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eDNA Reveals the Associated Metazoan Diversity of Mediterranean Seagrass Sediments

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Abstract: Anthropogenic impacts on marine ecosystems have led to a decline of biodiversity across the oceans, threatening invaluable ecosystem services on which we depend. Ecological temporal data to track changes in diversity are relatively rare, and the few long-term datasets that exist often only date back a few decades or less. Here, we use eDNA taken from dated sediment cores to investigate changes over approximately the last 100 years of metazoan communities in native (*Cymodocea nodosa* and *Posidonia oceanica*) and exotic (*Halophila stipulacea)* seagrass meadows within the eastern Mediterranean Sea, at two locations in Greece and two in Cyprus. Overall, metazoan communities showed a high turnover of taxa during the past century, where losses of individual taxa in a seagrass meadow were compensated by the arrival of new taxa, probably due to the arrival of exotic species introduced in the Mediterranean Sea from the Suez Canal or the Gibraltar Strait. Specifically, bony fishes (Class Actinopteri) and soft corals (Class Anthozoa) presented significantly higher richness in the past (before the 1980s) than in the most recent time periods (from 1980–2017) and some Cnidarian orders were solely found in the past, whereas sponges and Calanoids (Class Hexanauplia), an order of copepods, showed an increase in richness since the 1980s. Moreover, the Phyla Porifera, Nematoda and the Classes Staurozoa, Hydrozoa and Ophiuroidea were detected in *P. oceanica* meadows but not in *C. nodosa* and *H. stipulacea*, which led to *P. oceanica* meadows having twice the richness of other seagrasses. The greater richness resulted from the more complex habitat provided by *P. oceanica*. The combination of eDNA and sediment cores allowed us to reconstruct temporal patterns of metazoan community diversity and provides a novel approach to follow natural communities back in time in the absence of time series and baseline data. The ongoing loss of *P. oceanica* meadows, likely to be compounded with future warming, might lead to a major loss of biodiversity and the replacement by other seagrass species, whether native or exotic, does not compensate for the loss.

Keywords: environmental DNA (eDNA); CO1; Mediterranean Sea; sediments; seagrass; metabarcoding; metazoan; non-native

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1. Introduction

Since the beginning of the Anthropocene—a new geological period in which human impacts drive massive changes across the world [1]—ecosystems are changing globally at unprecedented rates mainly due to climate change, habitat destruction, and pollution [2,3]. Species respond to global warming by phenological adjustments [4–7], shifts in distributional ranges [8], acclimation and adaptation, and, where these responses are insufficient, population decline and species loss or extinction [9]. Our understanding of the extent of changes in biodiversity is, however, often hindered by the lack of reliable baselines and insufficient time series [10].

Traditional monitoring surveys (usually combined with historical data) have been essential to follow certain species through time, informing our understanding of long-term ecological patterns and processes of natural communities [11,12]. Unfortunately, most monitoring schemes started a few decades ago [13], well after most anthropogenic pressures began impacting biodiversity and shorter than natural climate cycles, making it difficult to determine drivers of changes in communities through time [14]. The lack of baseline data about natural communities preceding the rise of anthropogenic activities limits our understanding of past ecosystem states and the full consequences of such pressures and, therefore, our ability to inform decisions to implement and assess the success of conservation programs [15]. Moreover, traditional monitoring surveys usually measure a specific taxonomic group, are localized in a small geographic area, mainly in developed countries, and can be invasive and time consuming [16,17]. They are also based on the taxonomic identification of species, which is dependent on specialized taxonomists and can misidentify species due to phenotypic plasticity, cryptic species, juvenile life stages, [18] and changes in observers over time [16]. Hence, conservation efforts are in need of additional techniques for long-term and large-scale monitoring.

Environmental DNA (eDNA) involving analysis of the DNA that organisms leave behind is rapidly gaining momentum as a non-invasive approach enabling the assessment of ecosystem biodiversity from environmental samples [19,20]. The DNA contained in theses samples (e.g., soil, water and air) is extracted, amplified by PCR, and sequenced using a metabarcoding approach. One of the challenges of this approach is to amplify a short region of DNA, typical for degraded environmental DNA, but with enough taxonomic resolution. The genetic material present in the environmental sample can then be identified by comparing the sequences obtained to those in a reference database [21].

In aquatic ecosystems, the assessment of eDNA along sediment cores dated with radioisotopes have the potential to retrieve records of past biodiversity [19,22]. Hence, lake and marine sediments can act as natural archives providing a sediment geochronology where each layer has an estimated date and a specific group of organisms (identified through eDNA), allowing the assessment of changes in the community through time in relation to changing pressures and management actions [23]. Several studies have already applied eDNA to reconstruct past events, such as the colonization route of America by humans after the last glacial maximum [24], community changes in coral reefs [25], shifts in the plant community after the introduction of rabbits [26], and changes in the carbon sequestration rate in Mediterranean seagrass meadows after the invasion of an exotic seagrass [27].

Indeed seagrass meadows rank among the most valuable ecosystems on Earth [28], supporting an elevated productivity, carbon sequestration capacity, improvement of water quality, sediment stabilization, and biodiversity, among other ecosystem services [29], but have experienced a worldwide decline due to human activities [30,31] recently aggravated by anthropogenic climate change [32,33]. The abundance and diversity of the fauna and flora living in seagrass meadows are characteristically higher than in adjacent unvegetated areas [34] because (1) the seagrass photosynthetic activity produces organic matter that is used as the initial energy source for the food web [34] and raises pH offering refuge for calcifying organisms [35] and (2) the complex architectural structure (leaf canopy and network of rhizomes and roots) provides hiding places to avoid predation and habitat suitable for many organisms [34]. Understanding the dynamics of seagrasses and how associated faunal communities within these habitats change through time is important to understand their response to environmental change and assist predictions about the future of these ecosystems, as well as the scope to rebuild biodiversity losses through active seagrass conservation and restoration. This is particularly important in the Mediterranean Sea, which is home to extensive seagrass meadows, including the endemic and most widespread seagrass *Posidonia oceanica* [36,37], the native *Cymodocea nodosa* which is increasing its distribution in some areas [38], as well as the exotic (Indo-Pacific origin) seagrass *Halophila stipulacea* which has been found mostly in the eastern Mediterranean since 1923 [39] but is expanding into the western Mediterranean [40–42] and is forecasted to continue doing so [43]. However, the Mediterranean is highly impacted by the urbanization of the littoral zone since the 1960s, eutrophication, the exploitation of living resources, high warming rates (0.65 °C and 0.25 °C decade^{-1} in the western and eastern Mediterranean), and the rapid spread of exotic species arriving from the Atlantic though the Gibraltar Strait or from the Indo-Pacific Ocean through the Suez Canal, resulting in an extensive loss of coastal habitats [32,44,45]. Because the intensity of all these pressures is increasing, an assessment of long-term changes of the seagrass associated macrofaunal communities is needed to better inform management actions.

Here, we use environmental DNA retrieved from dated sediment cores in the eastern Mediterranean to (1) explore temporal changes in the seagrass-associated metazoan community through time and (2) compare changes in the metazoan communities across *P. oceanica*, *C. nodosa,* and *H. stipulacea* meadows. We do so by evaluating extracellular eDNA extracted from seagrass sediment cores dated with ^{210}Pb [27] from the eastern Mediterranean Sea (two sites in Greece and two sites in Cyprus) and assess metazoans from marine sediments using primer pairs targeting a section of the Cytochrome Oydase 1 (CO1) gene.

2. Methods

2.1. Sediment Sampling

We sampled 12 sediment cores from 8 seagrass meadows (4 *Halophila stipulacea*, 3 *Cymodocea nodosa*, and 1 *Posidonia oceanica*) from the eastern Mediterranean Sea. From Kalami (Greece), Maridati (Greece), and Limassol (Cyprus) we collected two sediment cores (PVC cylinders of 60 cm length and 9 cm diameter) of the *H. stipulacea* meadow and one core of the native *C. nodosa* and from Port (Cyprus) we collected two sediment cores of the *H. stipulacea* meadow and one core of the *P. oceanica* meadow. Immediately after sampling, cores were sliced in 1 cm thick segments and a subsample of 2 g wet weight from the centre of each slice was stored in 15 mL falcon tubes with RNA later for eDNA analysis. Sediment accumulation rates for the last century were determined using ^{210}Pb dating technique from another subsample of the same cores where eDNA was analyzed. However, robust sediment chronologies were only obtained for 9 out of 12 cores examined, since intense mixing processes precluded sediment dating for the meadows of *H. stipulacea* at Maridati and the meadows of *C. nodosa* at Limassol. Full details on core sampling description and age determination with ^{210}Pb are described in [27].

2.2. eDNA Analysis: Extraction, Amplification and Sequencing

Prior to field and laboratory work, we sterilized all equipment with 10% bleach and extractions of eDNA were performed in an area separated from the area used for PCR and library preparation. Extracellular eDNA from the seagrass sediment samples was extracted following the Lever et al., 2015 extraction protocol from 88 sediment samples from 12 cores as described previously [27], which gave similar results to commercial kits [46]. To sequence, we prepared a plate containing 88 samples of amplified extracted sediment DNA, 5 negative extraction blanks (containing reagents of extraction protocol and DNA-free water), one negative PCR blank (containing PCR mix and DNA-free water instead of sample template), and two mock samples, with a mix of metazoan DNA in different known concentrations from different metazoan species to validate the taxonomic assignments in the sediment samples. Mock sample 1 included DNA extracted from the tissue of 10 metazoans and mock sample 2 from 13 metazoans (Supplementary Table S1). For eDNA amplification and sequencing we used a primer pair that targets metazoans by amplifying a fragment of 310 bp of the mitochondria Cytochrome Oxidase subunit 1 (CO1) gene (Leray et al., 2013), with Illumina tags included: forward primer TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWGGWTGAACWGTWTAYCCYCC and reverse primer GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAIA-

CYTCIGGRTGICCRAARAAYCA. For each sample, five replicate PCR reactions were run on separate plates and then pooled to decrease PCR bias. PCRs were performed in 10 µL total volume containing 1 µL of 1:10 diluted sediment DNA, 5 µL PCR QIAGEN Multiplex master mix (QIAGEN, Valencia, CA, USA), 3 µL of PCR grade water, and 0.5 µL of each both forward and reverse primer (10 mM). The PCR thermal conditions for amplification in a thermocycler were: 15 min at 95 °C, followed by 5 cycles of 15 s at 95 °C, then 30 s at 46 °C and 30s at 72 °C, then by 35 cycles of 15 s at 95 °C, then 30 s at primer annealing temperature of 53 °C, then 30s at 72 °C, and a final extension at 72 °C for 5 min. PCR products were electrophoresed on 1.5% agarose gels and cleaned by AMPure XP magnetic beads (Beackman Coulter, Brea, CA, USA) according to the Illumina Metagenomic Sequencing library preparation guide.

Amplicons were indexed with Illumina Nextera XT index kits v2 (Illumina Inc., Thuwal, Saudi Arabia), cleaned again with AMPure XP magnetic beads following the Illumina Metagenomic Sequencing Library preparation protocol, and sequenced using MiSeq Illumina platform at King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia. Sequences were demultiplexed following illumina protocol. Primers were then trimmed from the forward and reverse reads using Cutadapt. Sequences were analyzed using the DADA2 package by dereplicating FASQ files, performing quality filtering (correcting for errors), merging forward and reverse unique reads, dereplication, and removing chimeras for generating the final amplicon sequence variants (ASVs) table. For taxonomic identification we used the Ribosomal Database Project (RDP) classifier tree along with the Insect package in R (classify function with sensitivity thresholds set to ping = 1 and threshold = 0.6; V.1.1.9) to make the assignments of the ASVs derived from dada2 package. The code is available from https://github.com/ngeraldi (accessed on 1 September 2021). We removed contamination from the samples using the R package decontam with a threshold of 0.5, which identifies as potential contaminants all sequences that are more prevalent in negative controls than in the samples. After this, extraction and PCR blanks were removed from the data set. In order to focus exclusively on metazoans, we removed all prokaryotes, fungi, phytoplankton, sequences unassigned at the Kingdom level and Metazoans not identified as any Phylum.

Sequencing depth was analysed using rarefaction curves and the number of reads were rarefied to 2000 metazoan reads per sample with the Phyloseq R package. Data was then transformed to presence/absence data for all analyses, as the correlation between true abundance/biomass of a species and eDNA signal strength obtained through metabarcoding is unreliable [47].

2.3. Data Analysis

To analyse temporal changes in metazoan communities' in seagrass meadows, we initially used 69 sediment samples, as 19 samples could not been reliably dated due to sediment mixing. Rarefaction to 2000 reads was performed to account for uneven sequencing depth among samples. This resulted in the removal of 22 samples and the subsequent removal of 20 ASVs no longer present after rarefaction, leaving a total of 123 ASVs and 47 samples. To analyse the data collected from different cores at different depths and ages, we divided the sample data into 4 time intervals: from 1998 to 2017, from 1980–1997, from 1960 to 1979, and from 1930–1959. These divisions were established to include at least two samples of each seagrass meadow in each time period and to account for uncertainties in dating. The periods cover the increase in urbanization since the 1960s [44], the steep increase in exotic species arrivals since the 1960s due to the intensification of global trade [48], and the increase in ocean warming since the 1990s [49–51].

Alpha diversity measures (Richness and Shannon diversity index) were calculated with the phyloseq package in R software for the three seagrasses and the four time intervals. In order to analyse differences in metazoans richness in seagrass meadows over time, we fitted a Linear Mixed effect Model (LMM) with a Restricted Maximum Likelihood method (REML) to the data using the lmer function in the lme4 package [52] in R statistical software as:

$$Richness \sim Seagrass + Time + Seagrass \times Time + (1|Site/Core) \quad (1)$$

where Richness was the response variable. Seagrass (with the factors *H. stipulacea*, *C. nodosa*, and *P. oceanica*) and Time (with the factors 1998–2017, 1980–1997, 1960–1979, and 1930–1959) were fixed factors and Core was nested in Site (Kalami, Maridati, Limassol, or Port) which was included as a random factor. We performed Beta diversity analysis with Nonmetric multidimensional scaling (NMDS) using euclidean distances with the package phyloseq in R in order to assess the community structure and effect of several metazoan phyla and classes.

3. Results

Average metazoan richness in seagrass meadows ranged from 6 ± 3 to 25 ± 5 ASVs. The diversity, measured by the Shannon diversity index, ranged from 1.4 ± 0.5 to 3.2 ± 0.2, with the lowest values observed in the older time period of the *C. nodosa* meadow (1930 to 1959) and the highest in the period from 1960 to 1979 of the *P. oceanica* meadow (Figure 1). The linear mixed effect model indicated significantly higher richness and diversity in *P. oceanica* meadows than in *H. stipulacea* and *C. nodosa* meadows (Figure 1 and Supplementary Table S2). Metazoan communities' richness and diversity in *P. oceanica* meadows was twice as high than in *C. nodosa* and *H. stipulacea* meadows. For example, for the most recent time period (1998–2017) richness and Shannon diversity in *P. oceanica* (19.1 ± 5.1 and 2.9 ± 0.3) were almost twice that in *C. nodosa* (11.3 ± 0.8 and 2.4 ± 0.1) and *H. stipulacea* (9.9 ± 1.1 and 2.2 ± 0.1, Figure 1). Specifically, the linear mixed effect model detected significant differences in metazoan communities across seagrass meadows for 5 out of 15 phyla (Cnidaria, Porifera, Equinodermata, Arthropoda, and Nematoda; $p < 0.003$) and for 6 out of 20 classes (Hydrozoa, Demospongiae, Hexanauplia, Homoscleromorpha, and Echinoida; $p < 0.002$) after Bonferroni correction, with *P. oceanica* showing significantly higher richness than *C. nodosa* and *H. stipulacea* (Figures 2 and 3, Supplementary Table S2).

Richness of metazoan communities, especially in *P. oceanica* meadows, showed a steep decrease since the 1980s to present, although the linear mixed effect model did not detect significant differences among time periods (Figure 1 and Supplementary Table S2). Mean metazoan richness remained elevated between the 1930s and 1980s, with the earlier period (1930–1959) exhibiting a mean richness of 12.7 ± 3.5 SVs, remaining high between the 1960s and 1980s with 12.4 ± 3.3 SVs. After the 1980s, mean richness decreased to 10.4 ± 2.1 during the period 1980–1997 and remained at this reduced biodiversity level at 10.9 ± 1.1 during the most recent years (1998–2017). Conversely, the Shannon diversity index remained stable through the different time periods.

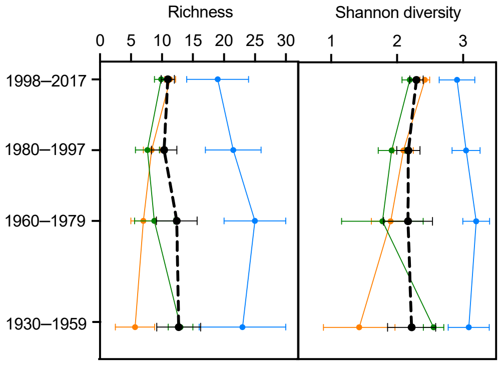

Figure 1. Alpha diversity measures (mean Richness and Shannon diversity index ± SE) of metazoan communities through time in seagrass meadows (*H. stipulacea* in green; *C. nodosa* in orange; *P. oceanica* in blue; overall of the three seagrass species in black). Dashed dark lines indicate non-significant differences between time intervals, considering the three seagrass species combined.

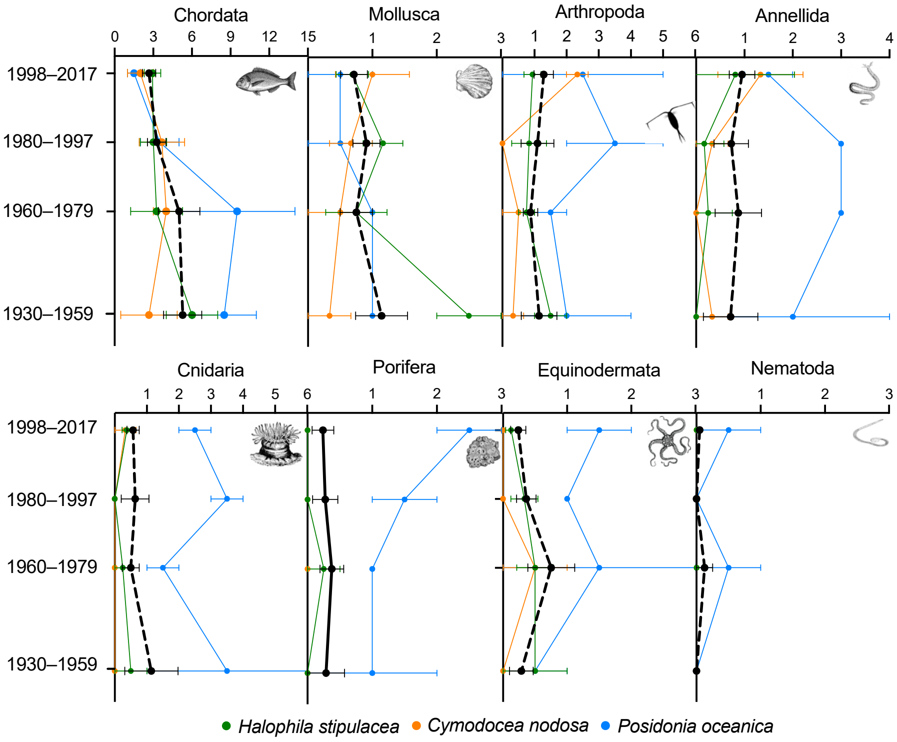

Figure 2. Temporal changes in richness (mean ± SE) in eight metazoan phyla within seagrass sediments (*H. stipulacea* in green; *C. nodosa* in orange; *P. oceanica* in blue; overall of the three seagrass species in black) from the eastern Mediterranean Sea (Greece and Cyprus). Solid dark lines indicate significant differences between time intervals and dashed dark lines indicate non-significant differences ($p < 0.003$ after Bonferroni correction), considering the three seagrass species combined.

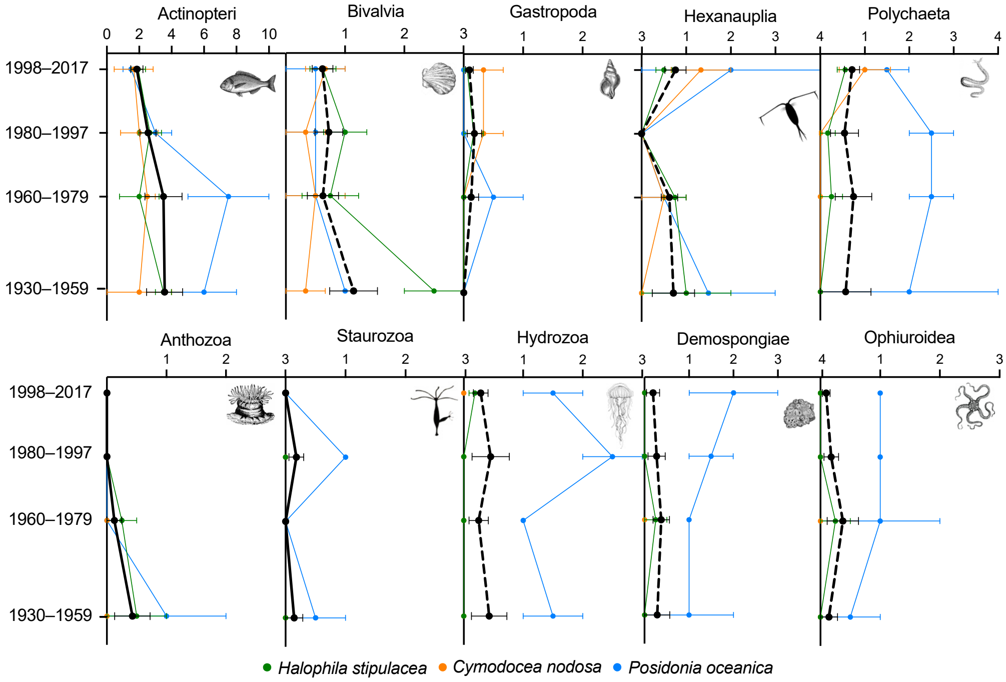

Figure 3. Temporal changes in richness (mean ± SE) in 10 metazoan classes within seagrass sediments (*H. stipulacea* in green; *C. nodosa* in orange; *P. oceanica* in blue; overall of the three seagrass species in black) from the eastern Mediterranean Sea (Greece and Cyprus). Solid dark lines indicate significant differences between time intervals and dashed dark lines indicate non-significant differences ($p < 0.002$ after Bonferroni correction), considering the three seagrass species combined.

Significant changes in seagrass metazoan communities over time were detected for 1 out of 15 phyla and for 5 out of 20 classes (Figures 2 and 3 and Supplementary Table S2) after Bonferroni correction ($p < 0.003$ for phyla and $p < 0.002$ for class). The Phylum Chordata did not show differences in richness through time, but at a lower taxonomic level the bony fishes (Class Actinopteri) had significantly higher species richness in sediments from before the 1980s (periods 1960–1979 and 1930–1959) than in those from the recent years (period 1998–2017 and 1980–1997) (Figures 2 and 3). Richness peaked in the time period from 1960 to 1979 (Figure 3). During this period the orders of bony fishes Labriformes and Acanthuriformes (absent in the period before) appeared in the samples while the order Spariformes (only present in the period 1930–1959) disappeared from all seagrass meadows. In contrast, the orders Perciformes and Lophiiformes were present during all time periods (Figure 4).

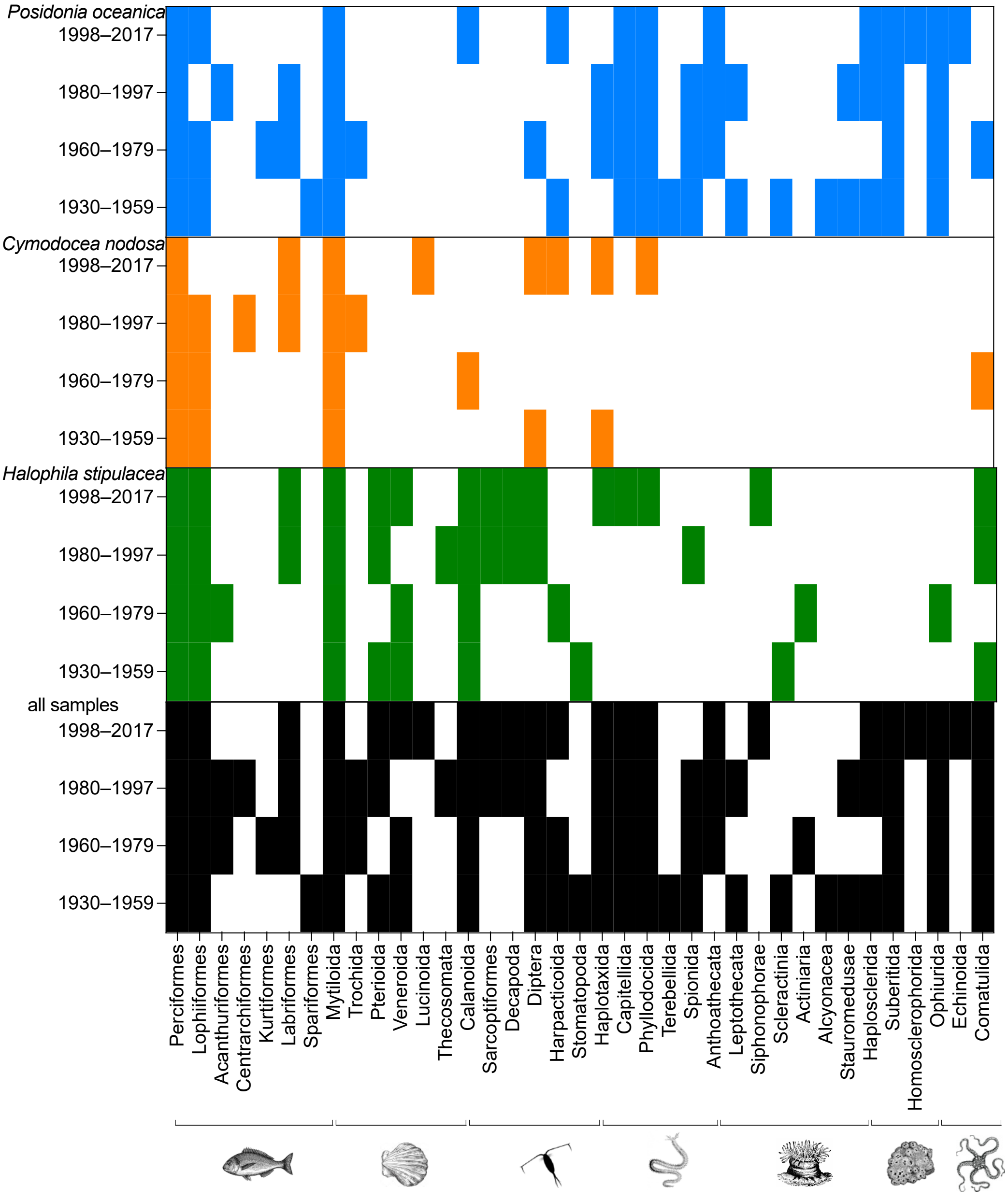

Figure 4. Heat map displaying the presence of metazoan orders through 4 different time periods within seagrass sediments (all samples in black; *H. stipulacea* in green; *C. nodosa* in orange; *P. oceanica* in blue) from the eastern Mediterranean Sea (Greece and Cyprus).

The Phylum Cnidaria also did not show differences in richness through time, but at a lower taxonomic level, the linear mixed effect model found significant differences through time periods for the Class Anthozoa and Staurozoa, but not for the Class Hydrozoa (Figure 3 and Supplementary Table S2). Several cnidarian orders were present only in older samples (mainly *P. oceanica*) and absent from the most recent periods (Figure 4), as was seen for the orders Stauromedusae (stalked jellyfishes), Leptothecata, and Alcyonacea (soft corals). Conversely, athecate hydroids (order Antoathecata) appeared in *P. oceanica* since the 1960's and siphonophores, an order of Hydrozoa, since the 2000s in *H. stipulacea* (Figure 4). All Cnidarian orders ($n = 7$) were present in *P. oceanica* (except for Siphonophores) and absent from *C. nodosa* and only three Cnidarian orders were present in *H. stipulacea* (Figure 4).

Conversely, the Phylum Porifera showed a significant increase in richness over time periods (especially for *P. oceanica*) and significant differences in richness between seagrass species (Figures 2 and 3). All identified orders ($n = 3$) belonging to the Phylum Porifera were only present in samples from *P. oceanica* and absent from *H. stipulacea* and *C. nodosa* meadows (Figure 4).

Similarly, Arthropods increased their richness in the modern periods (Figure 2, Supplementary Table S2), probably due to an increase in the Class Hexanauplia, which also showed higher richness with time, though this trend was not significant (Figure 3, Supplementary Table S2). The Class Hexanauplia in our seagrass sediments samples was represented by Calanoids, an order of copepods, and this order was present in all time periods of *H. stipulacea*, in *C. nodosa* solely in the period 1960–1979 and in *P. oceanica* in the most recent period (1998–2017) (Figure 4). Other Arthropoda orders (Sarcoptiformes and Decapoda) appeared in the two most recent periods (from 1980 to 2017) in *H. stipulacea* meadows and were completely absent in *C. nodosa* and *P. oceanica* (Figure 4).

Mollusk richness remained constant through time (Figures 2 and 3). However, the Gastropod order Trochida was absent from the most recent periods: in *P. oceanica* it was present only in the older time periods (1930–1959 and 1960–1979), in *C. nodosa* in the period 1980–1997, and completely absent in *H. stipulacea* (Figure 4). Conversely, the orders Pterioida and Veneroida (Class Bivalvia) and Thecosomata (Class Gastropoda) were only present in *H. stipulacea* (Figure 4).

Annellid richness was similar between time periods and seagrass meadows. The same pattern was observed for the Class Polychaeta (Figure 2, Supplementary Table S2). The polychaete order Terebellida was only present in seagrass sediments of *P. oceanica,* the order Spionida in *P. oceanica,* and *H. stipulacea.* The rest of polychaete orders, Capitellida and Phyllodocida, were present in all seagrass species, although in *H. stipulacea* and *C. nodosa* only in the present day (Figure 4).

Echinoderm richness was similar between time periods and differed significantly between seagrass species, with *P. oceanica* having the highest richness (Figure 2). The order Ophiuroida, an order of brittle stars, was present in *P. oceanica* during all the time periods, absent in *C. nodosa* and present in *H. stipulacea* only in the period 1960–1979. Conversely, the order Comatulida (Class Crinoidea), also known as feather stars, was absent in *P. oceanica*, present in *H. stipulacea* in the most recent time periods (1998–2017 and 1980–1997) and in *C. nodosa* only in the period 1960–1979.

Non-metric multidimensional scaling (NMDS) ordination method using Euclidean distances further confirmed differences in metazoan communities through time. The analysis revealed that the period 1960–1979 formed a wider group composed of samples presenting higher variation between each other. The samples from other periods tended to aggregate, indicating higher diversity in metazoan communities during the time period from 1960 to 1979 and simpler, more homogeneous metazoan eDNA communities thereafter (Supplementary Figure S1). However, the diversity of metazoans not only changed over time but the species composition of each period also changed. From 1930 to 1997 we identified 27–29 different orders in each time period and 35 orders in the most recent period (1998–2017): 9 orders were gained and 11 were lost from 1960 to 1979, 9 were gained and 7 were lost from 1980 to 1997, and 14 were gained and 8 were lost from 1998 to 2017 (relative to the period before) (Supplementary Figure S2).

The NMDS also indicated differences between the three seagrass species: *P. oceanica* samples formed a dispersed assemblage showing greater dissimilarity distance from each other and grouped separately from those from *H. stipulacea* and *C. nodosa*, which showed a tight clustering of the samples and displayed a closer proximity in metazoan eDNA community structure to each other (Figure 5). Significant relationships were found between the NMDS axes and 10 metazoan phyla ($p < 0.05$). The highest significant correlations for the NMDS configuration were in descending order for the Phylum Porifera, Cnidaria, Chordata, Arthropoda, Xenacoelomorpha, Echinodermata, Annelida, Gastrotricha, Nematoda, and Mollusca (Figure 5).

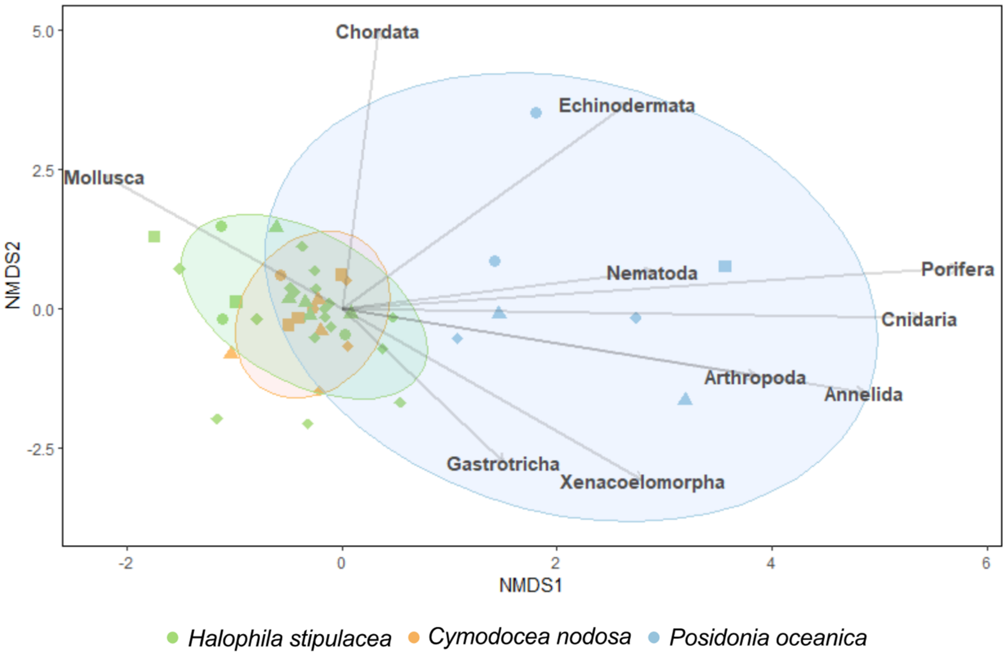

Figure 5. Nonmetric multidimensional scaling plot of temporal changes in metazoan communities within seagrass meadows (*H. stipulacea* in green; *C. nodosa* in orange; and *P. oceanica* in blue). Ellipses show 95% standard error around the mean the three seagrasses and dark grey arrows indicate significant relationship ($p < 0.05$) between the NMDS axes and different metazoan phyla. Greater length of arrows indicates greater r^2 value and is relative to the importance of the explanatory variable in the ordination, and the arrow direction indicates positive or negative correlations. Kruskal stress = 0.16.

4. Discussions

This study assessed changes in the metazoan community over almost 100 years in eastern Mediterranean seagrass meadows based on metabarcoding of eDNA using multiple dated cores and the CO1 region. eDNA provides a robust and reproducible approach to assessing community structure in marine habitats, and is rapidly gaining momentum [23]. Coupled with sediment chronologies, as done here, eDNA provide a tool to reconstruct changes in biodiversity over time.

The taxa identified within seagrass sediments reflect temporal trends in diversity in major marine metazoan groups with significant differences among time periods. It also demonstrates that the diversity of seagrass associated with the macrofaunal community is enhanced with increasing structural complexity of the habitat forming plant, from *P. oceanica*, which develops structurally complex meadows [53] compared to the other smaller seagrasses *C. nodosa* and *H. stipulacea*.

In general, metazoan communities in eastern Mediterranean seagrass meadows showed a step change in metazoan community in diversity and richness around the 1980s, as a result of a decrease in diversity through time in bony fishes and soft corals and a parallel gain of other taxa (e.g., phylum Arthropoda and Porifera) in the most recent periods. All the other metazoan groups remained relatively stable over time, showing a series of fluctuations between time periods. This decrease in diversity and richness in certain metazoan taxa might be related to anthropogenic pressures such as habitat destruction, overfishing and water degradation [54–58] likely to be increasingly compounded by warming [55,59,60], although disentangling the causal effects of this decline is complex and likely reflects the effects of cumulative pressures over time. The lack of time series in seagrass meadows make it difficult to relate our eDNA based findings to more traditional survey methods. However, there is anecdotal evidence that lends credence to our findings. The decrease in fish richness (Class Actinopteri) since the 1980s might be related to increased fishing pressure during these last decades, as increasing fishing pressure has shown to reduce fish abundance [61,62]. For example, the order Spariformes, a common group in Mediterranean seagrass meadows (*P. oceanica*), was observed only in the older periods and the absence of this group in the most recent years could be due to increased fishing pressure. Indeed, sparids increased in density and abundance with the closure of fishing in Marine Protected Areas (MPA's) [63]. However, while many species of Sparid are indeed targeted by fishing, they remain some of the most abundant species of fish in and around the meadows analyzed here [64–66]. Therefore, as some primer pairs show amplification bias for some taxa, with the preferential capture of certain species and the lack of amplification of other [47], this absence might reflect more of a methodological limitation than an empirical change in the ecosystem.

Conversely, other taxa increased their richness from past to present (Phylum Porifera and Arthropoda). We hypothesize that the loss of species richness through time might go unnoticed due to the arrival of exotic tropical species through the Suez Canal (due to its proximity) or through the Gibraltar strait. The Suez Canal has connected the Red Sea with the Mediterranean Sea since 1869. Therefore, many species we detected from the oldest periods could already be exotic. However, the increase in trade and globalization started in the 1960s, which accelerated the introduction of marine exotic species worldwide [48]. Indeed, the 1960s and 1980s showed the greatest diversity (especially for fishes and echinoderms) with the presence of nine orders not found in the period before. For example, the order Acanthuriformes appeared for the first time and persisted since then. Fishes belonging to this order are typically from tropical and subtropical coral reef ecosystems [67], suggesting their introduction from the Red Sea through the Suez Canal, since there are no native species from the order Acanthuriformes in the Mediterranean Sea. To date, eight acanthurid species, mostly with Red Sea and Indo-Pacific Ocean origins, have been observed in the Mediterranean Sea for the first time in the 2000s [68–73], although our data suggest they could have arrived earlier, sometime between the year 1960 and 1980. The strongest wave of exotic species arrivals in the eastern Mediterranean Sea occurred in 1998 due to an abrupt shift in the increasing eastern Mediterranean SST [74], although the arrivals of exotic species are expected to continue increasing due to further global trade [75]. In fact, the period in which we detected the highest gain of new species is the most recent one (1998–2017), where we observed 14 orders not identified before.

The diversity and richness supported by the endemic seagrass *Posidonia oceanica* was twice as high as the other two seagrass species, which is consistent with reports of this seagrass species playing a particularly important role in supporting biodiversity. Ref. [53] found a gradient of species richness from *P. oceanica* meadows, with the largest leaf canopy and rhizome substratum, to *C. nodosa* or *Z. nolti* with lower leaf surface and forming less dense habitats. Most studies on animal communities associated with Mediterranean seagrass meadows focus on *P. oceanica* [76–78] and demonstrate higher species richness compared to bare soft bottoms and shallow hard substrates [79]. This is mainly as a result of the increase in the habitat complexity, which is particularly evident in *P. oceanica* due to the strong differentiation in microhabitats among the various compartments of the system (leaf canopy, the rhizome layer, and the sediment mate-layers), which can facilitate microhabitat specialization of species [77]. In contrast, the phyla Cnidaria, Porifera, and Echinodermata were absent in *C. nodosa* meadows while Porifera and some Cnidarian orders were absent from *H. stipulacea* meadows but were all detected in *P. oceanica*. These metazoan taxa may be absent from *H. stipulacea* (2 leaves per shoot with length of 12–17 cm and width of 6–10 mm) and *C. nodosa* (shoots bear 3–6 leaves 30 cm long × 2–4 mm wide) because they have a lower fraction of surface area compared to *Posidonia oceanica* leaves (7–9 leaves per shoot 40–50 cm long and 5–9 mm wide) [80–82]. In addition, the rhizome layer could also constrain the presence of certain taxa, as this is characterized by a high temporal persistence compared to the high seasonal variability of the leaf layer [83], offering a hard substrate stability for rhizome colonizers, which is especially important for sponges. Ref. [84] found that most Porifera within *P. oceanica* live on rhizomes because of their very slow growth rate while the faster turn-over of leaves offer less time to colonize. This could explain why this taxon is absent in *C. nodosa* and *H. stipulacea* meadows as their rhizome layer is smaller, turns over faster and is largely belowground, while vertical rhizomes of *P. oceanica* extend into the water column as required by filter feeders to use as substrate.

Studies comparing *C. nodosa* and *H. stipulacea* associated fauna are scarce. Our data indicate similar values in metazoan richness and diversity in native *C. nodosa* and exotic *H. stipulacea* meadows. However, the diversity in *C. nodosa* may be expected to be higher than in *H. stipulacea*, as Cymodocea has longer leaves with more suitable habitat and leaf life span than that those of *H. stipulacea* [80] which may imply greater epiphytic cover, as leaves offer more available space for attachment and epiphytes have a better chance of establishing on leaves with a longer life span [85]. Indeed, there can be a higher number of epibionts on *C. nodosa* compared to *H. stipulacea* [86]. However, *H. stipulacea*'s root system had a greater meiobenthic copepod diversity [87]. The small canopy of densely packed leaves and shallow rhizomes of *H. stipulacea* presents a complex architecture and novel habitat with a different morphology compared to dominant native Mediterranean seagrasses species (*P. oceanica* and *C. nodosa*) that may provide new habitat and additional food sources to seagrass-associated fauna. Therefore, more comparative studies are needed to characterize the associated metazoan diversity of these two small seagrasses.

The metazoan diversity associated to *P. oceanica* meadows reported in this study might be underestimated, as only one meadow of *P. oceanica* was sampled and the core were taken from a highly disturbed and impacted meadow [27]. If more meadows of *P. oceanica* would have been sampled (from less disturbed sites), it is expected that the metazoan diversity would be much higher than in this study and much higher than in meadows of *C. nodosa* and *H. stipulacea*. Therefore, Posidonia meadows do seem to have the highest metazoan diversity within this seagrass trio in the Mediterranean Sea [53]. Unfortunately, not all seagrasses and their associated organisms respond equally to current anthropogenic pressures. *P. oceanica*, a long lived species of temperate affinity characterized by its very slow growing rates (1–6 cm year^{-1}; [88]) being particularly vulnerable to warming [32], with shoot mortality increasing during heat waves exceeding 28 °C at the end of summer [89]. Conversely, *H. stipulacea* supports rapid growth (0.5 cm day^{-1}; [90]) and turnover rates, as well as an upper thermal threshold of 38 °C, the maximum temperature experienced in its native Red Sea [90]. *C. nodosa* is of tropical affinity and has an intermediate growth rate and thermal thresholds [91]. In fact, *P. oceanica* is experiencing a well-documented regression due to habitat degradation and increasing summer heatwaves [32] and species distribution models project that the suitable habitat of the species could be completely lost by 2100 under the business as usual climate change scenario [92,93] while *H. stipulacea* will expand its distribution by 50% [43]. In fact, some meadows of *H. stipulacea* are currently growing on top of meadows that were *P. oceanica* or *C. nodosa* in the past [27]. However, the projected regression of *P. oceanica* is based on Western Mediterranean data and a recent study suggest that eastern populations of *P. oceanica* in Cyprus are more thermally tolerant than previously realized [94]. In any case, our results suggest that a shift from *P. oceanica* meadows to those dominated by *C. nodosa* and *H. stipulacea* with a warmer thermal affinity to ocean warming [90,91] will lead to a major loss of biodiversity.

The combination of environmental DNA and dated sediment cores used here to resolve changes in biodiversity over century time scale provides a novel approach to follow natural communities back in time in the absence of time series and baseline data. However, the use of eDNA involves limitations, as the sequences of metazoan are diluted relative to those of other eukaryotes such as fungi, diatoms, and many non-identified cellular organisms that are also retrieved when targeting the CO1 region [95,96]. The consequence is a low sampling depth of metazoans, so that the organisms detected are a subset of the metazoans present in the ecosystem, which would be retrieved using much higher sequencing depth or more selective markers. Hence, we were only able to describe the diversity patterns at the order level and the majority of sequences remained unassigned at genus or species level. This could be because DNA preservation in seagrass sediments is much lower than in frozen cores or other stable environments (anoxic lakes, deep sea) [97–99], or because successful taxonomic identification in eDNA studies rely on reference databases, and some seagrass associated species may not be in reference libraries. Moreover, multiple studies also show amplification bias of primer pairs for some taxa, with the preferential capture of certain species and the lack of amplification of other [47]. For example, Spariformes were only present between 1930–1960 and yet Sparids (e.g., Diplodus spp., Sarpa, Oblada, Sparus) are some of the most common species on and around seagrass meadows in the eastern Mediterranean today [64–66]. Conversely, the order Acanthuriformes appears since the 1960s and, while species within this order are present in Crete and Cyprus, they are in very low abundances, particularly compared to Sparids [64–66]. Therefore, it seems that the CO1 primer preferentially amplifies Acanthurids compared to Sparids. This could also be because the preservation of eDNA is not uniform among species taxa [99]. DNA degradation could also be higher in older and deeper sediment samples, although the metazoan community detected in our eDNA samples did not vary through time and certain taxa showed a higher richness in the past, arguing against progressive eDNA degradation through time in our samples.

Lateral mixing of sediments could also occur in seagrass meadows due to local currents [100], as well as vertical mixing due to leaching or through other processes such as storms, river plumes, or bioturbation [101,102]. Contamination during the sampling process could have gone unnoticed because we did not have ‘controls’ for this, even though strict precautions were taken to avoid it (e.g., sterilization of all equipment with 10% bleach before field and laboratory work). To minimize the effect of these biases we analyzed the eDNA from 9 dated sediment cores with clear ^{210}Pb-specific activity profiles and we excluded those samples related to mixing events (e.g., samples related to the massive sediment accumulation event rich in terrestrial plant sequences brought by an ephemeral stream discharged near one of the sampling sites of our cores; [27]). Additionally, the abundance of species is relative in eDNA samples, as changes in one species could affect another and, even though we rarefied sequences so all samples had similar reads and used presence/absence to reduce this artifact, perhaps the presence of low abundant species could still be affected by changes in other species.

Future studies in the reconstruction of past events in seagrass meadows with eDNA would benefit from longer sediment cores (to cover a longer time period) and by incorporating additional DNA markers to reconstruct historical trajectories of specific taxa, possibly using quantitative PCR to retrieve more quantitative assessments of DNA of target species [103].

Regardless of these challenges, our results successfully retrieved temporal trends of the metazoan community within seagrass meadows in the eastern Mediterranean Sea over the last 100 years combining eDNA with sediment chronologies. Our results demonstrate that species richness and diversity is higher in *P. oceanica* than in *C. nodosa* and *H. stipulacea* meadows and show a turnover of taxa, where taxa lost due to anthropogenic pressures are compensated by the recruitment of exotic taxa to the community. In the future, thermal limits of *P. oceanica* might be exceeded with warming. Unfortunately, the replacement of this endemic species by other seagrasses with a warmer thermal affinity [90,91] whether native (*C. nodosa*) or not (*H. stipulacea*) will not compensate for the species loss.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14070549/s1, Figure S1: Nonmetric multidimensional scaling plot of temporal changes in metazoan communities within seagrass meadows (*H. stipulacea* in green; *C. nodosa* in orange; *P. oceanica* in blue). Ellipses show 95% standard error around the mean of the four time intervals and and dark grey arrows indicate significant relationship ($p < 0.05$) between the NMDS axes and different metazoan phyla. Greater length of arrows indicates greater r^2 value and is relative to the importance of the explanatory variable in the ordination, and the arrow direction indicates positive or negative correlations. Kruskal stress = 0.16.; Figure S2: Number of metazoan orders in seagrass sediments identified for each time period (black dots). Columns indicate the number of orders either gained (blue) or lost (red) relative to the period before.; Table S1: Mock sample nucleic acid concentration (ng μL^{-1} per genus/ ng μL^{-1} of total sample × 100) and Sequence Variants reads (SV per genus/SV reads of total sample × 100) after filtering and taxonomy assignment of several marine metazoans.; Table S2: Linear mixed effect model analysis of of Metazoan communities richness through time in seagrass meadows (H.s.: *H. stipulacea*, C.n.: *C.nodosa* and P.o.: *P. oceanica*) with Site as a random factor, Core nested in Site and Seagrass and Time as fixed factors.

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