Environmental DNA as non-invasive monitoring tool for detection of marine species of the Levantine Basin of the Eastern Mediterranean Sea

Rebecca Valani

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE MASTER'S DEGREE

University of Haifa

Faculty of Natural Sciences

Leon H. Charney School of Marine Sciences

The Department of Marine Biology

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I

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Abstract

The world's biodiversity is declining at an alarming rate, thus efficient and non-invasive monitoring tools, are needed to assess its development. Metabarcoding of environmental DNA (eDNA) has accelerated rapidly as a high potential supplementary monitory tool to conventional methods. The true biodiversity of the Eastern Mediterranean Sea remains relatively unexplored. Thus, advanced monitoring before its further decline due to factors such as climate change, anthropogenic activity, and invasive species, remains crucial. This study aims to develop and assess the abilities of eDNA as a monitoring tool in the Eastern Mediterranean Sea. We investigated 4 sampling sites along the Israeli coastline of the Eastern Mediterranean Sea, for a total of 60 samples stretching from north (Achziv) to south (Ashdod). We found that 94% of the species identified in this study, are known to occur in the Mediterranean Sea and 70% are known to the Eastern Mediterranean Sea. We found significant differences in both richness and assemblage of ASVs among different sampling sites. We discuss the difference in biodiversity and phylum compositions, detected at the different sampling sites, which consists mainly of marine invertebrates. We test the accuracy of biological replicates and examine its importance within use of eDNA as a monitoring tool. Finally, we showed the importance of increasing sampling sizes, to increase the detectability rate. This study is amongst the first in the region to develop and highlight the use, and benefits of eDNA as a monitoring tool.

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Introduction

The world's biodiversity continues to decline, and an increasing number of species become endangered due to anthropogenic impacts (Thomsen & Willerslev, 2015). For decades, we have relied on so-called conventional methods for biodiversity monitoring and assessing the health of our ecosystems. These methods include visual surveys (such as diving or boat observational surveys), traditional surveys from research vessels including plankton nets and other measures of capture. Finally, we also rely on the data from the fishing industry, rather it is trawling or bycatch. These methods, are however invasive, demands many hours in the field and only allows for a small sample coverage. Further, the identification of species sampled by conventional methods, requires, and solely relies on the eyes of the expert, thus can be prone to human error (Ruppert el al., 2019). These often fail to describe the full aspect of biodiversity at a given site or successfully characterize the presence of rare, endangered, or migratory species. Therefore, this can challenge the efficiency of conservational efforts, as vital species might be overlooked. To estimate the state of the world's biodiversity, efficient and non-invasive monitoring tools are needed, more so now than ever. This battle against time is partly due to invasive, costly and time demanding efforts to monitor and detect vital habitats for endangered species across ecosystems.

eDNA is defined as short DNA fragments that an organism leaves behind in non-living components of the environment i.e., water, air or sediments (Foote et al., 2012). eDNA is derived from either cellular DNA, present in epithelial cells released by organisms to the environment through skin, urine, feces, mucus or extracellular DNA (Hajibabaei et al., 2007). As every organism on earth contains and leaves behind DNA in its environment (Díaz-Ferguson et al., 2014), eDNA metabarcoding allows for non-invasive monitoring of target species in their environment, by tracing their DNA and identify them down to the species-specific level (Ruppert et al., 2019). eDNA was previously defined as 'particulate DNA', used to describe plankton and microbial communities in sediments in the 1990's and 1980's, respectively (Díaz-Ferguson et al., 2014). eDNA was first introduced as a monitoring tool in marine waters in 2012, when scientists from University of Copenhagen were the first to utilize the method to detect fish fauna of commercial importance in Danish waters (Thomsen et al.,2012).

Their initial findings established the excellent ability of eDNA to detect verified species at a higher level, than any conventional method. In the last decade the use of eDNA has accelerated as a global tool to monitor biodiversity and detection of endangered and rare species across the spectrum of the animal kingdom (Andruszkiewicz et al., 2017; Port et al., 2016). eDNA has already been recognized to have high potential as a monitoring tool in ecological and conservational management programs, as it reveals populations of species, often never observed by the naked eye (Bakker et al., 2017). Thus, it has enormous potential in assisting with the reduction of loss of biodiversity (UNEP, 2011). Although eDNA is widely used worldwide, as a monitoring tool (Díaz-Ferguson et al., 2014), this is to our knowledge one of the first studies of its kind in the Eastern Mediterranean Sea.

When handled with care and the right precautions, eDNA can serve as a powerful monitoring tool of species on a large-scaled spectrum, as it provides thousands to millions of DNA sequences available for analysis (Thomsen & Willerslev, 2015). While eDNA has huge potential for being a groundbreaking supplement to traditional surveys, several factors can influence sample quality and sequencing results. Such factors include sunlight and temperature during storage of samples as well as a high-risk of contamination, through every step of the protocol. It is therefore essential to not only sample, but process samples with great care to allow for minimum risk of contamination and highest quality of sequencing results (Ruppert et al., 2019). Further, the origin, transport and decay of eDNA greatly influences the possible DNA retrieval from the given environment (Harrison et al. 2019). Likewise, the size and physiology of the species are also sources of misrepresentation of certain taxa (Souza et al., 2016).

The climate in the Eastern Mediterranean Sea is described as cool and humid winters, with dry and hot summers (Lionello et al., 2006). Large differences in oceanographic conditions within the Mediterranean Sea, heavily influence its biodiversity from West to East. With only two connections to other seas (the Atlantic Ocean in the west and the Red Sea to the southeast), the basin becomes very concentrated. As evaporation is highest in the eastern end, this results in decrease of water levels in the region and increase salinity (Coll et al., 2011).

This ultimately leads the Eastern Mediterranean Sea to be an oligotrophic environment and often regarded as a 'marine desert' with the warmest and most saline waters in the Mediterranean region (Rilov et al., 2010).

The biological production in the Mediterranean, follows a similar trend, with an all-time low in the Levantine Basin, due to its extreme conditions (Coll. Et al., 2011). This is predicted to only increase with climate change, anthropogenic threats and by further expansion of alien and invasive species. The marine biota of the Mediterranean Sea is heavily influenced by its invasion of species from the Red Sea. The Eastern Mediterranean Sea hosts the most alien species of the entire Mediterranean Sea, as the vast majority have been introduced through the Suez Canal (Galil et al., 2020). These species continuously change the biodiversity of the area, ultimately resulting in populations collapse of native species (Albano et al., 2021). The region has its own list of endemic species and contain various critical habitats and endangered species. With new species, particularly smaller ones, being regularly discovered, the true biodiversity of the Mediterranean Sea may be much higher than currently believed.

Additionally, many species could be going extinct, before they are even discovered. One area which remains relatively unexplored and unknown, is the Eastern Mediterranean, particularly the Levantine Basin (Coll et al., 2010). Recent and ongoing efforts have been deployed to fill the present knowledge gap of biodiversity in the Eastern Mediterranean Sea, such as those conducted by University of Haifa, by Morris Kahn Marine Research Station (https://med-lter.haifa.ac.il/index.php/en/) and by Israel Oceanographic and Limnological Research Institute. There is however, an urgent need for non-invasive and low-cost monitoring tools, to properly apply conservation and management approaches. Thus, eDNA could play a vital role in revealing critical marine biodiversity hotspots, that are otherwise less accessible with traditional methods. In this study, we will develop and test eDNA's ability to successfully detect marine species known to the region of the Levantine Basin and the Mediterranean Sea.

Working hypotheses

1. Environmental DNA can be used as a reliable and efficient monitoring tool for marine species in the Eastern Mediterranean Sea
2. Using eDNA, we can detect different species composition at different sampling sites
3. The species identified via eDNA, will reflect the known local and regional composition of species

Objectives

The objectives of this study, are three-folded and reads as follow:

1. To assess eDNA as a monitoring tool in the Eastern Mediterranean Sea
2. To screen for biodiversity of the coastline for various marine species using eDNA
3. To compare species identified with eDNA to known species in the region

Material and Methods

Study area

Figure 1: Map of the four sampling locations.

The Israeli coastline stretches over an area of 200 km of the Eastern Mediterranean Sea, from Rosh Hanikra in the north, to Ashkelon in the South. Sampling was done from 4 sampling sites (Figure 1), one site in the north in Achziv (33.0°N, 35.082°E), two sites in the center (Sdot Yam: 32.550°N, 34.896°E and Herzliya: 32.121°N, 34.694 °E) and one site in the south in Ashdod (31.672°N, 34.526°E). All samples were sampled in the winter of 2020.

Site	Station	Sampling date	Distance to shore (KM)	Length of transect (KM)	Temp (°C)	Sampling depth from the surface (m)	Depth (to seafloor) at sampling point (m)	Latitude (°)	Longitude(°)	Amount of seawater sampled
Achziv	A1	27/Jan/20	2.14	5.3	18	0	16	33.09	35.08	3x1 Liter
	A2	27/Jan/20	2.99		18	0	28	33.06	35.07	3x1 Liter
	A3	27/Jan/20	2.57		18	0	43	33.06	35.07	3x1 Liter
	A4	27/Jan/20	2.06		18	0	17	33.06	35.08	3x1 Liter
	A5	27/Jan/20	1.20		18	0	23	33.07	35.08	3x1 Liter
Sdot Yam	HS1	28/Jan/20	0.84	10.7	18	0	19	32.55	34.90	3x1 Liter
	HS2	28/Jan/20	2.00		18	0	28	32.56	34.88	3x1 Liter
	HS3	28/Jan/20	4. 25		18	0	48	32.56	34.86	3x1 Liter
	HS4	28/Jan/20	9.34		18	0	85	32.56	34.80	3x1 Liter
	HS5	28/Jan/20	11.58		18	0	119	32.56	34.79	3x1 Liter
Herzliya	HY1	09/Mar/20	8. 44	7.8	19	0	43	32.12	34.69	3x1 Liter
	HY2	09/Mar/20	8.15		19	0	42	32.14	34.71	3x1 Liter
	HY3	09/Mar/20	7.50		19	0	42	32.15	34.71	3x1 Liter
	HY4	09/Mar/20	6.47		19	0	42	32.16	34.73	3x1 Liter
	HY5	09/Mar/20	6.33		19	0	33	32.18	34.74	3x1 Liter
Ashdod	AS1	27/Jan/20	2.06	22.6	17	0	8	31.67	34.53	3x1 Liter
	AS2	27/Jan/20	2.93		17	0	13	31.70	34.54	3x1 Liter
	AS3	27/Jan/20	3.24		17	0	23	31.75	34.57	3x1 Liter
	AS4	27/Jan/20	0.24		17	0	19	31.85	34.66	3x1 Liter
	AS5	27/Jan/20	3.90		17	0	31	31.84	34.61	3x1 Liter

Table 1: List of sampling sites and conditions. The abbreviation HS shorts for Sdot Yam.

Sample collection

Samples were collected for the purpose of comparing the known biodiversity in the region, to the biodiversity in the samples. Three of the sample sets (Table 1) were sampled in late January and one sample was sampled in early March of 2020. From each site (Achziv, Ashdod, Sdot Yam, Herzliya), five sampling stations were determined and samples of triplicates of one liter of seawater as described by (Andruszkiewicz et al., 2017) from each of the four sampling sites, were collected.

The samples were sampled from the surface at each station. As seen in Table 1, the samples from Sdot Yam were sampled with an increasing distance to the shoreline and thereby also an increasing distance to the sea floor. All three replicates from each station at each site, is part of the analysis, of which this study is based on. The samples were collected in sterile single-use bottles. The samples were stored on ice, while at sea in a closed container, while being transported back to the research facility on land. A total of 60 liters of seawater (20 non-replicates) has been collected for the purpose of this study.

Filtration

The samples were filtered immediately upon arrival to the research facility or otherwise frozen down at -20°C, if immediate filtration was not possible. Each sample was filtered using single-use 'Nalgene Rapid- Flow Filters' (Thermo Scientific, USA). Due to the practical issues, the samples from Sdot Yam were not able to be processed upon arrival to the research station. The water bottles were instead, frozen down until the following morning where only then, they were filtered.

DNA extraction

All samples were extracted using SV Genomic DNA Purification system kit (Promega, USA) in accordance with manufacturer's instruction. Extractions were stored at -20°C until use, shortly after extraction.

PCR amplification

The primers used for this project was the 'mlCOIint' primers, which target metazoan diversity in a short fragment of 313 base pairs of the mitochondrial COI region (Leray et al., 2013). Tags of CS1 and CS2 were added to the primers in order to allow for NGS sequencing.

Primer sequences are as follow:
Forward: CS1_mlCOIint: ACA CTG ACG ACA TGG TTC TAC AGG WAC WGG WTG AAC WGT WTA YCC YCC
Reverse: CS2_jgHCO: TAC GGT AGC AGA GAC TTG GTC TTA IAC YTC IGG RTG ICC RAA RAA YCA

The PCR was carried out using 12.5 µL of Dreamtaq Green PCR Mastermix (Thermo Scientific, USA), 0.5 µL of each primer, 1µL of bovine serum albumin (BSA) and 10.5 µL of sampled DNA extract, adding up to a volume of 25 µL. One set of NTC and one set of positive controls were added to the PCR cycle.

Thermal conditions: 95 °C for 45 seconds; 10 cycles of: 95 °C for 15 seconds, 60 °C for 20 seconds, 72 °C for 20 seconds; 10 cycles of: 95 °C for 15 seconds, 55 °C for 20 seconds, 72 °C for 20 seconds; 10 cycles of: 95°C for 15 seconds, 50 °C for 20 seconds, 72 °C for 20 seconds, 15 cycles of: 95°C for 15 seconds, 65 °C for 20 seconds, 72 °C for 20 seconds; 72 °C for 2 minutes and store at 15 °C.

Positive and negative control

Two sets of negative controls (NTC) and two sets of positive controls were processed with the main samples and sent to NGS sequencing. The positive controls both contained DNA extract of the coral *Oculina patagonica*, which is commonly found throughout the Mediterranean Sea (Coll et al., 2011).

The two positive controls of *Oculina patagonica* produced a total of 23 ASVs and 20,099 reads (one sample with 6,891 reads and another with 13,208 reads). Both positive controls contained other species as well, that were otherwise not identified in the remaining samples. These species were Lionfish (*Pterois miles*) with 4 ASVs, (Lamellibranchia sp) 2 ASVs, (*Madracis pharensi*) with 1 ASV, (Pennaria sp) 2 with ASVs, (Denostrea sp) with 1 ASV and Choridariaceae sp) with 1 ASV. The detection of these other species in the positive control, can be due to lab contamination, especially regarding Lamellibranchia sp.

However, it should be noted, that *Oculina patagonica* was sampled from the open waters near the city of Haifa. It is a very common species in the area and therefore very likely, that the coral itself hosts several other species and/or is in close proximity to other species. Both negative controls were free of contamination.

Verification of PCR

As this is a new method in the region, several verification steps (various protocols of DNA extraction and PCR) were taken in the initial stages of the project's development, to ensure the validity of the methodology. Saltwater was sampled from an aquarium at a facility within the research station, which only contained the species of gilt-head bream (*Sparus aurata)*.
The samples were of duplicates of one liter.
The samples were processed and four PCR products, were sent to sanger sequencing. All four samples confirmed the presence of gilt-head bream (*Sparus aurata)*. Likewise, several negative controls throughout the verification process, were carried out, to solidify the validity of the method. Note, these samples were processed with a different set of primers (Andruszkiewicz et al., 2017), originally meant to be used throughout the entire project. However, due to unforeseen issues of the efficiency of these primers, it was necessary to switch to a different set of primers. Nonetheless, these samples verified the PCR, allowing us to continue with the method.

Test of primers

The primers ('mlCOIint') were prior to the sampling tested on several known species in Eastern Mediterranean Sea. Tissue extraction were processed of two sharks (*Isurus oxyrinchus* and *Carcharhinus plumbeus*) and two dolphin species, (*Tursiops truncatus* and *Delphinus delphis*). Extract of the sea urchin *Diadema setosum*, was further tested on the primers, to ensure the full cover range. The samples were sent to sanger sequencing and all samples came back confirmed, as the respective species. Thereby confirming the primers ability to identify a large taxonomical range.

Sequence and phylogenetic analysis

All 60 samples (3x5x4) as well as the two positive and the two negative controls were sent to NGS sequencing. Amplified products (amplicons) were prepared and sent for NGS sequencing at 'Research Resource Center, Genome Research Division' Chicago, US at the lab of Dr. Stephan Green. The sequence analysis was done according to Amplicon Sequence Variants (ASV). Sequences were compared to representative sequences in GenBank by BLAST using the BioEdit Sequence Alignment Editor. Only sequences which had ≥ 97% in identity percentage, ≥ 80% in Query cover and $\leq 1e^{-20}$ were included in the final analysis.
Statistical tests of Kruskal Wallis and Mann-Whitney U test, were performed to test for significant presence of phyla between the four locations, as data was not normally distributed.

Analyses of richness and assemblage of ASVs and examination of the triplicate verification method, was performed in Excel (Microsoft Excel, 2019). All successfully identified taxa, were thoroughly researched within literature and online databases, to confirm their presence within the region of the Mediterranean Sea. All resources used are specified for each individual taxon in table S1, in supplementary material. For estimation of beta-diversity parameters (Table 3), the count table was Hellinger transformed. To examine differences in composition of biota detected by the eDNA method, among sites we applied the permutational analysis of variance (PERMANOVA) method using R package 'vegan' (version 2.5-7) function 'adonis'. We applied a nested model with sampling points nested within sites and the analysis was based on Jaccard distances calculated pair-wise between samples; 999 permutations were used. We calculated pair-wise differences using the R package 'pairwiseAdonis' function pairwise.adonis2 using the same design. Bonferroni correction was applied to correct for multiple hypotheses testing. In addition, we inspected similarity among samples by non-metric multidimensional scaling (NMDS) analysis. NMDS was calculated with three dimensions based on Jaccard distance matrix with 1000 random starts. Stress value for the NMDS was 0.11. Finally, a Krona plot was used to show an overview of the distribution of different phyla identified.

Results

Raw sequence processing

The raw sequencing results consisted of 2,273 ASVs. However, final analyses were done from a total of 1,280 sequences. The remaining sequences were either of non-target taxa, not clearly identifiable taxa or could not be found within the NCBI database.

Differences among biological replicates

The results show that biological replicates (triplicates), did not identify the same ASVs in each replica. As many as 45 % of the identified ASVs were found in only one of the three replicates. Further, as few as 23,7 % were identified in all three replicates (Figure 2). Finally, it should be noted that no triplicates of ASVs were detected within the requirements at the sampling site of Sdot Yam (as seen in the figure 2), thus data from this site, was not included in this analysis.

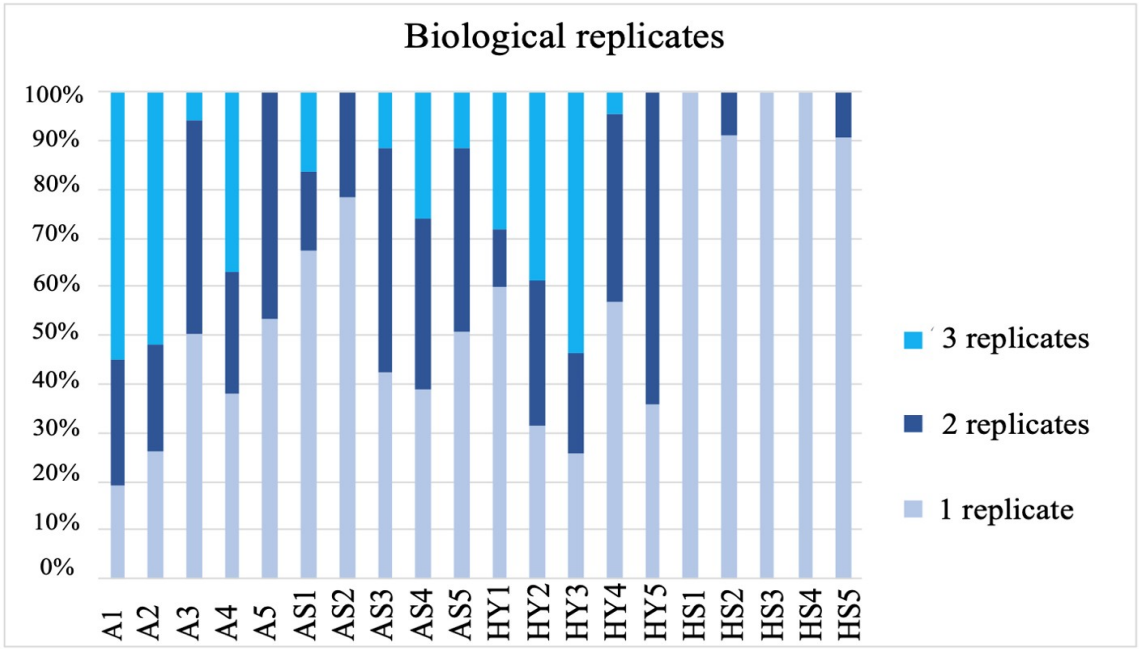

Figure 2: Graph displaying the percentage of identified ASVs found in 1, 2 of 3 replicates. HS labels represent the sampling site of Sdot Yam. The number following the letters indicate number of sampling station within each site.

Richness of ASVs

A total number of 386 unique ASVs (1215 repeated at sites/non-unique) were successfully identified using the NCBI BLAST database. Of the total amount of identified ASVs, 67.8 % were marine invertebrates. While 31.5 % are distributed within several algal taxa, vertebrates were only represented by 3 successfully identified ASVs, which make up 0.7 % of the total amount of ASVs. The ASVs (non-unique) were identified across the four sampling sites, divided into four stations of which the ASVs are distributed as follow: 47% Achziv, 28% Ashdod, 37 % Herzliya and 21% Sdot Yam (Figure 3). There 47 % found in Achziv is significantly higher in comparison to the remaining sites ($p<0.01$). As the same ASV could be found at different sites, the percentage exceeds 100% in total (Figure 3).

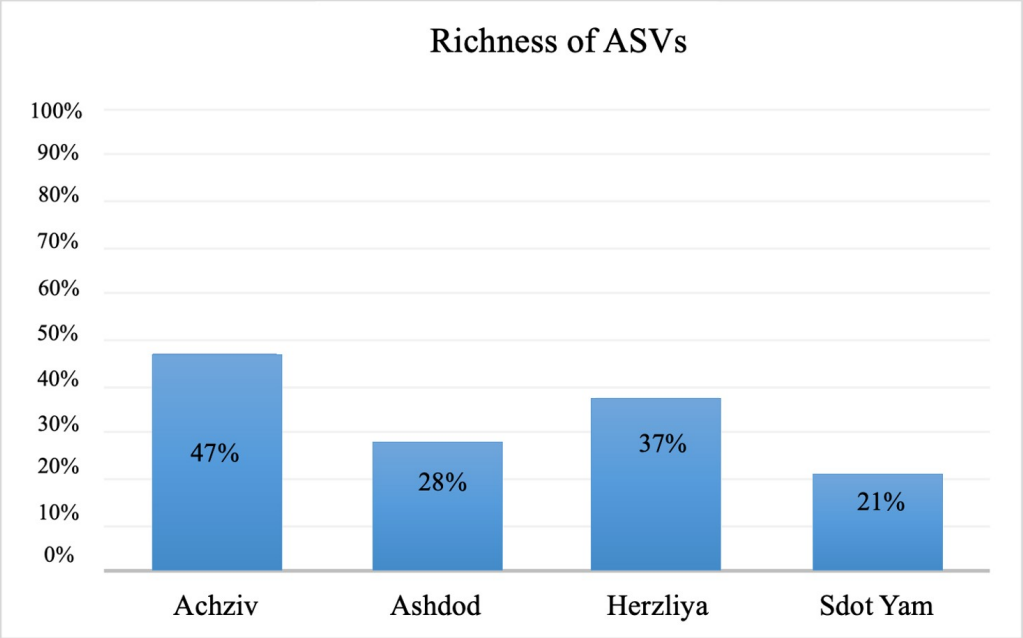

Figure 3: Percentage of unique ASVs identified at each of the four sampling sites.

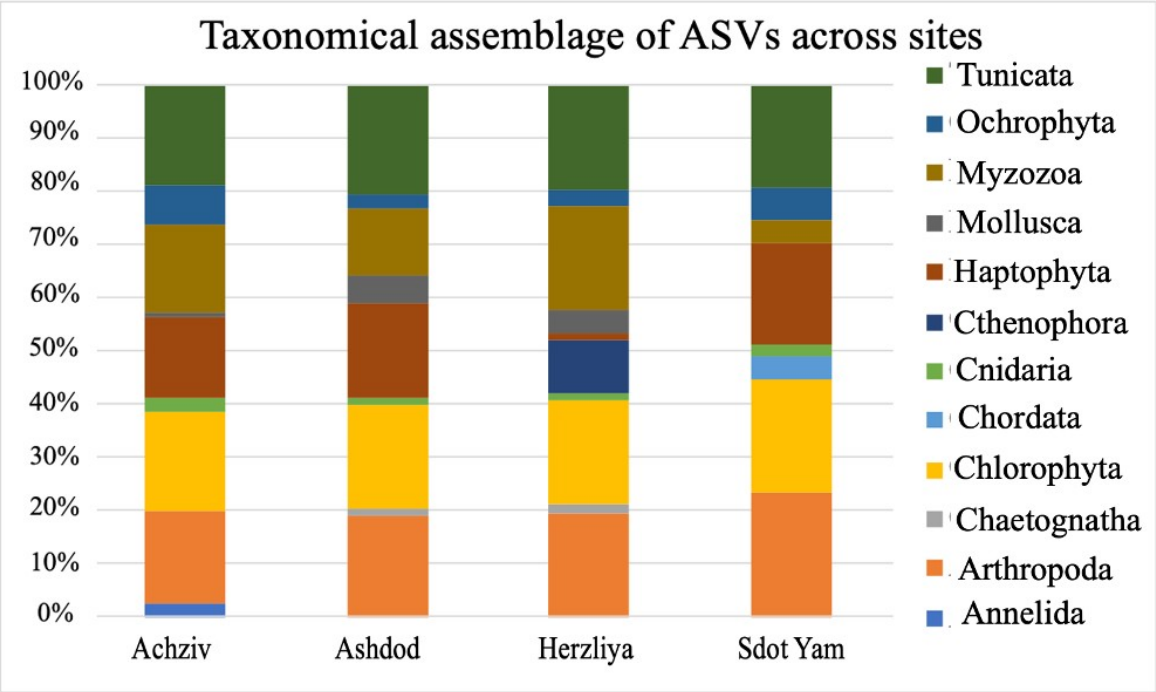

Figure 4: Assemblage of ASVs across the four sampling sites, taxonomically divided into identified phyla. Tunicata was classified as its own taxonomic class, as describe in the text.

Assemblage of ASVs

A total of 11 phyla and one subphylum (Tunicata) within the domain of Eukaryota were identified within the criteria mention in the methods. Chordata in this study is defined as vertebrates (fish, elasmobranch, reptiles, and mammals). Tunicata is defined in this study as a taxonomic group of its own. As it is only a sister group to vertebrates, although it is a subphylum of Chordata (Delsuc et al., 2016), it would be a misleading definition to include it under Chordata, in our analysis. There is a strong presentation of the phylum Arthropoda in all four sites as well as Tunicata and a general broad representation of various algal groups as seen in Figure 4.

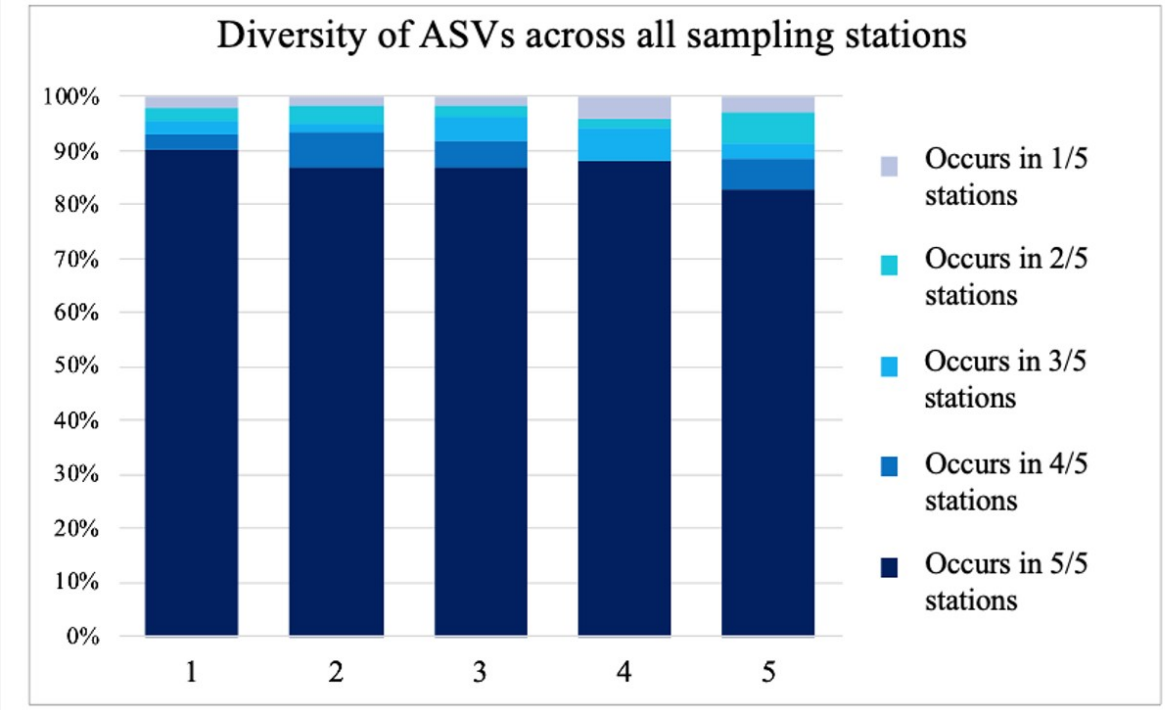

Figure 5: Overview of ASV diversity at all sampling sites and stations. All five stations from all four sampling sites (Achziv, Ashdod, Sdot Yam, Herzliya) merged in one graph, showing the overall diversity of ASVs identified at sampling sites.

Site Diversity

Large taxonomical overlaps are clear, as on average 87 % ASVs were found at all five stations, in all four sites (Figure 5). Whereas an average of only 3.23 % of ASVs occur in one of the five stations at the four sampling sites.

The three most dominating phyla identified for all four sites were by far Arthropoda (375 ASVs), followed by Tunicata (307 ASVs) and Chlorophyta (280 ASVs). The 3% that only occur in one out of five stations for the four sites, consists of Annelida, Ctenophora, Chordata, Cnidaria, Mollusca and Chaetognatha. Likewise, there were a few phyla that were only found in one or between two sampling sites (Figure 6).

Most noticeably was Annelida (16 ASVs), which was only detected in Achziv, Ctenophora (6 ASVs) was only found in Herzliya, as well as Chordata (3 ASVs) only being identified in Sdot Yam. Ashdod is the only sampling site, that does not have a unique identified phylum in this analysis.

However, despite the large taxonomical overlaps, the different species' community is significantly different, at each site. Further it is evident that the site sampling point play a significant role in the beta-diversity seen among the 4 sites (Table 3).

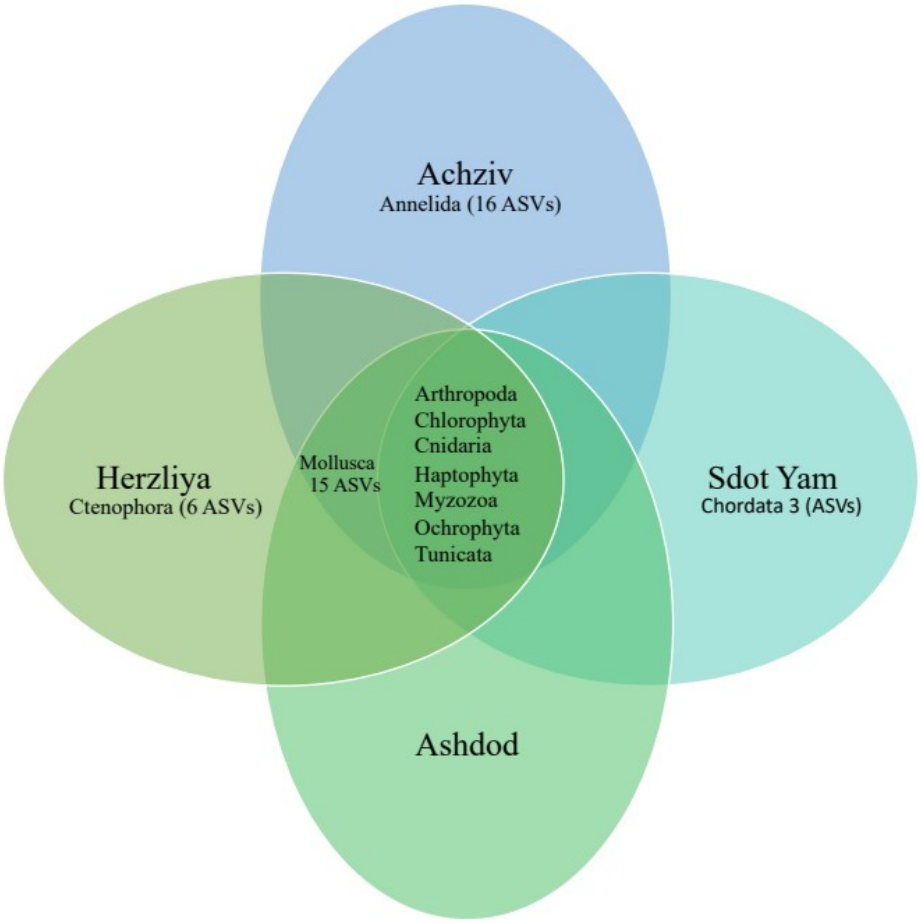

Figure 6: Venn-diagram of similarities and differences of identified ASVs, found between the four sampling sites.

Species diversity

The Simpson index was calculated for all four sites (table 2). The Simpson index range from 0 (low) to 1 (high).

Site	Simpson Index
Achziv	0.7817716
Ashdod	0.7751265
Herzliya	0.7710156
Sdot Yam	0.8596025

Table 2: Simpson index for all four sampling sites.

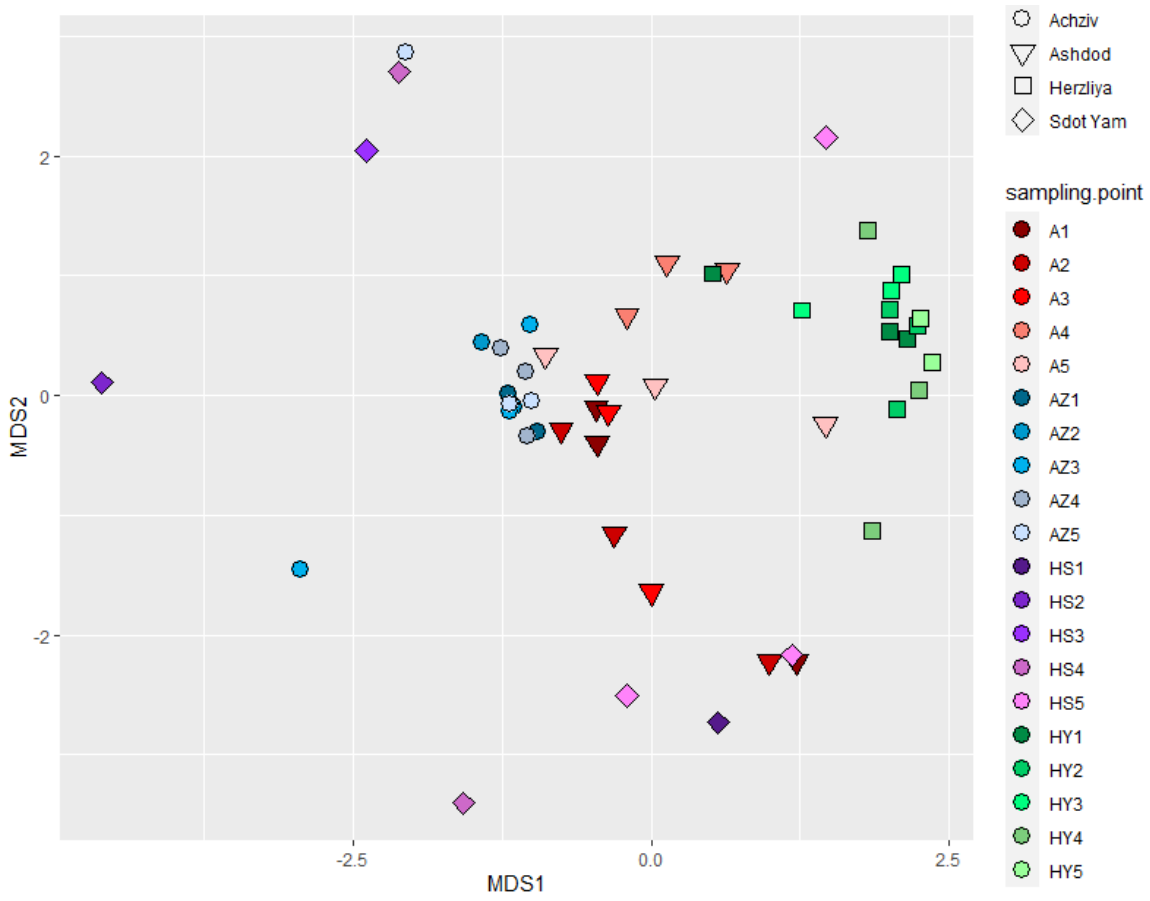

Figure 7. NMDS plot of the community diversity at all four sites with a stress value of 0.11. Each of the four symbols represents a sampling site with a corresponding color. The nuance of each color further presents the five sampling stations at each site. The letter code HS represents the sampling site Sdot Yam, as also shown in table 1.

Site	Achziv	Ashdod	Sdot Yam	Herzliya
Achziv		0.001	0.001	0.001
Ashdod	0.001		0.002	0.001
Sdot Yam	0.001	0.002		0.001
Herzliya	0.001	0.001	0.001	

Site sampling points	Achziv	Ashdod	Sdot Yam	Herzliya
Achziv		0.171	0.18	0.319
Ashdod	0.171		0.034	0.051
Sdot Yam	0.18	0.034		0.036
Herzliya	0.319	0.051	0.036	

Table 3. Results of statistical significance of beta-diversity among sites and sampling points.
The upper table shows the p value of the PERMANOVA test for the four different sampling sites.
The lower table shows the p value of the PERMANOVA test for the five different sampling points within each sampling site.

Taxa identified using eDNA

This study identified 53 taxa belonging to 11 different phyla and one subphylum (Tunicata). 50 taxa of the 53 (table 4) are known to the Mediterranean Sea and 70 % are known to the Eastern Mediterranean Sea and/or the Levantine Sea.

Further, out of the 53 taxa identified, 85 % was successfully identified to the species-specific level. This included endangered species such as the green sea turtle (*Chelonia mydas*), the invasive species Ctenophore *Mnemiopsis leidyi* and the Scyphozoan *Rhopilema nomadica*. The phylum of Arthropoda was the most observed phylum identified at all four sampling sites with 375 ASVs. This was followed by Tunicata with 307 ASVs and Chlorophyta with 280 ASVs (Figure 8). Ascidiacea, a class of Tunicata, was the only class found within the subphylum for all four sampling sites (table 4). Within the phylum of Arthropoda, all taxa belong to the subphylum of Crustacea. Within Crustacea, the most dominate group of organisms, was copepods with an overwhelming 99% of all identified species in that phylum. 20 different genera of copepods were identified of which 19 are confirmed to live in the Mediterranean Sea.

Phylum	Class/subphylum	Species
Annelida	Polychaeta	Sabellaria spinulosa (s)*
Anthropoda	Crustacea	Acartia clausii (s)**
		Acartia tonsa (s)**
		Anomalocera patersoni (s)*
		Balanidae (g)**
		Calocalanus minutus (s)
		Calocalanus styliremis (s)**
		Clausocalanus furcatus (s)**
		Clausocalanus lividus (s)**
		Clausocalanus paululus (s)**
		Euterpina acutifrons (s)**
		Lucicutia flavicornis (s)**
		Paracalanus denudatus (s)**
		Paracalanus indicus (s)*
		Paracalanus nanus (s)**
		Paracalanus parvus (s)**
		Paracalanus quasimodo (s)*
		Paracartia grani (s)**
		Parvocalanus crassirostris (s)**
		Pleopis polyphemoides (s)*
		Temora stylifera (s)**
	Maxillopoda	Balanus trigonus (s)**
Chaetognatha	Sagittoidea	Sagitta setosa (s)*
Chlorophyta	Mamiellophyceae	Bathycoccus prasinos (s)*
		Micromonas commoda (s)**
		Micromonas pusilla (s)*
Chordata	Actinopterygii	Pterois miles (s)**
	Mammalia	Homo sapiens (s)**
	Reptilia	Chelonia mydas (s)**
Cnidaria	Hydrozoa	Clytia gracilis (s)**
		Obelia dichotoma (s)*
		Pennaria disticha (s)**
	Scyphozoa	Aglaura hemistoma (s)*
		Ectopleura dumortieri (s)**
		Rhopilema nomadica (s)**
Ctenophora	Tentaculata	Mnemiopsis leidyi (s)**
Haptophyta	Prymnesiophyceae	Emiliania huxleyi (s)**
Mollusca	Bivalvia	Dendostrea (g)**
	Gastropoda	Creseis virgula (s)**
Myzozoa	Alveolate	Heterocapsa rotundata (s)*
		Karlodinium sp (g) **
		Protoperidinium (g)**
Ochrophyta	Bacillariophyceae	Minutocellus polymorphus (s)**
		Skeletonema menzellii (s)*
		Thalassiosira profunda (s)*
	Pelagophyceae	Pelagomonas calceolata (s) *
Tunicata	Ascidiacea	Ascidia (g)**

Table 4. List of species identified to the closest identifiable taxonomic rank. Each taxon is indicated to which level of identification it was identified: Species (s), Genus (g), Class (c). The taxa are further indicated to which area they have previously been observed: * known to the mediterranean, **known to the Eastern Mediterranean.

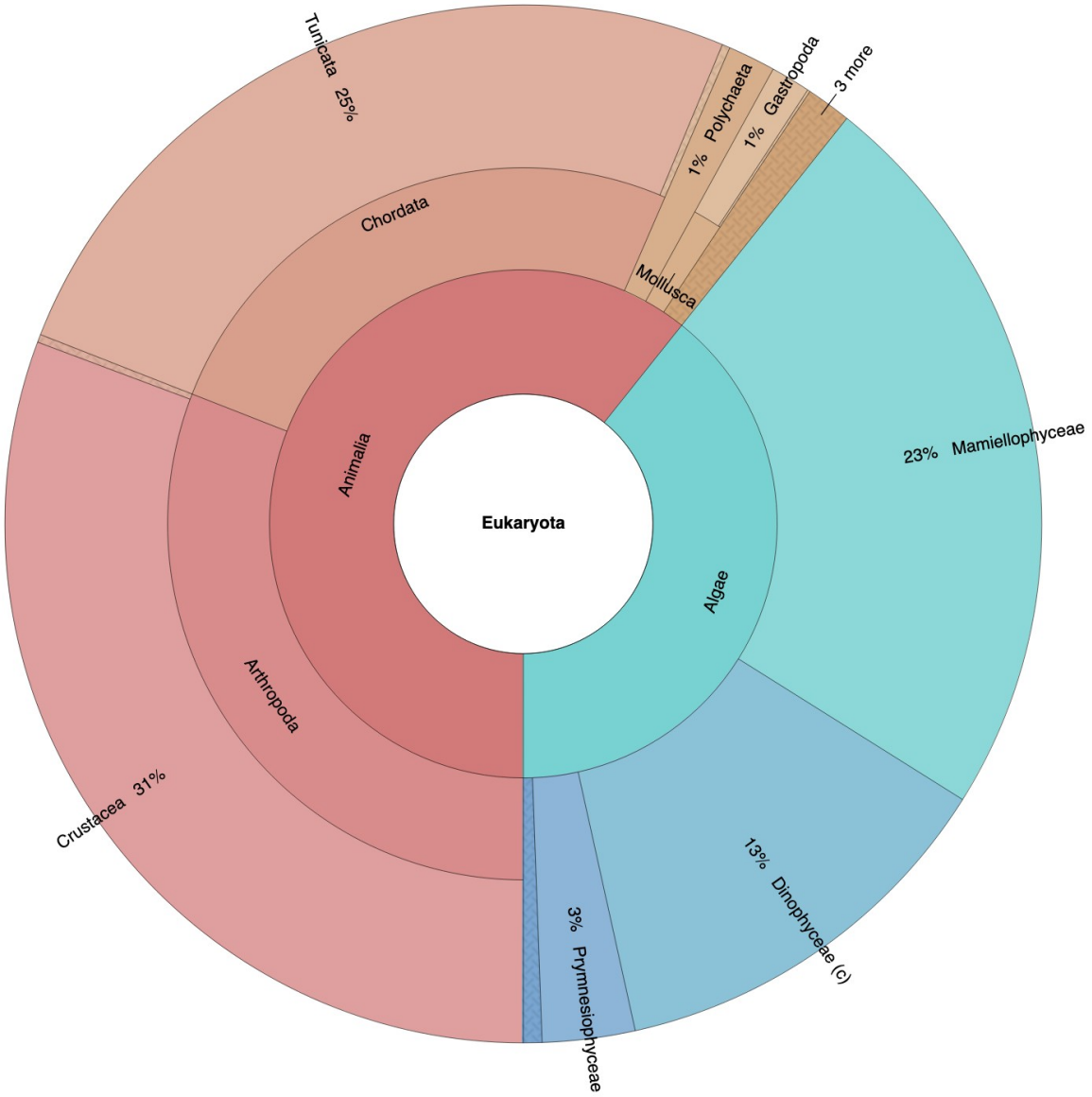

Figure 8. Krona plot showing taxonomic distribution of all identified classes and phyla. Identified classes with less than 1% of the total amount of ASVs are not visible in the plot.

Discussion

Richness of ASVs

There is a noticeable difference between the richness in ASVs among the sampling sites, most evident is the stark difference (Figure 3) between Achziv (47%) and Sdot Yam (21%). While several factors play in, when it comes to richness of ASVs in samples (Diaz-Ferguson et al.2014), such as light and temperature, the sampling processing is also likely to affect the outcome of the sequencing (Thomsen et al., 2015). Due to the practical issues, the samples from Sdot Yam were not able to be processed upon arrival to the research station. The water bottles were instead, frozen down until the following morning where only then, they were filtered. While this cannot be further validated as a cause for the low amount of ASVs, a study (Hinlo et al. 2017) found the retrieval of DNA to decrease, when stored in the freezer overnight. It is therefore very plausible, that the storage of this sample set affected the richness of the DNA in the sample set. Interestingly, despite having the lowest amount of ASVs, Sdot Yam still has a more distinct species composition compared to the three other sites. This is also evident in the Simpson biodiversity index (SDI) in Table 2, with a slightly higher SDI for the sampling site of Sdot Yam.

Site diversity

The results suggest according to the Simpson index (Table 3) a relative high biodiversity at the four sites, however with a high homogeneity among the five stations, at each site (Figure 4 & 5). This could possibly be due to the lack of heterogeneity that eDNA displays throughout the water column and insufficient mixing (by shaking) of the water samples prior to filtration (Andruszkiewicz et al., 2017, Port et al., 2016, Valsecchi et al., 2020). Finally, habitat preference among species is also known to influence homogeneity in detectability of eDNA (Klymus et al., 2017). The high diversity of species compositions for Sdot Yam, is surprising as the site with the highest presumable biodiversity is Achziv, as the site is in within a Marine Protected Area. However, many factors could play into this equation, one explanation could be the technical aspects of sampling at the given site.

As seen in table 1, while all samples were taken from the surface, the sampling at Sdot Yam, was done with an increasing distance to shore and consequently at an increasing depth to the sea floor. Perhaps, this could be part of the explanation as to why the species composition is higher and significantly different there.

The smaller difference seen in species composition among three of the sites (Achziv, Herzliya and Sdot yam) as seen in Figure 6, particularly the small presence of Ctenophora (6 ASVs) only detected in Herzliya. Ctenophora are more abundant towards the spring and the summer in the region (Gülsahin & Tarkan, 2013) and thus their site-specific detection could be explained by the different sampling date. The samples from Herzliya were done later in the season towards the spring (09/03/21), in contrast to the other sites, which were sampled in the end of January (table 1). The site-specific detection of Annelida in Achziv and Chordata in Sdot Yam, seem to more randomly associated with the sites in terms of detection, given the small aspect of this study. Thus, their detection does not seem to hold any apparent significance.

Identified taxa

There is generally a large knowledge gap of most invertebrates in the Levantine Sea compared to the western part of the Mediterranean Sea (Coll et al., 2010). Thus, confirming the presence of the species found in this study, is challenging and to some extent limited.

The absence of a species (never observed, described or confirmed), does certainly not exclude the possibility of its presence in the area. Many species, especially smaller invertebrates are still being discovered each year particularly in the Eastern Mediterranean Sea (Coll. et al., 2011).

Therefore, while the results of eDNA, should be thoroughly evaluated and its limitations considered, an identification of a new regional species, is very much possible. Out of the total amount of taxa identified, 95 % of them are known to live in the Mediterranean Sea.

An additional 70% have been observed in the Eastern Mediterranean Sea and/or in the Levantine Basin (table 4).

The remaining taxa, which are currently not known to the Eastern Mediterranean, could be due to various reasons. One reason could simply be that the species is new to the region, thus through eDNA its new geographical distribution has been revealed.
Another possible explanation could be insufficient molecular knowledge of the species; thus, it could not be properly identified through the genetic databases and/or misidentified.
Lastly, improper sequencing could also cause misidentification (Andruszkiewicz et al., 2017).

The results showed a dominance of the phylum Arthropoda in all four sampling sites. This represents well, the knowledge of it being by far the largest and most diverse phylum in animal kingdom, with 80% of all described animals in the world (Zhang et al., 2011). Further, Crustacea is one of the largest subphyla within Arthropoda, this is further evident in the results of this study with 99% of all species identified within this phylum belonged to the subphylum of Crustacea and more specifically to the group of copepods. As copepods are pelagic organism, it is predictable to find a large representation of them in this study, as the samples were exclusively sampled at the surface. This is further supported by the detection of the copepod *Paracalanus parvus*, which is highly representative in the Mediterranean Sea along the coastal regions and is one of the most abundant and most representative of the genus (Kasapidis et al., 2018). Similarly, Ascidiacea was exclusively represented with 100 % of all identified genera within this taxon. This is further well-represented, as Ascidiacea is by far the class, with most described species, making it the largest class within the subphylum of Tunicata (Coll et al. 2011). Thus, it can be argued that within the small scope of this project, the various findings, are arguable supported by what is known from the literature.

Biological replicates

Sampling biological replicates from the field is a relatively new verification method within eDNA. Previous studies have only included replicates of pooled samples or pseudoreplicates; a large volume sample, split into multiple subsamples (Andruszkiewicz et al., 2017).
The results from this study, the majority (48%) of ASVs were only identified in one out of the three biological replicates (Figure 2).

Additionally, no triplicates from Sdot Yam were identified within the given threshold applied to all samples. Therefore, while verifying the identification of the same ASVs in all three replicates, is important for data accuracy, this method might not be a true representative of that. One study, Andruszkiewicz (2017) found only 0-13 % of the identified ASVs were detectable in all three replicates. The results of our study are in comparison therefore, relatively decent with 23.7% of ASVs detectable in all three replicates. As mentioned earlier, a study found (Hinlo et al. 2017), that the retrieval of DNA decrease if the samples are stored in the freezer overnight. This could thereby not only have affected the richness of ASVs, but also affected the identification of ASVs in biological replicates, as DNA could have been lost in the process. Another study (Souza et al., 2016), found high variability in detectability based on series of replicates, the seasonal temperature as well as the seasonal activity of species of the time of sampling. The study concludes a series of replicates of 4-14 (cold season) or 10-32 (warm season) depending on the seasonal activity of the species of interest, are needed in order to achieve 95% probability of detection.

It has generally been shown that small water samples of many replicates, provides the most accurate detection (Ruppert et al., 2019). Lastly, the stochastic nature of the PCR process, could provide another explanation for the high variability in identified ASVs in the biological replicates to a certain extent. Therefore, for future studies of this kind, it would be advisable to sample a larger series of biological replicates of 1 liter or more, in order to increase the detectability rate.

Lack of vertebrate DNA

Although these primers are designed to target all taxa within metazoan diversity, it is noticeable that a surprisingly low number of ASVs (0.7 %) were identified belonging to the phylum of Chordata (green sea turtle and human DNA). Although vertebrates overall represent a small part of the animal kingdom, a larger representation in this study was expected. Marine vertebrates only make up approximately 4% of all taxonomic groups in the Mediterranean Sea (Coll et al., 2011).

Their biomass and density of DNA is less present in the ocean, compared to smaller and more abundant species (Deagle et al., 2014), particular in surface water (Djurhuus et al. 2018).

Therefore, it can be speculated that either there was a very limited amount of vertebrate DNA present in the water at the time of sampling, or vertebrate DNA was simply not in the water, that was sampled within the three liters of samples at each station.
Further, with the use of primers which target metazoan diversity, perhaps organisms with an overall smaller abundance in the ocean, and therefore a lower level of DNA present in samples, are not proportionally detected, as evident in other studies (Ruppert et al., 2019; Thomsen et al., 2012). It is apparent that studies, which focus on monitoring of marine vertebrates use primers that are specifically designed to detect their target-species, in order to avoid this issue (Andruszkiewicz et al. 2017, Baker et al., 2018, Bakker et al.2017., Port et al., 2016, Thomsen et al., 2012).

Limitations of eDNA

Environmental DNA can be used for a variety of different monitoring purposes, ranging from targeting single species or taxonomic groups by designing specific primers, to biodiversity surveys of entire ecosystems as it detects and compares species between sites. While eDNA holds huge potential and has been proven as a highly effective monitoring tool, it does have its limitations, as any science does. One very noticeable risk is that even if DNA from a particular species is in fact present in the water, eDNA is not guaranteed to provide the detection of that species, in the sequencing results (Pilliod et al., 2019). This could be due to several reasons, as mentioned above. It is therefore wise to invest in research that can further evaluate the limitations of eDNA, particularly when using the method for ecosystem monitoring.

There is currently a large knowledge gap, when it comes to understanding the range and precision of using eDNA for ecosystem monitoring. It is currently not possible to achieve precise quantitively measures of a species biomass, due to the random and unpredicted timing and behavior of shedded of DNA.
Similarly, while most studies within eDNA set the threshold of percentage identity at 97%, Klymus (2017) showed species that were present in an aquarium, were detected at a lower percentage, thereby raising the question where the threshold should be set for eDNA studies.

More can still be done to understand behavioral and seasonal tendencies. One way could be to test the method repeatedly in an enclosed environment (aquarium) with known species. It has previously been shown that eDNA is more sensitive to detecting biomass rather than abundance, in terms of reads (Klymus et al., 2017). Therefore, ideally, a small group of the same species with the same body size, could allow for more precise measurements, rather than a large group of different species with different body sizes. This is also due to the assumption, that the same species follow the same life stages and behavioral activity (Souza et al., 2016), thus the shedding of DNA is more likely to be unanimous. By adding more individuals of the same species to that enclosed environment, as the repeated sampling continues, it could perhaps be possible to better understand the quantity and behavior of shedded DNA within a controlled environment.

Other challenges include the uncertainty of knowing the origin of the DNA, whether it is of dead or living cells or at which live stage the organism shed the genetic material.
Choosing the right set of primers for an eDNA metabarcoding study, is crucial as this inevitably will influence sequencing results, to a lesser or greater extent (Ruppert et al., 2019). Likewise, obtaining the highest species diversity among sequencing results and conservation error trimming, presents an obvious tradeoff within bioinformatics (Coissac et al., 2012). eDNA as a method, is in its current state not suitable for estimation of biomass and abundance of species in ecosystems.
This is due to primer bias and PCR bias (mentioned above), which make estimation and comparison between taxonomic groups, too uncertain. Therefore, it is advised to use multiple primer sets to properly cover all groups across the tree of life, as some primers do not amplify certain taxonomic groups well enough, to provide a fair representation (Ruppert et al., 2019).

Likewise, relative abundance can only be compared among the same species or taxonomic group at different sites. However, due to the uncertainty and randomness of various factors that can influence the transport and decay of eDNA, such estimates should be considered accordingly.

eDNA is sensitive to sunlight, temperature, and other abiotic conditions in the ocean. Further, the DNA is estimated to break down within 7 days in saltwater (Diaz-Ferguson et al., 2014), and thus can only function as a temporary survey, which has both its advantages and disadvantages. On one hand, the fast degradation of eDNA allows for detection of species currently present in the environment. On the other hand, it only provides a short time window of species present in the given area (Thomsen et al., 2015).
eDNA undoubtably provides a powerful monitoring tool, however it cannot fully replace traditional surveys, due to its reliance on adequate sequencing homology, achieved through public databases (Cowart et al., 2018) and its relatively large knowledge gap as mentioned above.

An alternative method, which could allow for less abundant species to be proportionally represented in sequencing results. is Droplet Digital PCR (ddPCR) as described in Baker (2018). This method has so far only been used in a few studies of eDNA, yet seems to hold great potential to provide more accurate detectability (Miotke et al., 2015). This method fractions PCR reactions into over 20,000 droplets, by using oil emulsion (Doi et al., 2015). Using fluorescence dye in the PCR reaction, which targets the specific sequences, that have been bracketed by the primers. The ratio of DNA that is positively detected and the DNA that was negatively detected, is then individually counted, and compared. This method builds on the assumption, that the molecules of the target sequence, are among the 20,000 droplets and follow a Poisson distribution. Thus, this method does not include quantification through standard curves thereby eliminates any variance regarding each batch (Cao et al., 2016). Until more is known about detectability and accuracy of eDNA, a continued joined effort utilizing both methodologies (conventional and molecular), would be most efficient in terms of conservation and management approaches.
Overall, this study has demonstrated eDNA's ability to successfully identify marine species which are known to the region of the Mediterranean Sea.

Several modifications of the protocol are considerable, particularly in a warmer climate such as the Eastern Mediterranean Sea. These include implementing immediate filtration at sea, which will ensure the highest retrieval of DNA, as the samples are not stored until filtration. Further, increasing the sample volume could be beneficial, as in contrast to an aquarium, the open ocean contains much less concentrated DNA of the target species. Therefore, one might calibrate accordingly and increase the volume of water for each sample to several liters.

Although it has its limitations, we believe it can serve as a powerful supplement to traditional surveys, as it in many cases, allows for detection of species, which would otherwise go unnoticed. Overall, these results represent a very small area with a very small sample set, thus at best, only displays a shallow and limited survey of the sampling sites. Therefore, the true diversity might not be represented within the limited scope of this project, as much can be influenced by stochastic factors and bias. Finally, sampling sizes should also be considered, as naturally the more samples collected, the higher the likelihood of a larger diversity. Therefore, depending on the aim of a project, substantial sampling sizes and replicates could be essential for true representation of a site's diversity (Souza et al., 2016).

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Supplementary Material, Table S1. List of taxa and their source of geographical identification

Phylum	Class/subphylum	Species
Annelida	Polychaeta	Sabellaria spinulosa [1]
Anthropoda	Crustacea	Acartia clausii [2]
		Acartia tonsa [2]
		Anomalocera patersoni [3]
		Balanidae [4]
		Calocalanus minutus*
		Calocalanus styliremis [2]
		Clausocalanus furcatus [2]
		Clausocalanus lividus [2]
		Clausocalanus paululus [2]
		Euterpina acutifrons [2]
		Lucicutia flavicornis [2]
		Paracalanus denudatus [2]
		Paracalanus indicus [6]
		Paracalanus nanus [2]
		Paracalanus parvus [2]
		Paracalanus quasimodo [7]
		Paracartia grani [2]
		Parvocalanus crassirostris [8]
		Pleopis polyphemoides [9]
		Temora stylifera [2]
	Maxillopoda	Balanus trigonus [10]
Chaetognatha	Sagittoidea	Sagitta setosa [11]
Chlorophyta	Mamiellophyceae	Bathycoccus prasinos [12]
		Micromonas commoda [13]
		Micromonas pusilla [14]
Chordata	Actinopterygii	Pterois miles [15]
	Mammalia	Homo sapiens**
	Reptilia	Chelonia mydas [16]
Cnidaria	Hydrozoa	Clytia gracilis [17]
		Obelia dichotoma [17]
		Pennaria disticha [10]
	Scyphozoa	Aglaura hemistoma [18]
		Ectopleura dumortieri [19]
		Rhopilema nomadica [20]
Ctenophora	Tentaculata	Mnemiopsis leidyi [21]
Haptophyta	Prymnesiophyceae	Emiliania huxleyi [22]
Mollusca	Bivalvia	Dendostrea [23]
	Gastropoda	Creseis virgula [24]
Myzozoa	Alveolate	Heterocapsa rotundata [23]
		Karlodinium sp [23]
		Protoperidinium [10]
Ochrophyta	Bacillariophyceae	Minutocellus polymorphus [25]
		Skeletonema menzellii [26]
		Thalassiosira profunda [27]
	Pelagophyceae	Pelagomonas calceolata [28]
Tunicata	Ascidiacea	Ascidia [16]**

Table 5. Each number corresponds to a reference in the reference list below. * Indicates when taxa could not be confirmed in the Mediterranean. ** indicates when reference is assumed not needed.

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דנ"א סביבתי כשיטת ניטור לא פולשנית לזיהוי מינים ימיים באגן הלוונט במזרח הים התיכון

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תקציר

דנ"א סביבתי תפס תאוצה בשנים האחרונות כשיטת ניטור יעילה ולא פולשנית בכל רחבי העולם. מזרח הים התיכון הינו אחד מהאזורים הכי פחות נחקרים בים התיכון והידע על המגוון הביולוגי שבו לוקה בחסר. לפיכך, ניטור ומחקר המגוון הביולוגי באזור הינו בעל חשיבות עליונה על מנת לקבל את התמונה המלאה באזור, לפני ירידות נוספות במגוון. במסגרת המחקר, נאספו 60 דגימות מארבעה אתרי דיגום לאורך קו החוף הישראלי של מזרח הים התיכון מהצפון (אכזיב) ועד הדרום (אשדוד). 95% מהמינים הידועים כמיני ים תיכון מצויים גם במזרח הים התיכון, יחד עם 70% מהמינים אשר ידועים כי נפוצים באזור. בנוסף, מצאנו הבדלים משמעותיים הן בעושר והן בהרכבת ASV בין אתרי הדיגום שונים. עוד נידון ההבדל במגוון הביולוגי ובהרכבי הפילום שזוהו באתרי הדיגום השונים ומרכיבים בעיקר חסרי חוליות. אנו בודקים את הדיוק של שכפול ביולוגי ובוחנים את חשיבותם בשימוש ב- eDNA ככלי ניטור. לבסוף, הראינו את החשיבות של הגדלת גודל הדגימה, בכדי להגדיל את שיעור הזיהוי. מחקר זה מתפתח ומדגיש לראשונה באזור את השימוש והיתרונות של eDNA ככלי ניטור, מכיוון ששיטה זו יכולה לחשוף דפוסים משמעותיים, שלעתים קרובות מפוספסים בשיטות סקר מסורתיות.

דנ״א סביבתי כשיטת ניטור לא פולשנית לזיהוי מינים ימיים באגן הלוונט במזרח הים התיכון

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