems (n = 20) or not marine/coastal (n = 1), eDNA studies (free environmental DNA—here considered water-sampling studies in which metabarcoding targeted a broad range of taxa, including non-planktonic biodiversity; n = 35), sorted or mock samples, or other non-naturally occurring communities (ballast waters, cultures, or meso- or microcosmos; n = 20), reviews (n = 11), and those employing non-metabarcoding approaches (i.e., qPCR studies, metagenomics, metatranscriptomics, microsatellites; n = 40). Filtering the publications in the Web of Science yielded 74 studies meeting the accepted parameters. However, due to the relatively low representation, a Google Scholar search using the terms "coastal zooplankton metabarcoding" was conducted resulting in 2960 findings. To ensure comprehensive representation, similar searches were performed in additional sources, including the journals Environmental DNA, Metabarcoding & Metagenomics, and the Journal of Plankton Research, among others. This was important due to the relevance of these journals to the field, as they frequently publish key studies on eDNA and metabarcoding research, thereby enriching our review with the latest and most pertinent findings.

Overall, after careful inspection of the studies from the additional queries, a total of 103 publications were selected for further analysis. The majority of the publications were peer-reviewed studies, with the exception of three reports, which were also considered in this review [23–25]. For each publication, we extracted information regarding the sampling locations and methods employed, types of nets, and mesh sizes used, samples' preservation methods, sample processing techniques prior to genomic content extraction (including mesh material and pore size—these were specific in the case of pre-processing by filtration), DNA extraction methods, molecular markers and primers used for zooplankton communities' amplification and sequencing, as well as the sequencing platforms used. It is important to note that the review did not cover bioinformatic pipelines for post-sequencing analysis; however, for further information, see Hakimzadeh et al. [26]. We chose to emphasize the methodologies employed throughout the analytical chain of the DNA metabarcoding approach prior to sequence processing, as these steps hold the greatest potential to significantly impact the results and may exhibit variability across different laboratories. However, given that (i) applying rigorous data quality filters, (ii) using appropriate clustering algorithms for Operational Taxonomic Units (OTUs) or Amplicon or Exact Sequence Variants (ASV or ESV—hereafter mentioned as ASV), and (iii) assigning a taxonomy against the curated databases are all critical in enhancing the reliability and reproducibility of the outcomes and accurately characterizing the taxonomic diversity in zooplankton samples, we have also included a review of the clustering approaches used for metabarcoding-generated sequences and the subsequent taxonomic assignment methods.

3. General Overview

Notably, the implementation of DNA metabarcoding for assessing marine and coastal zooplankton communities has seen a steady rise (Figure 2). This approach has notably enhanced the taxonomic characterization of these intricate and morphologically challenging-to-identify communities. However, it has only been since 2020, in the past few years, that we have witnessed a significant surge in its adoption. For example, between 2013 and 2019, an average of 5.4 studies were published per year, whereas in the subsequent three-year period (2020–2022), the average soared to 19.3 publications per year, nearly quadruplicating the number of studies published up to 2019 (Figure 2). Although the exact reasons for this rapid adoption of DNA metabarcoding in assessing complex zooplankton communities remain unclear, the growing focus on zooplankton monitoring has been apparent over the recent decades (as exemplified by works such as Chiba et al. [27]). The advancements in technology and improvements in metabarcoding pipelines have likely played a significant role in driving the increased interest in taxonomic characterization using this method. Similar trends have been observed in the realm of environmental DNA (eDNA) publications [28–30]. Notably, around one tenth (n = 11) of the studies reviewed here were published in the ICES Journal of Marine Science during 2021, under the theme, Patterns of Biodiversity of Marine Zooplankton Based on Molecular Analysis, except one (accounting for 10.6% of the total number of the publications reviewed). Furthermore, a similar number of studies have been found published in Frontiers of Marine Science, although over an 8-year period.

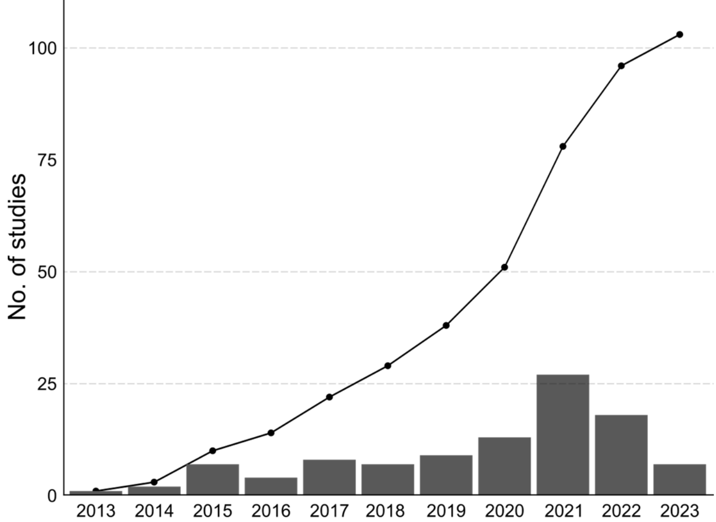

Figure 2. Cumulative (line) and exact number (bars) of publications, during the last decade (for 2023, only studies published until May were included), of naturally occurring marine and coastal zooplankton communities assessed through DNA metabarcoding.

The use of DNA metabarcoding for assessing marine and coastal zooplankton communities has not been evenly distributed globally. Most of the publications reviewed herein focused on samples from the northern hemisphere, with 81 publications, whereas only 15 were conducted in the southern hemisphere (Figure 3). The sampling efforts in the Atlantic Ocean were predominantly focused along the European coast, particularly in the North and Mediterranean Seas, as well as along the northern American coast. Conversely, the studies in the Pacific Ocean covered a broader geographic area, although clusters of sampling sites were notably observed along the coasts of North America, Korean Peninsula, Yellow Sea, the southern Japan, and New Zealand. In the Indian Ocean, fewer publications were found, with three main focal points: the eastern coast of South Africa, the eastern coast of the Red Sea, and the eastern Indian Ocean, while in the Arctic, the few studies highlighted sampling at, e.g., the Chukchi [31] and the Barents Seas [32], as well as along the coast

of Svalbard, and the Arctic Canadian and Norwegian coasts [32–34]. Notably, only two publications targeted Antarctic zooplankton communities, in the Ross Sea [35] and around the Antarctic Peninsula [36].

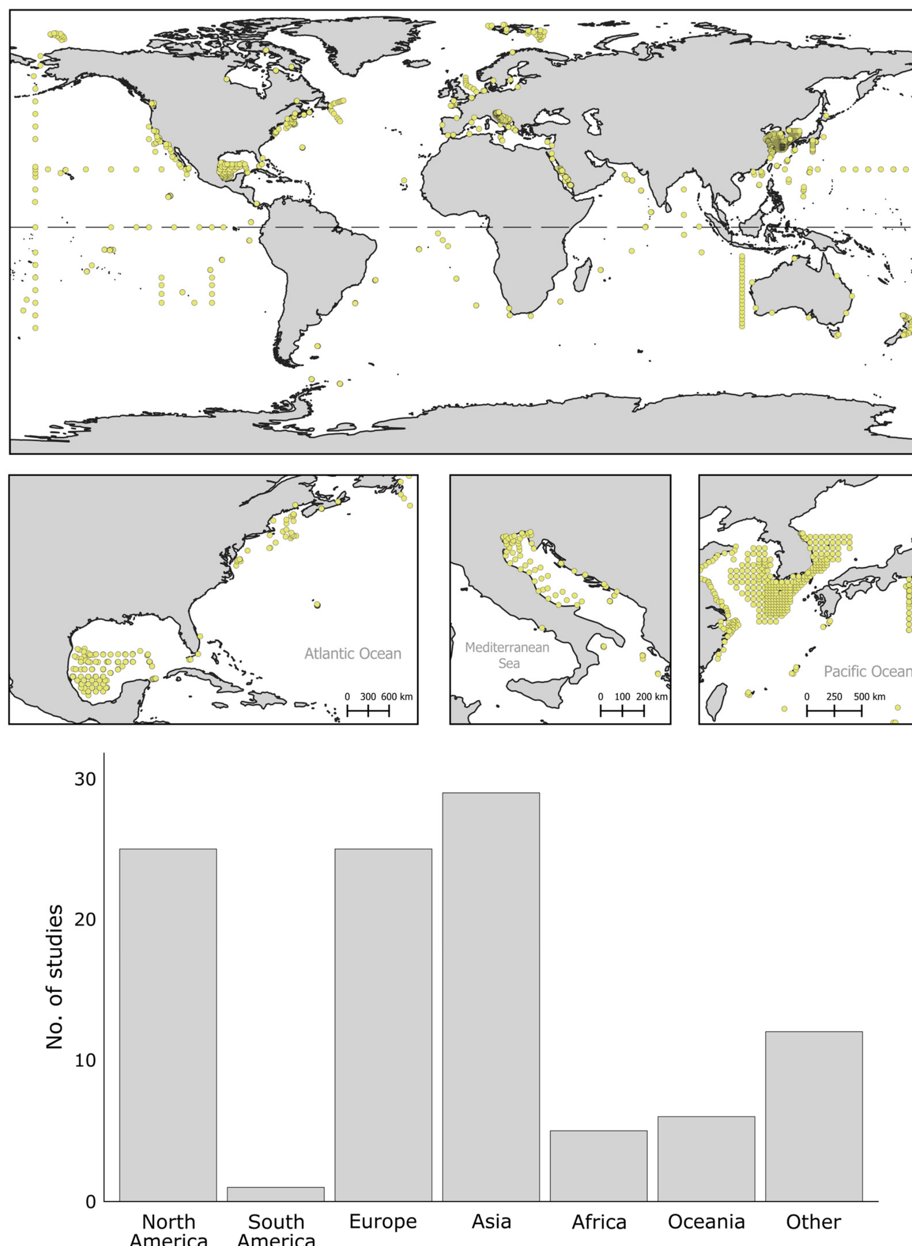

Figure 3. Sampling points from all publications reviewed and that have reported their coordinates (**top**). Sampling sites of several publications are not represented due to unavailable coordinates (for further details see Table S1, in Supplementary Material). Overall number of publications found for each geographic region employing DNA metabarcoding to assess zooplankton communities (**bottom**).

The geographical bias towards the northern hemisphere has been extensively documented in prior studies [37–39] in regard to the use of DNA-based tools in biodiversity monitoring. It has been noted that the dearth of research in the southern hemisphere is largely attributed to the socioeconomic constraints and inadequate infrastructural support for implementing DNA-based monitoring, particularly in the countries of the Global South, with African nations being notably affected [37,39].

4. Zooplankton Sampling Strategies

In zooplankton sampling, two main strategies emerge: the first involves the direct collection of water samples followed by subsequent filtration (either on-site or off-site), while the second entails direct sampling of targeted organisms through a sampling device, such as a plankton net to concentrate zooplankton biomass—a filtering-associated method [40].

Sampling water offers enhanced control and reliability concerning the volume and depth of samples collected along with a reduced risk of potential contamination from the surrounding water column levels. Furthermore, during water filtration, the monitoring for clogging becomes more manageable [40]. However, the volume of water sampled is significantly restricted compared to the filtering-associated methods [9]. Conversely, the latter method allows for the direct filtration of planktonic organisms from the water column through larger volumes of water and can be easily deployed from vessels of various sizes [40,41]. Each approach offers distinct advantages over the others. Nevertheless, the direct filtration of zooplankton from the water column emerges as the preferred choice due to its potential for greater cost-effectiveness, logistical efficiency, and time saving factors [41]. Indeed, most of the reviewed publications use either simple plankton nets (SPNs; n = 78) or multiple sample instruments (MSIs; n = 15). While net hauls enable greater vertical or horizontal water column integration in zooplankton sampling [42], thereby enhancing the likelihood of capturing rare and low abundance taxa [43], the water sampling methods ensure reliable sampling at deeper levels of the water column (as demonstrated in [44–46]). In addition, these methods offer a more efficient means of capturing small-sized taxa often overlooked by net hauling due to the fluid disturbance [47]. These factors appear to have influenced the selection of either Niskin bottles (n = 5) or CTD apparatus (n = 2) for water sampling in the remaining studies (Figure 4). Furthermore, water pumping consists of an alternative approach for sampling plankton (n = 4) (also conventionally used for morphology-based studies) [40]. Similar to water sampling with CTD and Niskin bottles, it allows the discreet collection of water at specific depths, but with the advantage of collecting larger volumes compared to volume-limited containers (e.g., bottles). Additionally, when coupled with a net, the pumped water can be filtered in situ [48,49], potentially offering logistical advantages over methods such as Niskin bottles and CTD. However, such approach is usually depth-limited (since it is considerably costly to pump water at extreme depths) and constricted to collecting micro- to mesozooplankton [40].

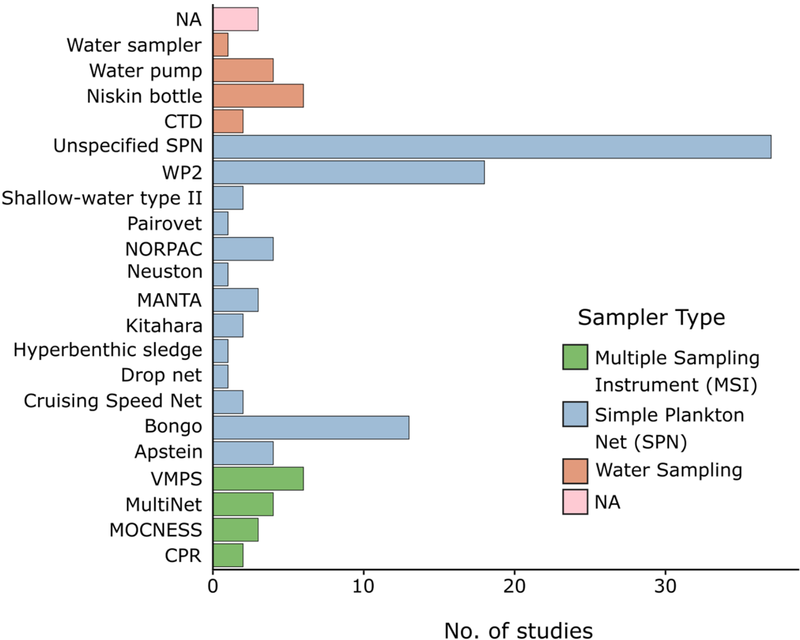

Figure 4. Number of publications using each type of sampler type for sampling marine and brackish zooplankton communities for DNA metabarcoding analysis.

The use of Niskin bottles in conjunction with SPNs have been reported to facilitate the sampling of larger eukaryotic zooplankton (>200 μm), while the Niskin bottles allowed for the capture of smaller-size fractions (ranging from 0.22 to 20 μm and 20 to 200 μm) [44,45]. Depth was not a limiting factor, as it was determined by the sampling location; therefore, vertical stratification of communities was not relevant to the study's aims. Instead, the focus was on characterizing the broad size range of zooplankton communities. On the other hand, only a single Niskin-specific publication reported to have collected depth-specific samples [50]. Similarly, publications that have reported the use of CTD apparatus also aimed to assess the effect of the sampling depth [47]. For instance, Qihang et al. [47] used CTD rosettes to collect 40–50 L of water at various depths. However, all the water samples underwent pre-filtration during the sampling processing, restricting the sampling of organisms smaller than the filter mesh size (200 μm). Conversely, Sun et al. [46] provided a vague description of their water sampling methodology, mentioning the use of a CTD instrument without specifying the water sampling equipment employed, and only noting the volume of water sampled (8 L). The reports on water pumping have also shown that it is not typically associated with a specific depth spectrum, other than for sampling surface or near-surface water [49,51,52]. The only exception was a water pump on board a subaquatic vehicle, which sampled pelagic larvae near the near-bottom abyssal plain [48].

A broader range of plankton nets has been documented for zooplankton sampling in DNA metabarcoding studies (Figure 4). In this study, we classified these approaches into two groups: Simple Plankton Nets (SPNs) and Multiple Opening-Closing Nets with Environmental Sensing Systems or Multiple Sampling Instruments (MSIs), following the categorization outlined in the Zooplankton Methodology Manual by the ICES [40]. However, for simplification, we have consolidated the MSIs into a single category in this review. While SPNs have emerged as the preferred method for sampling zooplankton communities, the selection of sampling methods is influenced not only by the technical advantages but also by logistical, funding, and time-related considerations. Across the 78 publications reviewed, twelve specific types of SPNs have been identified. However, many studies using SPNs employ simplistic designs and often did not specify the exact type of plankton net used (Figure 4). Instead, they commonly refer to it using generic terms such as "net", "standard plankton net", "plankton net", "traditional plankton net", or "zooplankton net" or provided descriptions on the size or shape of the net or mouth of the net (e.g., [53–57]). In a few studies (e.g., [58]), the authors opted to provide a more general description, referring to "zooplankton tows" without specifying the use of a particular net or plankton net (for a comprehensive overview, see Table S2 in the Supplementary Material).

Among those studies that did specify the use of a plankton net for sampling zooplankton communities, the WP2 net was the most employed, accounting for approximately 23.1% of the SPNs, followed by Bongo nets constituting around 16.7% of the SPNs. Other options were mentioned in 1–4 publications (Figure 4). Additionally, the reviewed literature identified four distinct MSIs, with a more even distribution of usage, as each method was reported once to thrice, indicating the absence of a clear trend.

The diverse array of plankton nets available in today's market is a response to a wide spectrum of sampling challenges that have been addressed for over a century. These challenges encompass factors such as mesh size, the water volume, the need for precise net closure timing, depth measurement during sampling, tow speed, and the prevention of organism avoidance and escapement [59]. The recent years have witnessed the introduction of newer models aimed at addressing the specific needs. For example, a portable Cruising Speed Net (CSN) has been developed [60,61] to offer a more accessible and efficient method for sampling the surface plankton assemblages without causing damage or introducing bias, particularly at higher cruising speeds (approximately 5 knots).

The WP2, named after the Working Party 2 in Zooplankton Sampling [59], was developed to establish standardized size-specific field equipment for sampling the upper 200 m layer. Since its inception, the WP2 has gained widespread adoption, particularly in Europe [40,62]. In contrast, the Bongo net, originally introduced 57 years ago [63] and later

redesigned [64], was specifically designed to sample with an unobstructed mouth opening, thereby minimizing the avoidance-related biases. Consequently, it has become the primary equipment used in Northwest Atlantic continental shelf surveys [62]. Thus, we suggest that the prevailing use of WP2 and Bongo nets for sampling marine and coastal zooplankton communities in metabarcoding-based analyses may be attributed to a disproportionate global focus. This is evident from the higher number of publications originating from these regions (see Figure 3). However, it is worth noting that another region-specific standardized plankton net—the North Pacific Standard net (NORPAC; [65])—has been described in the literature for sampling zooplankton (Figure 4). Nonetheless, NORPAC nets have only been mentioned in a series of three widespread zooplankton surveys conducted in the Pacific Ocean [66–68], and one small-scale study along the northern coast of Japan [69].

Overall, MSIs facilitate a more stratified and systematic collection of multiple plankton samples throughout the entire water column. Among the MSI systems reviewed in the literature, all reported enabled successive sampling of plankton from various depths in the water column. The Multiple Opening/Closing Net and Environmental Sensing System (MOCNESS) offers a method for sampling across the horizontal and oblique profiles of the water column [40]. In a comparative study conducted in Norwegian fjords, MOCNESS demonstrated superior performance in zooplankton biomass collection across the entire sampled depth spectrum, expected for lower depths (0–100 m), where MultiNet and even WP2 retained similar to or greater zooplankton biomass [70]. Both MOCNESS and MultiNet samplers offer exceptional flexibility in sampling planktonic communities allowing for vertical, horizontal, or oblique sampling. However, MultiNet excels specifically in vertical sampling and is typically lighter than MOCNESS. In contrast, the Vertical Multiple-Opening Plankton Sampler (VMPS) shares a similar sampling concept, but is tailored for vertical profiling [71]. From the literature reviewed here, the Continuous Plankton Recorder (CPR) stands out as a particularly unique MSI. It is employed for horizontal water surface profiling at higher speeds, reaching up to 25 knots, and uses a wounding silk mechanism to filter the incoming water (with 10 cm corresponding approximately to 10 nautical miles) [40]. This instrument has been integral to zooplankton surveys for nearly a century, enabling high-speed tows from any vessel across extensive transects. Notably, it offers the advantage of being deployable on commercial or private ships without requiring specific funding for scientific expeditions. However, it faces limitations, including the lack of calibration against more commonly used samplers [72] and the inherent issue of the low abundance achieved, as much of the water passes through the sampler mesh [73–75]. This limitation is particularly relevant for DNA metabarcoding analysis, as it may lead to an under-representation of the plankton species.

Despite the efforts to tailor sampling methods to enhance performance, none are universally suitable for a broad range of zooplankton sizes, spanning from mesozooplankton to macrozooplankton [70]. However, certain studies have highlighted the varying performance among samplers in specific circumstances [9,60,70,73,74]. The implementation of multiple complementary samplers would indeed be an ideal approach for the comprehensive monitoring of marine and brackish zooplankton communities. However, such an approach often faces logistical, resource, time, and funding constraints. Despite these challenges, several reviewed publications have described the use of multiple types of samplers, such as Niskin bottles coupled with a SPN [44,45]; water pumps and SPNs [49]; or multiple SPN deployments [36,56,57,60,69,76–78]. Additionally, the combinations of SPN with MSI have also been employed in a few studies [66,79,80] (further details can be found in Table S2; Supplementary Material).

It has been highlighted that the mesh size stands out as one of the most critical factors influencing the biomass and species composition of zooplankton communities, directly impacting the sample quality [70]. Other factors such as towing speed, patchiness, and avoidance also play significant roles [9,41,59,60]. In fact, the use of a 150 µm mesh net has previously been recommended for coastal zones with neritic zooplankton [70], underscoring the importance of the mesh size selection in the sampling methodologies.

Indeed, this further supports the requirement for the assessment of its influence, more particularly when samplers of the same type are to be deployed under similar conditions, always with sampler flexibility, logistical effort, and the associated costs in mind, for more reliability in long-term monitoring efforts comparison [81]. Nevertheless, it is relevant to note that sampler choice—and the associated mesh size—are overall highly reliant on the study's aim, the conditions of the study area, the available resources, the targeted taxa, and the sampling design, e.g., either day or night and vertical, horizontal, or oblique tows [76,82].

In regard to mesh sizes, it has been observed that a significant preference exists for larger pore dimensions when collecting zooplankton for DNA metabarcoding analysis. Specifically, a 200 µm mesh has been the most used (n = 25), followed by 500 µm (n = 9). The majority of the employed SPNs featured a mesh size ranging from 20 to 200 µm, accounting for approximately 83.3% of the cases. Conversely, SPNs with mesh sizes exceeding 200 µm were described in approximately 25.6% of instances. In the case of MSIs that have been documented, the smallest mesh size reported was 62 µm [42], although in certain instances, larger dimensions were employed, reaching up to 333 µm [83] (Figure 5). Nevertheless, the frequent reliance on 200 µm mesh size nets has been found to frequently underestimate the small copepods within the mesozooplankton community. Small-sized meshes are often prone to clogging due to the accumulation of debris. Therefore, meshes greater than 100 µm have been recommended for more efficient operation, particularly in environments where planktonic debris is less abundant in the water column [84,85]. Conversely, water sampling techniques commonly implement small-sized meshes, as evidenced by all the publications reporting meshes ranging from 0.2 to 20 µm (Figure 5). Nonetheless, due to the limited sampled water volume, mesh clogging poses a greater limitation to both SPNs and MSIs.

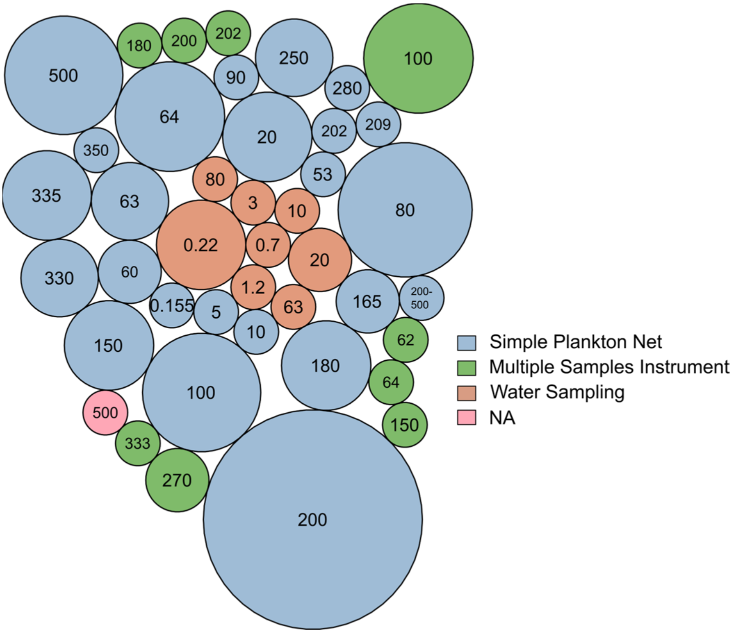

Figure 5. Proportion of the reported mesh size (in µm) for sampling naturally occurring zooplankton communities' studies using DNA metabarcoding. Bubble size is proportional to the number that each method/mesh size has been reported in the publications analyzed. For further details see Table S2 (Supplementary Material).

5. Preservation of Zooplankton Samples

The preservation of zooplankton samples with formalin, traditionally employed for morphological identification, is not recommended for DNA-based taxonomic characterization due to its known tendency to alter and degrade DNA [86,87]. However, a recently

developed protocol has been published, suggesting that it may be possible to effectively extract DNA from formalin-fixed plankton samples for DNA metabarcoding purposes [88].

Conversely, ethanol has emerged as the preferred choice for preserving the genetic material from plankton samples prior to DNA extraction, as indicated in ca. 60.2% of the reviewed publications (Figure 6A; also referenced in [87]). Ethanol serves as a cell dehydrator, safeguarding the DNA from degradation by precipitating proteins, which may otherwise contribute to the genetic content breakdown. However, its acidic nature poses challenges when the preservation of morphology is imperative, especially for calcifying organisms. Nonetheless, when combined with ammonium hydroxide, ethanol has been demonstrated to yield consistent results in sequenced richness, community structure, and composition, while effectively preserving the calcifying structures [89,90].

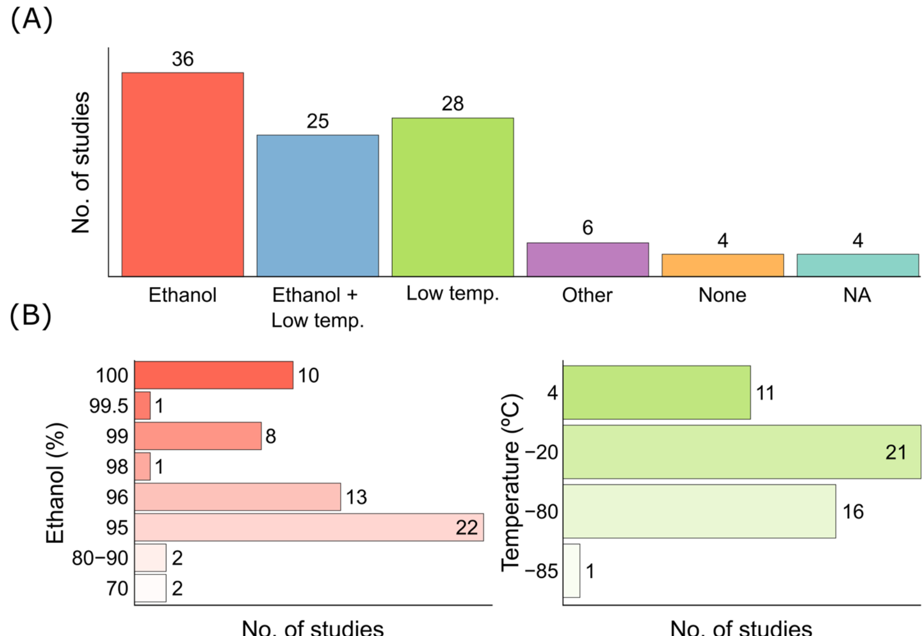

Figure 6. Methodologies used for preservation of zooplankton samples prior to DNA extraction (**A**). The "other" category includes methodologies with a singular study reporting its employment (e.g., the combination of ethanol with ammonium hydroxide (NH_4OH), the combination of RNA later with sub-zero temperature, and the use of guanidinium thiocyanate buffer, Longmire's lysis buffer, and DESS). Detailed insights on the ethanol solutions of different percentages and temperature treatments employed in zooplankton samples preservation (**B**).

High-ethanol-content solutions have been overwhelmingly favored overall [87,91,92], with concentrations of 95% or higher being employed for sample preservation in 55 publications (Figure 6B). On the other hand, the ethanol concentration in preservative solutions has been found to significantly affect the preservation of DNA, for instance, in insects [89]. Moreover, the effects of the ethanol % on effective DNA preservation may vary depending on the species [89,92]. Indeed, a lower ethanol content (as low as 70%) has been reported, which, according to Stein et al. [92], may not affect the sequencing of the full COI barcode region. Still, it is crucial to replace the ethanol within the first 24 h of sample preservation, as the water content expelled from the cells dilutes the ethanol, reducing its concentration and compromising its preservation effectiveness. Approximately 40.3% of the publications referring to ethanol-based preservation methods complemented it by storing the samples at low temperatures (Figure 6A). Specifically, sample storage at −20 °C has been the most adopted option, followed by 4 °C and −80 °C, with 15, 10, and 1 report, respectively. Around 21.4% of the publications reported to have opted to freeze or flash-freeze the zooplankton samples. In contrast to ethanol-based preservation, −80 °C (or lower) was the most common temperature choice for freezing, with 16 publications, compared to only 6

using −20 °C (Figure 6B). Additionally, few merely mentioned to have kept zooplankton samples on ice [93–96] or dry ice [97] until reaching the laboratory for further processing on the same day. Another temperature-based preservation method encompassed desiccating zooplankton samples with a heat treatment [98], displaying OTU clustering results comparable to those obtained via flash freezing. Similarly, using zooplankton mock communities, heat-drying treatment has been shown to result in DNA quantity, sequence depth, and recovered species richness very similar to those in regard to ethanol-preserved samples [32]. However, low-temperature conditions can be logistically limiting in some areas. Inherently, solely freezing zooplankton samples, particularly with non-flash-freezing temperatures, is followed by the potential risk of degradation occurring during the process of freezing and thawing, which could affect the sample integrity until the samples are fully (un)frozen.

RNALater also stands out as an effective preservative due to its ability to denature proteases and RNases, which are enzymes that degrade proteins and RNA, respectively. However, it has been noted to have a diminishing effect on the DNA content downstream [99], potentially explaining its limited mention in the reviewed literature. Brandão et al. [100] utilized Guanidinium Thiocyanate Buffer (GTB), known for its efficacy in lysing cells and denaturing nucleases, to preserve sieved zooplankton samples. The authors reported the efficient lysis of plankton samples stored at room temperature for a week using this method. Longmire's lysis buffer [101] was also reported to have been used to preserve zooplankton samples [34] and was found to be efficient for long-term preservation at room temperature [102,103]. Still, at room temperature, DNA may lose quality [104]. According to the reviewed publications, this may have been mitigated by also freezing the zooplankton samples at −20 °C. Additionally, Geller et al. [23,24] opted to preserve samples with DESS (salt-saturated DMSO buffers containing EDTA), which has been extensively employed for preserving other taxa (e.g., meiofauna) [87,105].

Four publications did not report any method of sample preservation. Two documented in situ sample processing immediately after collection [61,106], while the remaining indicated that the samples were transported to the laboratory, implying no preservation of the zooplankton samples [107,108]. Certainly, conducting in situ sample processing can be considered the most preferential approach, as it minimizes the introduction of potential biases or contamination from preservatives. However, the transportation time between the collection site and the laboratory can be crucial, since DNA degradation may occur. Hence, under such conditions, it is recommended, at least, to use low temperatures (e.g., ice) to limit the putative degradation. Moreover, a small fraction of the reviewed publications did not report any preservation method or indicated the absence of one (approximately 3.9%). While infrequent, such omissions can potentially compromise the replicability of the results.

6. Zooplankton Sample Processing Prior to DNA Extraction

The processing of samples constitutes a pivotal step in the DNA metabarcoding pipeline, wherein the bulk zooplankton biomass is separated from the fluid matrix, whether it be a preservative or water. Hence, the chosen processing methodology can significantly impact the biodiversity recovery, especially concerning rare and low-abundant species [49]. For instance, a review of the environmental DNA (eDNA) pipelines for detecting non-indigenous species (NIS) highlighted that the pre-processing of samples can heavily influence the biodiversity reports [28]. However, no specific remarks have been made regarding its influence on zooplankton studies.

In our current review, we identified two primary processing approaches used for characterizing marine and coastal zooplankton through DNA metabarcoding: sample filtration (n = 50) and sample centrifugation (n = 29) (Figure 7). Both methodologies have been employed in one study [54], while three other publications chose alternative approaches for processing zooplankton samples: homogenization [42,109], decantation [58], and evaporation [110] (represented as "Other" in Figure 7). Still, approximately 16.5% of the publications (17 in total) did not provide descriptions on how the zooplankton samples

were processed before DNA extraction. Additionally, a smaller percentage, around 3%, reported that the samples were not processed, such as those preserved in GTB [100], or those that directly used 10 mL of the original samples for DNA extraction [111].

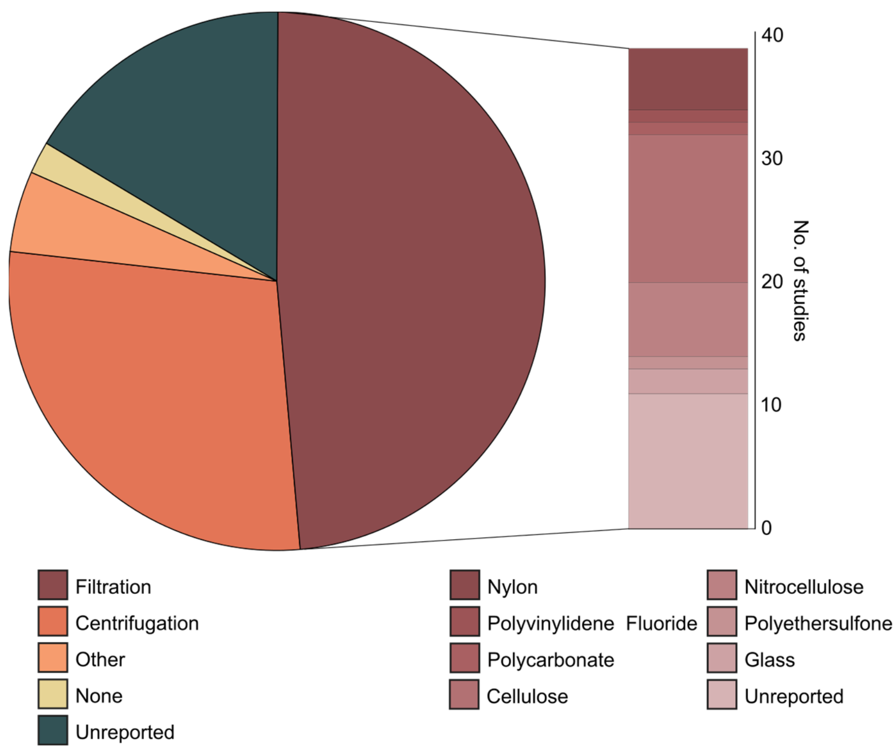

Figure 7. Main methodologies used for sample pre-processing before DNA extraction of zooplankton communities, e.g., for capture/concentration of zooplankton and ethanol/water excess removal. The "other" category included methodologies rarely reported (e.g., decantation, evaporation, homogenization, and the combination of both filtration and centrifugation). The outer layer illustrates the proportions of the filter's material type used for processing zooplankton samples through filtration.

Sample filtration facilitates the complete separation of both components by retaining the biological content within the entire sample volume. However, clogging can occur, especially with highly concentrated samples or when using filters with a small-sized mesh. Furthermore, small-sized taxa collected via SPN and MSI techniques may potentially pass through the filter during the filtration process, although the probability of occurrence might be low, when the mesh size employed is small. On the other hand, when the samples are centrifuged, the volume typically used is often much lower compared to what can be filtered. Alternatively, if similar volumes are processed, it may require multiple centrifugations of the same samples, which can be time consuming. Further steps are also usually involved, such as evaporation, especially with ethanol-based sample preservation methods. However, the risk of losing small-sized taxa is practically inexistent, though repeating centrifugation may increase the risk of cross-contamination. To the best of our knowledge, no study has been identified in the current review that comprehensively compares all the observed methodologies for processing zooplankton before the DNA metabarcoding analysis. Nevertheless, we acknowledge that each method may inherently introduce method-related biases. For example, time-consuming processes like filtration and evaporation (even after centrifugation) could potentially lead to DNA degradation.

Based on the literature that selected filtration as the method for processing zooplankton samples prior to DNA extraction, seven types of filter materials have been reported: nylon-based (n = 12), cellulose-based (n = 5), nitrocellulose-based (n = 1), polycarbonate-based (n = 6), polyvinylidene fluoride-based (n = 2), polyethersulfone-based (n = 1), and glass-based filters (n = 1). However, for most cases, no description has been provided regarding

the material of the filter used (Figure 7). To date, no evidence has been found regarding the influence of the filter's material on the recovered sequenced data through the DNA metabarcoding of zooplankton samples, which may explain the observed lack of reported information. However, such influence has been emphasized in an eDNA study, where cellulose-based filters were demonstrated to outperform glass fiber- and polycarbonate-based filters [112,113]. Indeed, there is a possibility that the material composition of the filters used for processing the samples could influence the downstream results. Further, research is imperative to thoroughly test this hypothesis and gain a deeper understanding of its potential impact.

7. DNA Extraction

Currently, there is a wide range of commercial kits available for DNA extraction, alongside numerous non-commercial protocols for extracting the genomic content from zooplankton samples [87]. However, the former has generally been preferred for the DNA extraction in the metabarcoding-based taxonomic characterization of complex marine and coastal zooplankton communities, accounting for approximately 84.5% of the cases, with non-commercialized protocols comprising around 15.5% (Figure 8). Similar trends have been observed for other taxa [28,38,87,113]. In our literature survey, we found a considerable diversity of non-commercialized protocols, but no specific trend toward any protocol. However, up to the present, the protocol from Corell and Rodríguez-Ezpeleta [114] has been employed three times for extracting the DNA from marine and coastal zooplankton [115–117], followed by a protocol from Aljanabi and Martinez [44,45,118] and Bucklin [80,119,120], and by those who developed their own protocols [77,121], each mentioned twice. On the other hand, the DNeasy Blood & Tissue Kit (Qiagen), the DNeasy PowerSoil DNA Isolation Kit (Qiagen/MoBio), and the E.Z.N.A. Mollusc DNA Kit (Omega Bio-Tek) were highly cited in the reviewed pipelines, in 26, 14, and 9 publications, respectively (Figure 8). Comparable results have been previously reported by van der Loos and Nijland [87], although the analysis encompassed plankton as a whole (phyto- and zooplankton). The prevalence of the DNeasy Blood & Tissue Kit has been consistently noted in previous studies [28,38,87,122] and further endorsed for the metabarcoding assessments of aquatic eukaryotes [123]. However, commercial kits have limitations such as restricted reagent volumes and optimization for a limited range of sample volumes [114], in addition to being generally more costly.

Furthermore, while the implementation of a single DNA extraction protocol in single studies has been a common practice, overall, some exceptions have been observed. For example, Abad et al. [44,45] described the use of a non-commercial protocol for the smallest-sized zooplankton, while using the DNeasy PowerSoil DNA Isolation Kit for the remaining samples. Similarly, Coguiec et al. [33] predominantly employed the E.Z.N.A. Mollusc DNA Kit for DNA extraction. However, when faced with issues related to DNA extraction kit availability, they resorted to using the DNeasy PowerSoil DNA Isolation Kit.

Undoubtedly, DNA extraction is one of the most critical steps throughout the entire metabarcoding pipeline. Its importance cannot be overstated as it lays the foundation for obtaining reliable results that accurately represent the sampled content. Indeed, this step in the metabarcoding workflow primarily influences, though not exclusively, the genomic content, quality, and overall purity of the extracted DNA. Given that metabarcoding relies on DNA for identification, the quality and quantity of the extracted DNA from planktonic metazoans are heavily dependent on the approach employed, which can greatly impact the performance of the resulting taxonomic screening of these highly intricate marine communities. Furthermore, low abundance and rare species are particularly vulnerable to these factors, as they may be subsequently underrepresented or even completely overlooked [124]. For instance, in the current review, only Coguiec et al. [33] reported a 44% decrease in diversity after changing from the E.Z.N.A. Mollusc DNA Kit to the DNeasy PowerSoil Kit. Conversely, Abad et al. (2016) acknowledged the potential introduction of a technical-related bias during the DNA extraction step, recognizing that the performance of

DNA extraction approaches can vary significantly among the different taxonomic groups. Furthermore, comparing the recovered biodiversity between the samples for which DNA was extracted using different protocols or kits should be approached with caution. Indeed, the DNA-based identification of multiple taxa within highly complex communities can be significantly influenced by the choice of DNA extraction kit, as observed in microbial communities [125].

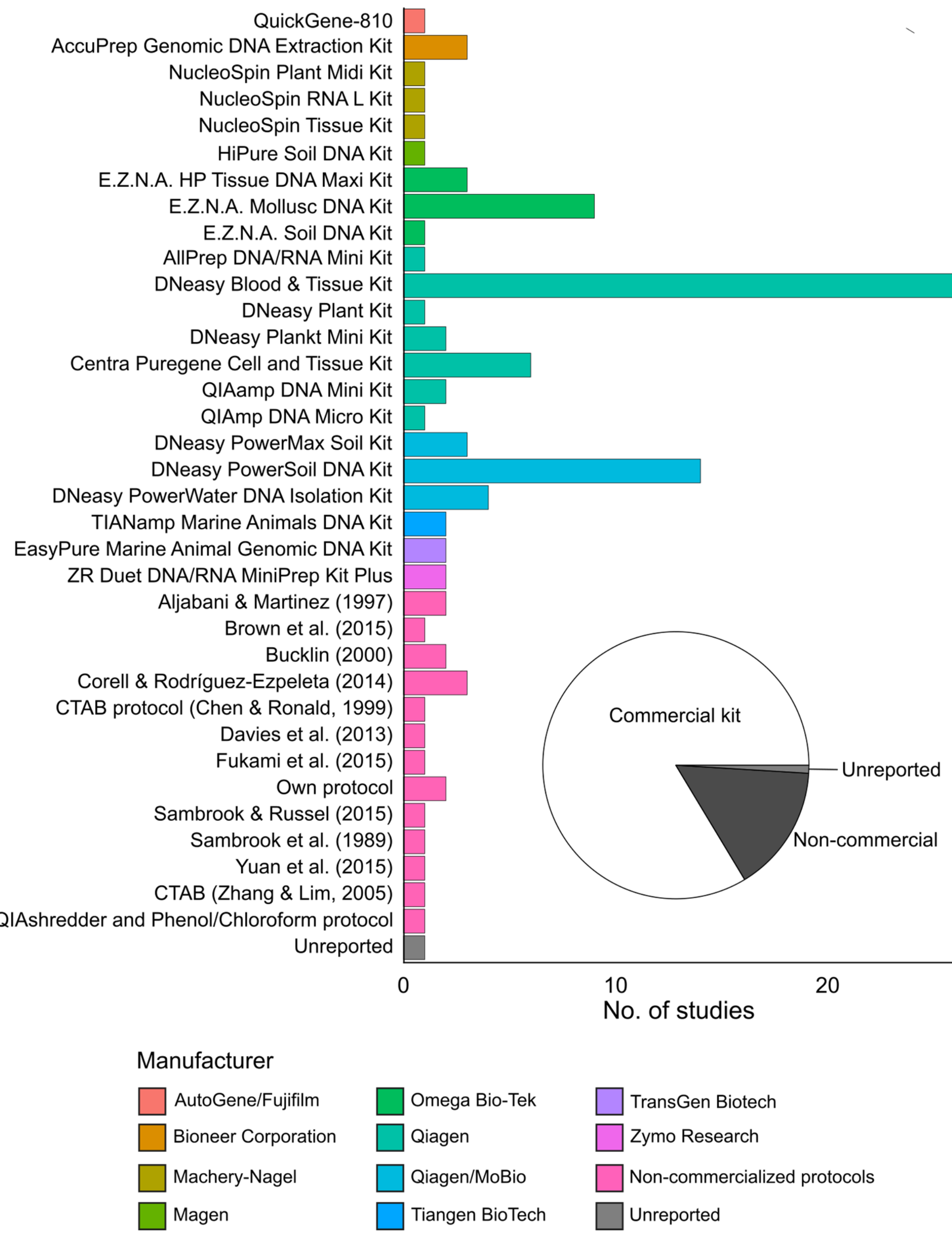

Figure 8. DNA extraction methods employed for assessing naturally occurring zooplankton communities through DNA metabarcoding, in marine and coastal ecosystems, encompassing both commercial (i.e., kits) and non-commercial protocols.

Nevertheless, commercial DNA extraction kits' market is rich and competitive; thus, the most efficient extraction kit is still debatable for zooplankton use. This highlights the need for the comparison of several kits, conducting a cost-effectiveness analysis (as pricing

may limit the DNA extraction in certain regions), and evaluating the non-commercial methods for the metabarcoding-based characterization of zooplankton communities.

8. Molecular Marker and Primer Choice

Undoubtedly, the choice of the genetic marker to be amplified and the primers to be employed are equally crucial factors to consider in the metabarcoding workflow. These decisions significantly influence the accuracy, specificity, and comprehensiveness of the taxonomic attribution process. Certainly, the targeted loci for amplification and sequencing should exhibit sufficient variability for interspecific discrimination, while also containing well-conserved sequences for primer binding [91]. This balance ensures both accurate taxonomic identification and efficient PCR amplification.

Indeed, several molecular markers, known as DNA barcodes, have been extensively discussed over the years to standardize the taxonomic identification across various taxonomic groups [126,127]. Among these, the mitochondrial cytochrome c oxidase subunit I (COI, or CO1, or COX1; hereafter referred to as COI) gene, with a length of 658 base pairs, has been designated as the standard DNA barcode for the DNA-based identification of metazoans [17,128]. It has been widely used in global projects and initiatives, such as the International Barcode of Life (iBOL) consortium. Indeed, other mitochondrial markers have been employed for taxonomic identifications and phylogenetic assessments, such as the 12S and 16S rRNA genes. However, their use for broad taxonomic identification has been hindered by the prevalence of indels (insertions and deletions), which greatly limit the sequence alignment [129]. Nonetheless, they have still been used for the identification of specific taxonomic groups, such as fishes and hydrozoans [58,130,131].

Indeed, COI displays several advantages, including uniparental inheritance, high mutation rates, a large number of sequences in reference databases, a lack of recombination and introns, and a low incidence of indels [132–135]. These features have facilitated species-wide identifications and contributed to its high representativity in databases and reference sequences [17,136,137]. However, despite COI's widespread use as the standardized marker for the universal identification of metazoans, other molecular markers have been proposed and recommended. These alternatives address issues such as the difficulties in primer design for COI (primer affinity), the presence of pseudogenes, the inability to discriminate recently diverged species, hybrids, and highly genetically conserved taxa, as well as instances of the biparental inheritance of mitochondrial DNA [111,138–140]. The recommended alternatives include various regions of the nuclear 18S and 28S rRNA genes, as well as the mitochondrial 12S and 16S rRNA genes, cytochrome B, ND6, and ND4 (Figure 9). Each of these markers offers unique advantages and may be more suitable for specific taxonomic groups or research objectives.

To date, the 18S rRNA gene (18S) has emerged as the dominant molecular marker in the metabarcoding-based screening of marine and coastal zooplankton communities, with approximately 61.2% of the publications reporting its use, followed closely by COI (approximately 54.4%). This trend is consistent with the previous findings [87,141,142]. Despite COI being standardized for metazoan identification, the 18S gene has historically been employed for characterizing marine microbial eukaryotes due to its moderate to high specificity to zooplankton, as well as its broad species coverage and capability for species identification [143–145]. Indeed, while the 18S rRNA gene benefits from an extensive reference database, it has been found to be too conservative for efficient species-level discrimination [31,146]. Consequently, several other molecular markers and inter-loci regions have been explored, but none have been used more than once, except for the mitochondrial 16S rRNA (16S) and the nuclear 28S rRNA (28S) genes (Figure 9). These markers have demonstrated greater taxa-specific recovery, making them valuable alternatives in metabarcoding studies. For example, the 16S rRNA gene has been particularly used for the specific detection of fishes and crustaceans [109,143,147], as well as for the detection of NIS, including mollusks, arthropods, bryozoans, and ascidians [58]. Additionally, Berry et al. [147] targeted a sequence for the universal identification of zooplankton taxa using

the 16S marker. Further, Kim et al. [131] targeted two regions of the 12S rRNA gene and developed a primer targeting a 12S–16S segment specifically designed for fish species identification. Furthermore, in another study, Kim et al. [148] devised a primer targeting a sequence segment between cytochrome B and ND4 for cephalopod-specific detection. Additionally, the D2 region of the 28S rRNA gene has been widely employed for characterizing Copepoda assemblages [66,68,149–154], although Tang et al. [146] observed it to be considerably more conserved. This conservation may contribute to underestimating the species richness within a community. These studies highlight the versatility and utility of the different molecular markers for the taxonomic identification in metabarcoding research.

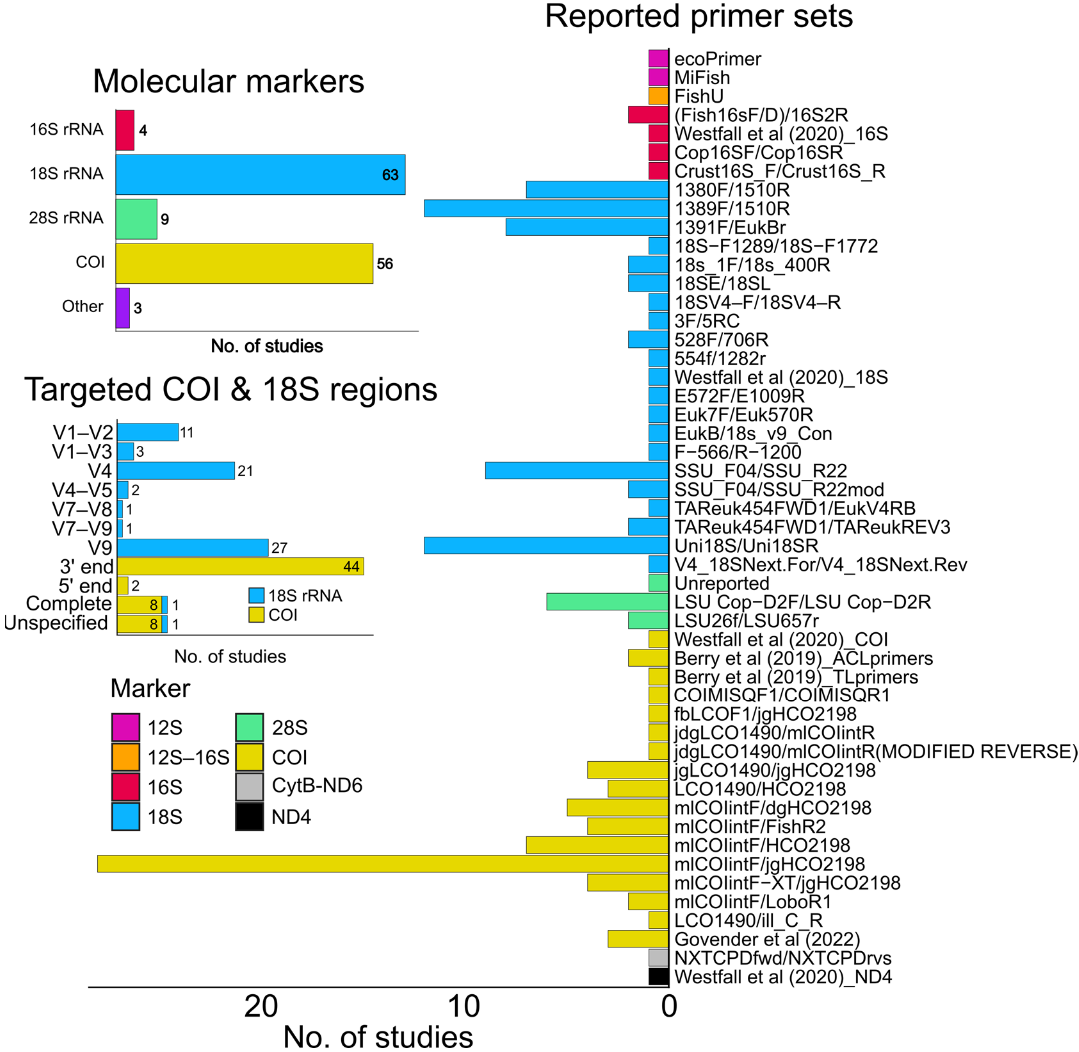

Figure 9. Molecular markers, targeted regions, and respective primers employed to assess naturally occurring zooplankton communities through DNA metabarcoding in marine and coastal ecosystems. Further details on the sequenced 18S and COI loci are also illustrated.

The complete sequencing of COI and 18S rRNA genes in marine and coastal zooplankton screening has been notably scarce, with only eight and one publications reporting their use, respectively. Instead, there has been a prevalent use of region-specific amplification and sequencing for both markers (Figure 9). Various hypervariable regions of the 18S rRNA gene have been targeted, with primers amplifying both the V9 and V4 regions being the most adopted, accounting for approximately 73% of the 18S reported publications (n = 27 and 21, respectively). Additionally, around 17.5% of the 18S publications have focused on the V1–V2 region. Other hypervariable regions were also found to have been

targeted to characterize zooplankton communities: V1–V3 [96,109,147], V4–V5 [155,156], V7–V8 [48], and V7–V9 [69]. Despite the diverse selection of hypervariable regions, they all seem to exhibit high nucleotide divergence, rendering them potentially suitable options for species-level identifications [31,157]. However, V9 stands out for its established length (around 150 bp) and the abundance of available data in reference databases.

However, it is worth noting that both the V4 and V1–V2 regions, which have been frequently sequenced, demonstrate a higher degree of consistency in the primers used during the amplification step. For the V4 region, the Uni18SF/Uni18SR primers (400–600 bp) [14] have been predominantly selected, accounting for approximately 57.1% of the V4-based publications. Conversely, for the amplification of the V1–V2 region, the SSU_F04/SSU_R22 primers (approximately 450 bp) [158] have been exclusively used, with the exception of two studies which employed a modified version of these primers [47,50].

In the first stages of Illumina technology, sequencing was limited to the V9 region due to its small size (approximately 150 bp) [159]. This limitation likely explains the still considerable number of reports of using this region. However, once the technology was improved and the sequencing of larger DNA fragments was possible [160], this opened doors for sequencing other 18S regions, such as V4 and V1–V2 (both around 450 bp) and its employment as a region of interest for targeting zooplankton communities.

The COI-3′ mini-barcode region has been dominant among the most targeted fragments, primarily due to the widespread use of primers such as mICOlintF/jgHCO2198 [161,162], which account for approximately 65.9% of the publications focusing on the 3′ region. Additionally, the reverse primer, HCO2198 [163], has been used to a lesser extent, in approximately 22.7% of the publications. It is worth mentioning that the degenerate version of the HCO2198 reverse primer (dgHCO2198) has also been considerably used (five publications). These primers amplify shorter fragments than the full barcode region, facilitating full-length sequencing with high-throughput sequencing techniques [91,162].

The preference for the COI-3′ mini-barcode region may stem from its superior performance across a broad range of metazoan phylogenetic diversity [162]. This region enables the identification of a greater number of exclusive genera/species compared to the COI-5′ region [164]. Despite the high variability of COI, which makes it a suitable molecular marker for species-level discrimination, identifying regions that are conservative enough for the design of universal primers suitable for DNA metabarcoding, across a wide range of taxonomic groups, remains challenging [165,166]. Nevertheless, several publications have reported the development and use of newly designed taxa-specific COI primers [58,78,109]. These customized primers have been employed, for example, to enhance the detection of NIS [58]. Additionally, they have been designed for the specific identification of cnidarians, copepods, and mollusks [109,147], or prawns, shrimps, and crabs, in zooplankton samples [56,78,167].

In the realm of metabarcoding studies, the significance of employing multiple molecular markers has been widely emphasized, particularly in the examination of zooplankton communities. Our review underscores the common adoption of a multi-marker approach, with approximately 27.2% of the publications using more than one molecular marker (further details are available in Table S3 of the Supplementary Material). Indeed, employing taxa-specific primers targeting the same marker concurrently with universal primers may present a viable approach.

9. Sequencing Platforms

A total of seven sequencing platforms have been used for assessing marine and coastal zooplankton communities through DNA metabarcoding (Figure 10). Illumina MiSeq has emerged as the most adopted platform; however, this has not always been the case, as sequencing platforms have evolved over time. Prior to 2019, pyrosequencing with Roche 454 dominated the number of reported publications. However, following its discontinuation, in mid-2016, Illumina MiSeq has progressively become the preferred choice, a similar trend noted by Santoferrara [168]. The preference for both, MiSeq and

pyrosequencing, likely stemmed from their superior read accuracy in microbiological communities, with the added advantage of generating longer reads to some extent [93,169].

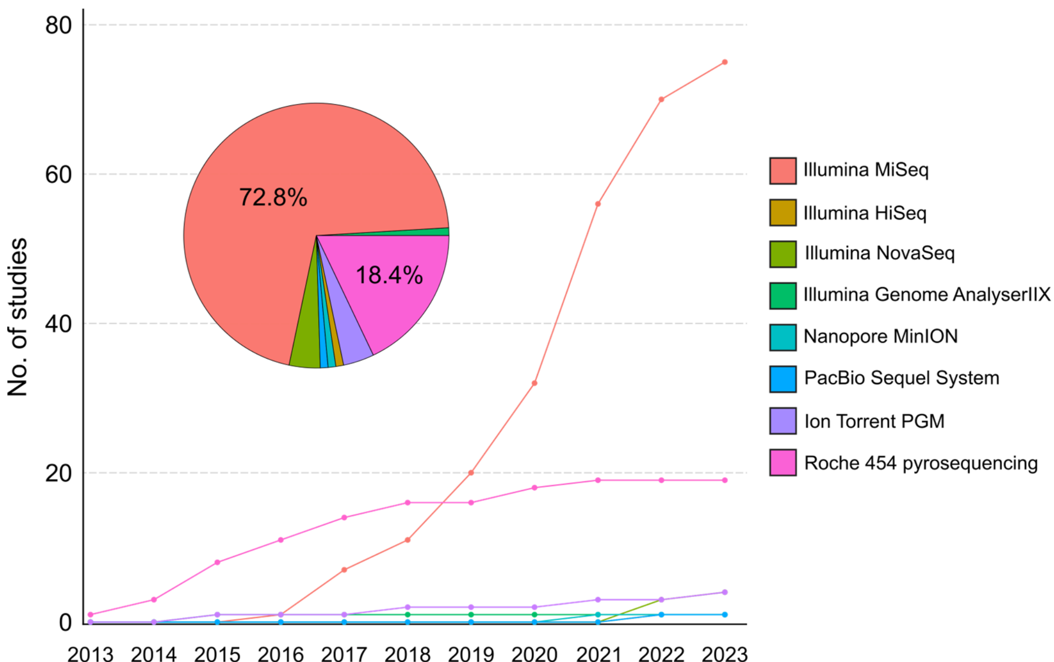

Figure 10. Sequencing platforms used for assessing naturally occurring zooplankton communities through DNA metabarcoding in marine and coastal ecosystems. Both proportions (pie chart) and cumulative number of reports in the last decade (line plot) are illustrated. All sequencing platforms are similarly color-coded as represented on the right.

MiSeq operates on a sequencing-by-synthesis (SBS) mechanism, employing fluorescently labeled reversible terminator nucleotides. These nucleotides are applied to clonally amplified DNA templates fixed on the surface of a flow cell. On the other hand, PGM also utilizes the SBS method, but with a different approach. DNA templates are first clonally amplified by emulsion PCR on the surface of microbeads, akin to the process in 454 pyrosequencing [93]. Illumina MiSeq is recognized for its higher potential throughput capability, but not for its sequencing speed, an area where PGM performs better [169,170]. Indeed, for other biological groups, such as bacterial mock communities using the 16S rRNA gene, both sequencing platforms generally showed good agreement [171–173]. Similar conclusions were drawn with arthropod mock communities, where the sequencing platforms did not significantly influence the species recovery. However, MiSeq was noted to provide better-quality sequences [173].

The most recent addition to Illumina's sequencer lineup reported for marine and coastal zooplankton metabarcoding is the NovaSeq 6000, also using an SBS approach. Four recent publications have documented its usage [42,47,50,95]. However, no direct comparison has been made with previous sequencers. Nonetheless, NovaSeq has demonstrated significantly greater sequencing depths, reaching up to a 700× coverage, resulting in the recovery of more diversity compared to MiSeq. Even when compared at similar sequencing depths, NovaSeq has consistently outperformed MiSeq in regard to diversity recovery, possibly attributed to the improved hardware, processing software, and flow cell technology [174].

Indeed, SBS technology has emerged as the dominant force in the market, providing practical solutions for achieving ultra-high sequencing depth and pair-end sequencing of short- and mid-sized amplicons, reaching lengths of up to 500–600 base pairs. However, this approach is generally PCR-dependent, which contributes to the increased sequencing costs and time consumption. On the other hand, single-molecule real-time sequencing

(SMRT), developed by Pacific Biosciences, emerged to address the several limitations of the second-generation technology. Further details can be found in Kchouk et al. [175] and Hu et al. [176]. In summary, this approach is based on the natural process of DNA replication, using phospholinked nucleotides that, once incorporated during sequencing, release fluorophores. The release of these fluorophores is then detected and used to identify the individual bases [177]. The implementation of SMRT sequencing coupled with nanopore technology formed an approach that has demonstrated superior results compared to its predecessor. Overall, MinION (Oxford Nanopore) uses an engineered protein as a pore (nano-scaled pore—nanopore), embedded in an electrically resistant membrane, immersed in an ionic solution. Once voltage is applied, ionic current passes through the nanopore, and as a molecule moves through the pore, it causes shifts in the ionic current in a detectable way. These shifts are detected by sensors, enabling the real-time identification of the molecule, or in this case, the DNA base [177,178]. This advancement represents a significant leap forward in sequencing technology, enabling researchers to conduct sequencing experiments with unprecedented speed (usually not requiring PCR amplification), accuracy, and efficiency (both cost and timewise). For further information, see Hu et al. [176].

The surveyed literature has revealed that only two studies have been published using a third-generation sequencer to assess marine zooplankton communities via metabarcoding, employing the MinION (Nanopore) platform [155], and the PacBio Sequel System (Pacific Biosciences) [35]. While no direct comparisons to more traditional next-generation sequencing platforms were made, the authors discussed both the strengths and limitations of MinION sequencing. For instance, they noted that only 40% of the reads were considered to be of high quality for analysis, a finding comparable to other nanopore studies but potentially lower than the standard sequencing approaches [155]. However, bioinformatic pipelines were shown to be able to compensate for the considerable high error-rate associated with nanopore sequencing [179–181]. Indeed, a recent direct comparison between MiSeq and MinION was performed, using zooplankton samples, which depicted much more acceptable error rates from the latter (around 4%), while achieving a similar zooplankton composition to MiSeq sequencing, and showing that nanopore sequencing is capable of generating indel-free results [182]. Indeed, since the introduction of MinION to the market, in 2014, the error rate has been the main barrier to its wide adoption. However, several versions have been released with improvements in pore chemistry and subsequently accuracy. In fact, more recent versions apparently have been reported to provide >95% accuracy [177]. Additionally, the portable nature of the MinION platform makes it ideal for remote surveys, such as those conducted in open waters to assess zooplankton communities, while providing fast results [182]. On the other hand, in Lee et al. [35], the PacBio platform was used alongside Illumina MiSeq, achieving a comparable species-level identification % of pooled reads. However, some taxa-specific limitations were noted, particularly for small-sized organisms like Copepoda. Still, the plankton net mesh size may have played a role, as discussed by the authors. Nevertheless, it is imperative to use mock communities for comparison to assess the potential taxa-specific biases introduced by the sequencing platform. Additionally, PacBio sequencing was noted to be more costly and is therefore recommended for the development of DNA reference databases, while MiSeq sequencing is better suited for ecological surveys [35], or monitoring purposes.

Up to today, no study has been found comparing the two most recent sequencing platforms—NovaSeq and MinION—using marine and coastal zooplankton communities. However, a recent metabarcoding study targeting terrestrial invertebrates (and vertebrates) already performed such comparison, where, although the number of resulting reads varied considerably between the sequencers, the recovered biodiversity was concurrent [183].

10. Sequence Clustering Algorithms and Taxonomic Assignment

The bioinformatics pipelines involved in the processing and taxonomic assignment of zooplankton sequences were not the focus of this review. However, the key steps in

these pipelines, particularly clustering and taxonomic assignment, are highly relevant to the topic. While sequence data processing is imperative for cleaning the dataset of noise (e.g., low-quality sequences, sequencing errors, chimeras, among others), the strategies used for sequence clustering and taxonomic assignment are crucial factors that most influence the depiction of zooplankton diversity.

Most of the reviewed literature reported clustering zooplankton sequences into molecular OTUs (MOTUs, hereafter referred to as OTUs), accounting for approximately 78.6%. This approach has traditionally been used in the DNA metabarcoding assessments across several taxa. Clustering molecular OTUs allows for the translation of these clusters into taxonomically meaningful units in metabarcoding-based community analysis, serving as a proxy for species. Additionally, clustering can function as an error-filtering step by grouping "noise-sequences" with error-free sequences [184]. Over the years, several algorithms have been developed, each employing different approaches, such as relying on global sequence similarities or on the maximum number of differences between reads (network-based clustering) [26,185]. For metazoans, species-level OTU clustering is generally considered the default, with a 97% sequence similarity threshold [162], though this can vary depending on the context or targeted taxa. Indeed, several of the reviewed publications used clustering with a 3% dissimilarity threshold (e.g., [23–25,31,49]). However, OTU analysis can be prone to bias, as genetically similar species (<3%) may cluster together [143,186]. In fact, 18S rRNA has been found to be highly conserved, meaning that OTUs often do not correspond to different species. Alternatively, clustering sequences into ASVs (100% similarity threshold) can help mitigate this issue by providing higher discrimination power. In the reviewed literature, two multimarker-based studies opted to process COI and 18S reads differently—using OTUs for COI and ASVs for 18S, respectively [47,90]. Further, the ASV analysis of COI can further resolve intra-specific variation (haplotypes). However, the use of OTUs versus ASVs remains debated, as the choice may depend on the specific goals of the study [57].

The taxonomic assignment in the reviewed literature was assessed based on three parameters: method/algorithm of identification, DNA reference databases, and taxonomy cross-reference. Two main methods of sequence taxonomic assignment were considered: alignment-based approaches, such as BLAST [187], and sequence composition-based approaches, such as "Wang's method"—the Naïve Bayesian classifier [26,188]. In general, the alignment-based approaches assign taxonomy by aligning reads against the DNA reference database, while the sequence composition-based methods do not require any alignment. Instead, the sequence composition-based methods use machine learning techniques, requiring a training dataset—typically the DNA reference database—from which sequence compositions are learned. Once trained, the classifier can assign taxonomy based on the most probable match. Due to the inherent dependency on database completeness and the lack of direct comparison in sequence composition-based approaches, these methods may be more sensitive to database patchiness [189]. Overall, most of the studies employed an alignment-based classification of the resulting reads, ca. 75.7%, compared to 37.9% for the sequence composition-based approaches. Two publications reported using both methods. One used a sequence composition-based approach (scikit-learn classifier) for 18S and BLAST for COI [190], while the other employed machine learning-based methods for classifying both 18S and COI, but also used BLAST for COI classification [106]. Thirteen databases were reported as references for the taxonomic classification of zooplankton sequences. One case did not report the use of the taxonomic assignment approach or DNA reference database, as no classification was required, and the analysis was performed at the OTU-level [49]. The NCBI (GenBank; www.ncbi.nlm.nih.gov/genbank/), SILVA (www.arb-silva.de/), and BOLD systems (v4.boldsystems.org/) were the most commonly used reference databases, cited in 56, 25, and 14 studies, respectively. PR2 followed with eight mentions. However, several studies (approximately 21.4%) opted to develop custom databases based on the existing sequence data from other DNA reference databases for the specific purpose of their study. Only one publication reported the development of a custom database for the

purpose of taxonomic assignment, in which original sequences were also included [78]. For further details, see Table 1. Finally, no information was found regarding taxonomy check/cross-referencing—or any indication that it was performed—in most of the reviewed publications (n = 80). Those that did cross-reference the assigned taxonomy displayed a preference for the World Register of Marine Species (WoRMS; www.marinespecies.org), cited in approximately 15.5% of the studies. Other sources were mentioned only once or twice, including BOLD, SeaLife Base (www.sealifebase.se), the Belgian Register of Marine Species (BeRMS; www.marinespecies.org/berms/), or various literature sources.

Table 1. DNA reference databases used for the taxonomy assignment of marine and brackish zooplankton sequences.

Database	Seq Source	No. of Studies	Reference
NCBI GenBank	NA	56	-
SILVA	EMBL database	25	[191]
BOLD Systems	NA	14	[192]
PR2	NCBI GenBank EMBL database WGS-EMBL	8	[193]
MIDORI	NCBI GenBank	6	
CO-ARBitrator	NCBI GenBank	3	[194]
MLML COI DB	Private	3	[24,25,194]
MZGdb	NCBI GenBank BOLD Systems	3	[195]
V9_PR2 (and V2)	PR2 (18S V9) SILVA (prok. 16S)	3 (1)	[36,98]
DUFA-Leray	NCBI GenBank BOLD Systems	2	[32,33]
ArCop	NCBI GenBank BOLD Systems	1	[31]
SilvaMod	SILVA	1	[196]
StreamCode	-	1	[57]
Custom	NCBI GenBank BOLD Systems SILVA PR2	22	-
None	-	1	-

11. Final Considerations

In the present review, although several protocols have been adopted through the metabarcoding workflow for characterizing the zooplankton communities in coastal marine ecosystems, we observed some major trends. Overall, these include the following: (i) the preservation of zooplankton samples in high ethanol-content solutions, which could or could not be accompanied by low-temperature storage; (ii) the use of commercial kits for the extraction of the genomic content, followed by (iii) amplification and sequencing of a single molecular marker—and subsequent primer set—through the MiSeq platform (Figure 1). Indeed, around one fourth of the publications (n = 25) here analyzed employed such protocol. However, there is a margin for the improvement to reach reliable and reproducible workflows for comprehensive monitoring. Indeed, three reports were considered in the present review, which provide a snapshot of the ongoing use of zooplankton communities for monitoring through taxonomic characterization using DNA metabarcoding. Overall, all the major trending approaches were employed, with the exception of preservatives, where an alternative to ethanol-based solutions, was chosen [23,25]. However, the use of ethanol-based preservatives appears to be the most consensus approach (Figure 6), provided that proper maintenance is ensured to maintain high ethanol concentrations [89,92]. Still, under certain conditions, alternatives such as GTB and DESS may also be considered [87,100,197,198]. Additionally, the use of multiple molecular markers (and in some cases multiple primer

sets) provides a more thorough detection of, e.g., low abundant species, or with low affinity or higher mutation rates in the primer binding regions. However, the study aims are a key factor to consider, as species-level identifications may not always be required. For assessments at the OTU or higher taxonomic levels, 18S rRNA genes may be a better option, as they are already widely tested and commonly used (Figure 9). The choice of the targeted region within the molecular marker is also imperative in a thorough zooplankton characterization. For instance, no agreement has been yet made for 18S. Indeed, V1, V2, V4, and V9 display similar high nucleotide divergence, which is appreciated for taxonomic identification [31,157], although the latter has been overall depicted to outperform the other regions in species discrimination, but not to a degree of exclusive implementation [199]. Furthermore, Illumina MiSeq has been the trending sequencing platform throughout zooplankton metabarcoding assessments.

Nevertheless, while DNA-based monitoring has reached a stage that provides reliable assessments of overlooked and cryptic biodiversity in planktonic metazoans for the detailed monitoring of marine and coastal ecosystems, a lack of standardization has been observed overall. Additionally, several trending approaches have been documented, most of which lack substantial support. For instance, no significant influence has been described on how zooplankton samples are processed before DNA extraction. Indeed, the processing of large samples (number and size wise) may be time consuming when centrifuging and be more prone to putative cross-contamination. Filtration itself is dependent on the filter mesh size, which can result in clogging with large volumes or highly turbid/concentrated samples, potentially leading to further DNA degradation. Additionally, while the filter material used for eDNA samples has been discussed as an influential step in the metabarcoding pipeline [112,113], no similar effort has been observed regarding bulk samples, including zooplankton. Although, theoretically, no significant influence is expected (since the targeted genetic material does not interact with the filter in the same way as environmental DNA), we recommend further investigation to determine if different filter materials have downstream effects on the recovered biodiversity or DNA quality of zooplankton.

12. Future Directions

Although consistent workflows have been identified throughout the reviewed literature for the characterization and assessment of complex, naturally occurring marine and brackish zooplankton communities, a deeper understanding of each step in the entire pipeline (from sampling to sequences analyses) is still needed. Hence, we recommend that future investigations focus on the following: (i) the sampler choice and mesh size for effective and comprehensive DNA metabarcoding-based monitoring—even though such can be highly dependent on the project's aim and target taxa [81], the assessment of the whole spectrum of the zooplankton community may be valuable for, e.g., ecological status assessment, NIS detection, among others; (ii) comprehensive assessment of the preservative alternatives and potential cross-comparison of the resulting data between the different approaches; (iii) a similar assessment to that described in (ii) is also recommended for zooplankton sample processing, particularly the reliability of omitting this step, e.g., as described in Brandão et al. [100] (Figure 7); (iv) compare different DNA extraction methods from zooplankton samples—indeed, a wide range of taxa constitutes metazoan plankton communities; therefore, assessing the effectiveness and reliability of the data generated from different kits/protocols is crucial; (v) regarding the targeted genetic markers, the reports observed herein (Figure 9) may provide a basis for selecting the most appropriate. However, it is recommended to conduct preliminary assessments to determine the best strategy for monitoring surveys—whether to use universal primers and/or taxa-specific, target specific genetic regions or the entire marker, and evaluate the inter-comparability of the resulting data; (vi) while some consensus has emerged in favor of sequencing zooplankton DNA extracts using Illumina MiSeq (Figure 10), considerable effort is still needed to assess the reliability and cost-effectiveness of the new third-generation sequencing platforms (e.g., MinION and PacBio) and NovaSeq [35,42,47,50,95,155]; and

(vii) finally, the bioinformatics pipelines still require significant refinement, particularly in assessing inter-pipeline comparability. It is worth noting that, although the present review provides an analysis of the state-of-the-art DNA metabarcoding of marine and coastal zooplankton samples, several recommendations, may also be considered for freshwater zooplankton communities.

To conclude, while a minority of studies omitted the descriptions of certain steps (Figure 1) and Supporting Information, we advocate for future studies to clearly outline the methodologies employed. Only through such transparency can we improve the replicability of the protocols and work towards achieving standardization across local, regional, and even global monitoring efforts focused on marine and zooplankton communities.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/jmse12112093/s1, Table S1: Detailed information on the sampled sites of the reviewed literature in the present study; Table S2: Description of the sampler used for collecting marine/brackish zooplankton for DNA metabarcoding analysis, according to the reviewed literature in the present study; Table S3: Details of the molecular marker, loci, and primer used by each reviewed study. (*) All the COI regions described represent the mini-barcode regions.

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