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Review

An Overview of DNA-Based Applications for the Assessment of Benthic Macroinvertebrates Biodiversity in Mediterranean Aquatic Ecosystems

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Abstract: The loss of aquatic biodiversity is increasing at a rapid rate globally. There is a worldwide effort to protect, preserve and restore aquatic ecosystems. For efficient biodiversity monitoring and reliable management tools, comprehensive biodiversity data are required. The abundance and species diversity of benthic macroinvertebrates are commonly used as indicators of the aquatic ecosystem condition. Currently, macroinvertebrate species biodiversity assessment is based on morpho-taxonomy, which could be enhanced by recent advances in DNA-based tools for species identification. In particular, DNA metabarcoding has the potential to identify simultaneously many different taxa in a pool of species and to improve aquatic biomonitoring significantly, especially for indicator species. This review is focused on the current state of DNA-based aquatic biomonitoring using benthic macroinvertebrates in the Mediterranean region.

Keywords: aquatic ecosystems; biomonitoring; eDNA; macroinvertebrates; Mediterranean ecoregion; DNA metabarcoding

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1. The Importance of Aquatic Benthic Macroinvertebrates as Ecological Indicators

The global biodiversity is decreasing regularly, affecting the ecosystems stability, sustainability, and several functions [1,2]. This decrease can be both due to human activities and environmental changes. For monitoring the biodiversity loss, it is first necessary to evaluate the species distributions and population sizes on a relevant ecological scale [3]. Aquatic ecosystems, including freshwater, transitional, and marine environments, are going through declines in biodiversity at rates greater than those in terrestrial ecosystems [4,5]. For this reason, many legislative framework directives are trying to be implemented in an effort to record and preserve the aquatic biodiversity and improve the ecosystems' quality status. It is critical that we improve the effectiveness of these strategies to halt or reverse the biodiversity loss [3,6,7]. In Europe, two of these Directives are the Water Framework Directive (European Commission, Directive 2000/60/EC—WFD) and the Marine Strategy Framework Directive (European Commission, Directive 2008/56/EC—MSFD).

Bioindicators include natural species that respond to environmental changes and can be used to assess the health status of ecosystems [8]. Benthic aquatic macroinvertebrates are widely used as indicators of ecosystems health [9–11] because they have limited mobility, perform their life cycles in water and respond to anthropogenic pressures. In fact, they cannot escape the pollution and are able to show the effects of different stressors over time [12–14]. Due to their long life cycle and a large number of species and phylogenetically diverse, they have always been suitable candidates for biomonitoring, either based on the presence or absence of certain species or on the numerical or taxonomic abundance [8]. Currently, macroinvertebrate species biodiversity assessments are mainly based on morpho-taxonomy.

Macroinvertebrates Biodiversity Assessment in Aquatic Mediterranean Ecosystems

Macroinvertebrates biodiversity assessment studies with conventional methods have several times been applied in Mediterranean ecosystems for evaluating the ecological status of aquatic ecosystems.

The Mediterranean Basin is one of the most significant biodiversity "hotspots" in the world [15] with a remarkable amount of endemic species [16]. It is estimated that the Mediterranean Sea is inhabited by more than 8500 species of macroscopic marine organisms, which corresponds to between 4% and 18% of the world marine species [17]. However, it is essential to mention that all the Mediterranean ecosystems are under different kinds of pressures, such as degradation, species loss due to human activity [16] and introduction of non-indigenous species [18] which are aggravated by a lack of adequate legislation. The conservation value of the Mediterranean Basin is recognized worldwide [15,16] and the conservation efforts could be facilitated with the use of new biodiversity assessment tools.

The ecological status of small Mediterranean rivers using benthic macroinvertebrates and macrophytes as indicators has been evaluated [19]. The ecological status of Mediterranean streams of the Balearic Islands, using an Invertebrate Multimetric index (INVMIB) has been successfully assessed [20].

The response of several macroinvertebrate metrics along perturbation in order to develop a multimetric index have been developed for ecological assessment of Mediterranean flatland ponds. The index included five measures and was able to differentiate the ecosystems condition with more than 86% efficacy [21]. In Italy a study focusing on macroinvertebrate diversity and conservation status of Mediterranean ponds was conducted analyzing the variation in macroinvertebrate community structure within and among ponds [22]. A study conducted in a Mediterranean lagoon investigated the adequacy of simplified sampling procedures, based on the selection of large body-size macroinvertebrates, for evaluating the ecological status of the ecosystem. It was proved that, the larger body-size macroinvertebrates seem to be advantageous for accurate, rapid and cost effective biomonitoring [11]. The macroinvertebrates sampling effort on ecological descriptors and indicators in perturbed and unperturbed conditions of Mediterranean transitional waters and streams have also been investigated [11,23].

However, aquatic macroinvertebrates morphological identification, especially at the species-level resolution, could be challenging because some immature stages lack necessary diagnostic features [24,25]. In addition, macroinvertebrates present high diversity, in comparison with other groups of species, making the identification effort time and cost-consuming [26]. DNA-based applications are emerging as an alternative or complementary tool for the study of biodiversity [27,28]. Over the last few years, research on molecular tools has progressed and indicated that their use can yield a finer taxonomic resolution and more accurate, cost-effective, and rapid aquatic macroinvertebrate species' biomonitoring, in comparison to the traditional morphology-based monitoring [27,29,30].

2. DNA-Based Applications for Aquatic Macroinvertebrate Species Identification in Mediterranean Ecosystems

Molecular approaches include DNA-barcoding, DNA-metabarcoding and eDNA-metabarcoding and are very promising for the study of biodiversity in order to complement morphology-based identifications. DNA-barcoding refers to the amplification and sequencing of a short genomic DNA fragment of a species in order to assign a species-specific molecular barcode. The most commonly used DNA barcode region for benthic macroinvertebrates is the gene that codifies for subunit I of the mitochondrial cytochrome c oxidase enzyme (COI) [31].

DNA metabarcoding provides species identification by DNA barcodes from a pool of organisms simultaneously. In fact, in DNA metabarcoding bulk samples are used and sequence data for standard taxonomic marker genes are generated via high-throughput sequencing [32]. Environmental DNA is referring to DNA metabarcoding of an environmental sample, such as soil, sediment or water. It seems like a promising tool for conservation projects and biomonitoring applied on aquatic ecosystems, since it can also detect the DNA that originates from feces, saliva, urine and skin cells of animals existing or visiting the water body [33].

Different studies compared the morphology-based and metabarcoding-based approaches for marine invertebrates monitoring [29,34]. A DNA metabarcoding protocol for marine littoral hard-bottom communities characterization was applied in two National Parks in Spain (one in the Atlantic Ocean and another in the Mediterranean Sea). Two molecular markers were used, COI and V7 18S. COI yielded higher taxonomic resolution, while 18S was not recommended for species-level resolution. The researchers embrace the use of DNA metabarcoding for marine benthic communities, with some adjustments, as for example the molecular marker that was found here to be more successful [35]. Environmental DNA metabarcoding has been applied in two hard-bottom communities of NW Mediterranean, by sampling water at increasing distances from these communities and then comparing them with the findings of direct sampling methods and DNA metabarcoding. Both methods could differentiate the two communities. The presence of benthic DNA in the water column confirmed that eDNA is sensitive enough to detect ecological differences. However, only a small portion of the marine benthic communities was detected in the water samples, especially a bit further from the benthic habitat [36].

In the North Adriatic Sea eDNA metabarcoding was applied for assessing the impact of three offshore gas platforms targeting benthic and planktonic eukaryotes with five different markers (18S V1V2, 18S V4, 18S 37F, 16S and COI). At close proximity from the platforms, biodiversity changes due to the platforms activities and perturbations were observed. In particular, AMBI index values, based on the occurrence of benthic macroinvertebrates' species, were the highest at the closest stations to the platforms, which indicate that these stations were the most subject to several stressors. The ribosomal V4 marker yielded more taxonomically assigned OTUs in water samples in comparison to the mitochondrial and foraminiferal ones [37].

2.1. The Primers Bias in Metabarcoding Applications for Aquatic Macroinvertebrates

Finding the primer pairs and PCR conditions that most accurately recover the organisms present in an environmental sample is important for the applicability of metabarcoding in biomonitoring.

Different pairs of primers and different gene regions lead to different results in terms of species identification in a pool of DNA [38]. Choosing the appropriate primer set is essential, because otherwise low DNA concentrations or even entire taxa may not be detected, leading to false-negatives [39]. The barcode coverage of the Barcode of Life Data Systems (BOLD) database on marine and freshwater macroinvertebrates is considerably limited [40]. However, since most of the existing barcoded macroinvertebrates are presented with a COI barcode, most of the barcoding studies target this specific mitochondrial region. The universal primers pair LCO1490/HCO2198, which was firstly introduced by Folmer et al. [41], has been extensively used for macroinvertebrates barcoding.

Afterwards, different primer pairs targeting only the mtDNA COI region of metazoans are tested and the mlCOIintF primer combined with the jgHCO2198 reverse primer had higher success rates in DNA metabarcoding studies because they amplify a shorter fragment useful in next generation sequencing [38].

Moreover, it has been verified, both in silico and in vitro, that high primers degeneracy leads to obtain the DNA amplification of larger number of taxa [28,42]. The BF/BR COI metabarcoding primers for freshwater macroinvertebrates detected more taxa than the standard "Folmer" barcoding primers [42]. The BF/BR primers pair is designed for long amplicons of freshwater macroinvertebrates, but this might fail when targeting samples of highly degraded DNA [43]. On this purpose, the primers pair fwh1/fwh2 have been developed [43] and a reverse primer called EPTDr2n, with 3′-specificity towards macrozoobenthic taxa, was useful for eDNA studies targeting macroinvertebrates [44]. This primer has been evaluated in vitro and silico using PrimerMiner. PrimerMiner is an R package, which can be used for primers' evaluation and manual design or in combination with bioinformatic tools for primer development [45].

2.2. The Completeness State of DNA Barcode Databases for Aquatic Macroinvertebrate Species

The success of molecular approaches (DNA-barcoding and metabarcoding) for macroinvertebrate species identification using the COI gene as a marker relies on the completeness of the reference libraries [18,46–48]. The two big public and widely used barcode reference libraries are BOLD and the NCBI GenBank [49]. Until now there is a significant amount of sequences in the databases, but they still need improvements and supplementations. GenBank is much larger, found to contain more than 212 million sequences until 2019, however we can only consider GenBank as a sequence repository, while BOLD can also act as a curation tool [50,51]. Besides, until today no European DNA barcode reference database for aquatic indicators exists [52]. In addition, databases require stricter standards and quality control, including the motivation for data providers and managers to better curate their data after the initial release [30]. A trend that has recently raised and seems very promising for dealing with this issue is the gap-analysis surveys [53,54] and reference libraries construction on a local scale, for example on a regional or ecosystem scale [55]. Regarding aquatic benthic macroinvertebrates, a list of 1565 species of the Mediterranean Apulia Region was examined by Specchia et al. [54] for the gap analysis of COI barcode sequence in the principal reference libraries, named BOLD Systems and GenBank. A DNA barcode was available for 58% of the listed species and the phyla with the larger gaps in the reference libraries were Nematoda, Entoprocta and Porifera [54] and Figure 1.

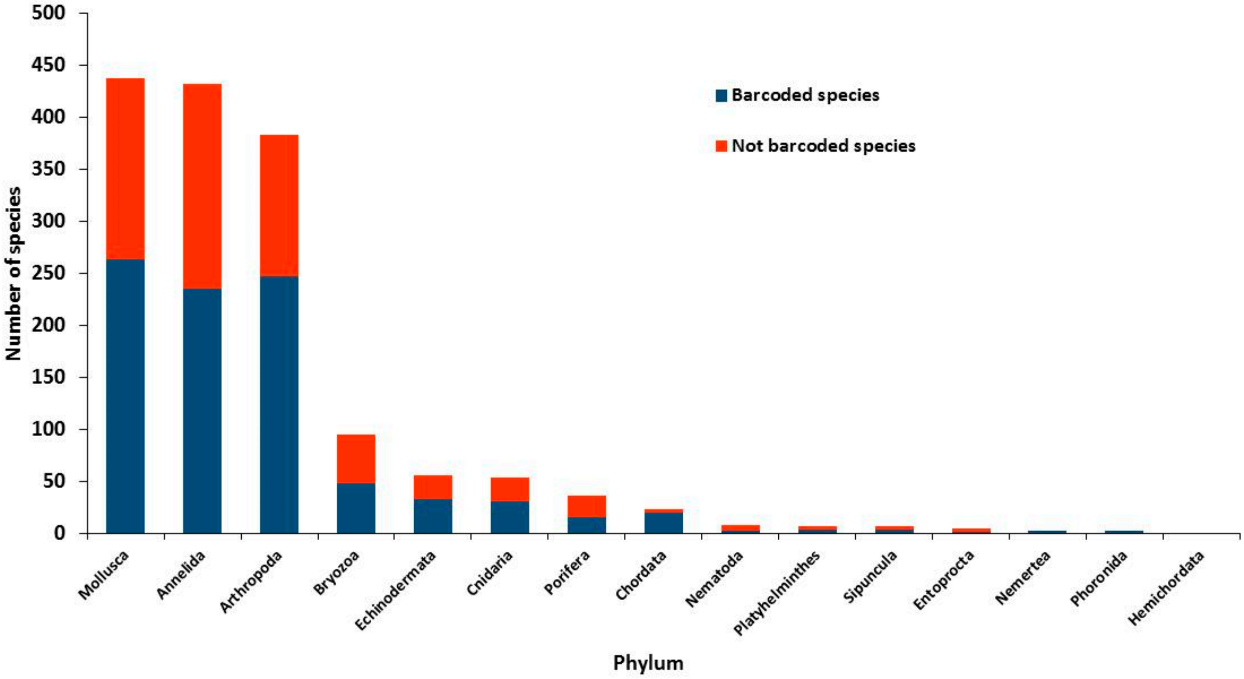

Figure 1. The number of barcoded and not barcoded macroinvertebrate species for each phylum retrieved from the aquatic ecosystems of Apulia Region (Southeast of Italy).

The gap analysis studies are very important to support the real applicability of metabarcoding in a given region at present and to direct experimental efforts towards obtaining the barcode of specific taxa.

3. eDNA Metabarcoding Application for Detection of Invasive Macroinvertebrate Species in Mediterranean Ecosystems

Metabarcoding approach can be an effective method for the detection and the identification of macroinvertebrate species at low densities, such as rare, endangered, threatened and invasive species [56,57]. Specifically, the precise understanding of invasive species distribution is a key requirement for conservation management [58]. Invasive species are characterized by a rapid adaptation to new environments, affecting biodiversity and ecosystem functioning, as well as ecosystem services and human well-being, like agriculture and fisheries [59]. In general, biological records are important to document the presence of both alien and native species that are rare or recently arrived, and eDNA can become a beneficial tool for monitoring biological invasions [60,61]. Identifying invasive macroinvertebrate species in early life stages and at low densities is very important for tracking, controlling and eradicating invasions [33,62–64]. The applicability of DNA-based methods was confirmed for invertebrate non-indigenous species detection in the Mediterranean Sea. Marine invertebrates collected from the Bay of Biscay (Atlantic Ocean) and the Gulf of Lion (Mediterranean Sea) were barcoded and 19.3% of them were non-indigenous species [65]. The application of a simplified eDNA protocol in the Bay of Biscay revealed three invasive invertebrate species [66].

In addition, in some instances, COI variation can be analyzed to reveal the geographic source region and pathway of invasion [47]. Inferentially, molecular genetics can be crucial for defining cryptogenic and invasive species, giving us the opportunity to detect them before entering the taxonomic hierarchy.

4. Discussion and Conclusions

Metabarcoding as a complement to the traditional species identification methods presents great potential for application in aquatic ecosystems biomonitoring surveys. Especially the incorporation of the non-invasive eDNA method in conservation and biodiversity assessment efforts seems valuable. Yet, the absolute application of these tools for studying the macroinvertebrate dynamics in different Mediterranean aquatic ecosystems requires a certain level of optimization and standardization. The eDNA method currently has some limitations. In this regard, the potential failure of the eDNA metabarcoding method to distinguish between living and dead organisms, as well as not providing information on size and stage of development should be considered [3]. Another issue that arises, especially in marine environments, is the eDNA decay. Persistence/decay is dependent on the source and state of the eDNA [67] and its interaction with the abiotic [68,69], biotic [70] and physical [71] properties of the environment. Persistence times are also dependent on the transport flows [72]. Riverine eDNA last for hours and is regularly transported, lake eDNA can last for a month or so, marine is much more variable as the transport dynamics are the most chaotic [73]. The persistence of eDNA is exactly the key point in biomonitoring and conservation efforts, as DNA fragments can be found even after a certain amount of time has elapsed. In particular, for many macroinvertebrate species, for which DNA extraction is difficult due to their chitinous exoskeleton and, consequently, the concentration of DNA extracted in metabarcoding is usually low, eDNA is a promising alternative.

Beyond the technical issues that appear, the unreliability of the existing reference databases must be overcome. Furthermore, the critical point of their application is to ensure that continued application of the current methodologies is used to draw increased reliability. Combating all these challenges will introduce a new era of rapid, efficient, and reliable environmental status and biodiversity assessment.

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