Reviewer 1 (Jean-David Grattepanche)

Basic reporting

• English needs to be checked as some sentences are not clear (e.g., L98-100: do you mean "remains rather unclear"?; L114-118: the sentence is too long and needs to be split for better understanding; L124-126: I not sure I understood this sentence; L134: replace target by objective; etc).

Thank you for the observations, we performed these changes as suggested

• I think the title should be rephrased for clarity, something like "Increased parasites contribution in microbial eukaryotic community of the Aegean coastal water".

Thank you for noticing this we rephrased to "**Increased parasite contribution in coastal microbial eukaryotic communities**"

• NGS (next gen sequencing; L 101) is an old terminology and should be replaced by HTS (high-throughput sequencing)

Thank you for your comment we changed the terminology

• Also there are many typos. Here are few of them: L211: "Similariites", L 286 & 353 & 358 "MICE"?, L365 "Syndiniales ,and mixotrophs while", L 366 with.mixotrophic, L418 "cilates", L426 "because of to", L435 "dinoflaggelate", L439 "Prorocenrum"

Thank you for your comments. We performed the respective changes

• There is many supplemental information, some should most likely be included in the main text (e.g. Fig S2 cited six times)

Thank you for this suggestion but Fig. S2 is cited four times and these results are supplementary to our main findings so we do not believe that they could serve in the main text.

• There is not figure 6 (L 442) and Table 3 (L334, 337)

This was also a typo. The correct is figure 5b and Table 2 (Table S6 in the updated version) .

• Please homogenize OTU notation (OTU59 or Otu00059)

We homogenized OTU notation.

Experimental design

• Can the authors better explain the sampling strategy? There is seven coastal site (not nine as mentioned in Table 1) and five stations per site? and three depths per station, so 105 samples (or 135 if really nine sites). So, what are the 112 samples mentioned? the map Fig S1 can be useful in the main text to understand distance between sites. Kodias or Kontias ?

Some samples (Table S1) were collected in replicates. For this reason instead of 105 samples we totally collected 112.

It is Kodias, thank you for noticing that. We changed Table 1 respectively.

• Can the authors justify the use of the v2-v3 primer? Most of the works on microbial eukaryotic community has been done using the V9 primer (de Vargas et al 2015) or the V4 primer (Stoeck et al 2010, Pernice et al 2016). How do your primer set compare to the others?

The primers were selected in 2016 since the same samples were used for phytoplankton community analysis in the same area (Spatharis et al., 2019). The use of these primers by Genitsaris et al. 2015, successfully detecting the majority of eukaryotic groups in the English Channel further supported our choice.

I still think some discussion should be included in the manuscript, especially to broader the conclusions by comparing with other studies.

• The bioinformatics is not really clear and not common for eukaryotic work. Please see Pernice et al 2016, or Christaki et al 2023 for common practice. L193 you mention SILVA but I am not sure for what and then L202 you mentioned PR2 (not PR4). Generally, the

sample need to have the same depth to be compared. You calculated Bray-Curtis similarities using Hellinger transformed abundance, so you actually using Manhattan distance. To use Bray-Curtis similarities index you should use untransformed data with the risk of uneven sampling depth or rarefy your samples before calculation. The same should be applied to compare alpha-diversity.

Thank you for this comment. SILVA was used for the alignment and PR2 for the taxonomic classification that is the common practice when using mothur (alignment and then taxonomy). Christaki et al. 2023 have used completely different methods since they analyzed ASVs, while in Pernice et al. 2016 they performed pyrosequencing and also used SILVA and PR2.

We agree with your comment regarding Bray-Curtis and Hellinger, thus we recalculated all bray-curtis similarities with non-transformed data. (l.219-220/l.277-282)

While I agree that Pernice et al 2016 used a similar approach, the method is at least 7 years old and things are changing rapidly in bioinformatics and in regard of knowledge on protists. As mentioned by the reviewer 2, a clustering approach at 97% does not look appropriate for protists, because some clades (e.g., dinoflagellates) will show little variation in their SSU sequences and therefore you will collapse several taxa in one OTU. Now, with your goal of looking at difference in taxa composition between habitats, you ran the risk of losing signal using this approach and you should acknowledge this possibility in the discussion.

Validity of the findings

• More details about the environment are needed to conclude that each sample or gulf represented a different niche (L225-226 and L329-331). Levins' niche index is interesting here but needs to be used on contrasted environment (the R of the index means differing environments). A top generalist needs to be observed in more than 15 samples and so can be present in only 1 site? Did you remove the replicate samples before performing this analysis? I think some work/analysis showing the difference between samples, depth, stations, sites are therefore needed (e.g., PCA), especially given the communities are grouping/clustering by site for each depth. What about the depth? Are the communities sampled at different depths for a station, for a site, and across sites similar?

- The way B is calculated does not take environmental conditions into account but only niche specialization. Therefore we do not agree that specific analysis of environmental factors is needed in order to define different niches. We added a description for this on lines 236-239..
- What is the R of the index? We are sorry but we cannot find it. Okay. There is a more recent version. $Bn[j] = (1/R)/ \sum_{i=1}^{N} p_{ij}^2$ (Feinsinger et al. 1981. A simple measure of niche breadth. Ecology 62(1):27-32). In this version, number of sample and number of different environments are separated (see following comment).
- A top generalist has a value >15 but this does not mean that it needs to be observed in more than 15 samples.
- No we did not remove the replicate samples. Not removing the replicate will increase the weight of some samples over others. Is the analysis similar without the replicate samples?
- Clustering by site was not that evident. As shown in Fig. S2a within gulfs similarities were quite high only in surface. Also in Fig.1 although communities look differentiated the stress is very high and this was also mentioned in the manuscript l. 284-285. Also we have also mentioned the environmental factors that are important for this grouping in the manuscript (l. 285-289). Using hierarchical clustering you can group samples by similarity (e.g., Donoso, K., F. Carlotti, M. Pagano, B. P. V. Hunt,

R. Escribano, and L. Berline (2017), Zooplankton community response to the winter 2013 deep convection process in the NW Mediterranean Sea, J. Geophys. Res. Oceans, 122, 2319-2338, doi:10.1002/ 2016JC012176.), which can be more meaningful that considering all sample as an ecological niche/habitat regarding physico-chemical conditions. By the way, adding the stress on figure 1 could be useful for the readers.

• Some clarifications are needed about OTU classification as abundant or rare. Are you considering 1% in the whole dataset or 1% in a sample? Are you considering the raw data or the transformed data? If it is 1% in the whole dataset, how can be relative abundance of the 25 abundant OTUs less than 5%? IF it is 1% in a sample, how you classify an OUT with 2% in a sample and 0.1% in another?

Percentages are calculated in the whole dataset and this is how OTUs were characterized as abundant, rare etc. We clarified this now (l. 235-237: "OTUs were classified as abundant or rare based on total relative abundances of on non-transformed number of reads and on the thresholds…"

• Can you explain the difference between nanograzers and nanoheterotrophs? Why are pico- and micro-heterotrophs missing in figure 6?

Nanograzers feed on nanoplankton while nanoheterotrophs feed on picoplankton(bacteria). These classifications as also cited in the text were based on Genitsaris et al. 2015 and 2016.

Thanks for the clarification. Use of pico-, nano- and micrograzers does make more sense to me as in Genitsaris et al (2016).

There is no figure 6 but if you mean figure 5 these groups are not there because there were no significant correlations. Ok thanks.

• L405-407: a better analysis of environmental parameters, as suggested above, can help with this aspect.

This conclusion is based on previous studies (cited above) characterizing the east compartment more niche rich compared to the west compartment

Ok, but this contradicts your reply to my last comment.

• I do not understand your network analysis. The idea is generally to summarize the interaction in an ecosystem, but you decided to only consider OTUs differentially abundant across sites. Main players such as OTUs abundant at all sites, or without abundance variations across sites were excluded from the analysis. This aspect should be more discussed, especially the lack of (L481) "clear interactions between phytoplankton species and their grazers".

As mentioned in the introduction "In this study **we tried to define taxa that are important for differences** detected in the marine microbial network across the different gulfs **studied as well as their characteristics in terms of trophic level** (autotrophs, mixotrophs, heterotrophs, parasites), **niche breadth** (generalists, specialists) and abundance (abundant, rare). **Our target was to elucidate whether these taxa and their trophic and ecological characteristics differentiate between different depths, as well as their associations. "** This is the reason why we focused only in the differentially abundant species between gulfs

The use of the network still does not make sense to me. I understand that you identified the OTUs differentially present in your samples and hypothesized that their environments are responsible of their presence/ecology. But:

1- How can you identify difference with the 97% cutoff? Some close relatives can be differentially present in your samples.

2- Did you consider the replicate in this analysis? Are the same OTUs present in your replicates?
3- As you have mentioned in the text, removing the abundant OTUs, you may observed not only direct but also indirect connection.

• L479-480: “explaining microbial network differences observed across the different gulfs studied”. You performed network per depth but not per site, so how can you have studied the difference between gulfs?
Thank you for noticing this. As you can see we do not refer to our networks (Fig.5) but to the protistan network in general that is presented in the whole manuscript. Ok, that was not clear to me, maybe using protistan community instead of network can help clarify the sentence.
• Some results are presented (e.g., L276-277) but not discuss.
We do not move to the ecological interpretation of the dominance of specific groups as such patterns change with time and in our study we did not aim in temporal variabilities. See my previous comment. Can you clarify?

Increased parasite contribution in coastal microbial eukaryotic communities

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Abstract

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Background-Aim

Protistan communities have a major contribution to biochemical processes and food webs in coastal ecosystems. However, related studies are scarce and usually limited in specific groups and/or sites. The present study examined the spatial structure of the entire protistan community in seven different gulfs and three different depths in a regional Mediterranean Sea, aiming to define taxa that are important for differences detected in the marine microbial network across the different gulfs studied as well as their trophic interactions.

Methods

Protistan community structure analysis was based on the diversity of the V2–V3 hypervariable region of the 18S rRNA gene. Operational taxonomic units (OTUs) were identified using a 97% sequence identity threshold and were characterized based on their taxonomy, trophic role, abundance and niche specialization level. The differentially abundant, between gulfs, OTUs were considered for all depths and interactions amongst them were calculated, with statistic and network analysis,

Results

It was shown that Dinophyceae, Bacillariophyta and Syndiniales were the most abundant groups, prevalent in all sites and depths. Gulfs separation was more striking at surface corroborating with changes in environmental factors, while it was less pronounced in higher depths. The study of differentially abundant, between gulfs, OTUs revealed that the strongest biotic interactions in all depths occurred between parasite species (mainly *Syndiniales*) and other trophic groups. Most of these species were

generalists but not abundant highlighting the importance of rare species in protistan community assemblage.

Conclusion

Overall this study revealed the emergence of parasites as important contributors in protistan network regulation regardless of depth.

Keywords: coastal systems, Protist communities, parasites, 18S rRNA

Introduction

Single-celled eukaryotes play an important role in numerous essential ecological and biogeochemical processes within marine ecosystems globally, serving as primary producers, predators, decomposers and parasites [Sherr & Sherr, 2002]. Despite their importance, the distribution of protists within the pelagic environment as well as the role of protistan interactions within marine food webs remains rather unclear. The emergence of high-throughput sequencing (HTS) in the last decades has enabled more in depth studies on the distribution, metabolic activity, trophic status and biological interactions of marine protists, mainly focusing on over depth and/or seasonal changes [Giner et al., 2020; Ollison et al., 2021; Yeh et al., 2022]. Most of the published literature is focusing on depth and/or seasonal changes in oceanic samples reaching depths of up to 4000 m [Giner et al., 2020]. Other studies also working in open ocean sites have tested protists dispersal in ocean surface showing that only 0.35% of the operational taxonomic units (OTUs) detected are indeed cosmopolitan implying dispersal limitations [Burki, Sandin & Jamy, 2021; De Vargas et al., 2015] mostly set by ocean currents for large heterotrophs and by environmental conditions for small-bodied phototrophs [Sommeria-Klein et al., 2021].

Although the importance of unicellular eukaryotes, has been recognized as highly relevant to several Marine Strategy Framework Directive descriptors, notably D1 (Biological Diversity), D4 (Food webs) and D5 (Eutrophication) (Caruso et al. 2016), such studies in coastal systems are limited [Genitsaris et al., 2015; Massana et al., 2015; Skouroliakou et al., 2022], mostly focusing in temporal changes, while over space and depth studies are lacking. Understanding the drivers behind protistan distribution and interactions in coastal ecosystems is crucial since these systems tend to be very

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different from oceanic environments, being much more influenced by terrestrial inputs, adhering to very different hydrodynamic processes and being subject to very different light regimes.

In order to answer questions related to protist variation over space and depth (related to light penetration) and to highlight the trophic interactions driving these changes, we studied protistan community composition across different coastal sites and functional depths (surface, depth of Secchi disc, and 2x depth of Secchi disc), from seven different gulfs in the Aegean Sea (regional Mediterranean Sea), using 18S rRNA sequencing. Studies on protistan community composition on the specific area are scarce and limited and are focused on specific protistan groups separately; i.e. ciliates [Giannakourou et al., 2014; Meziti & Kormas, 2022]. Studies using novel metabarcoding techniques investigating the whole community are scarce and are focused on specific sites, i.e., Thermaikos Gulf [Genitsaris et al., 2020; 2022] or groups, i.e., phytoplankton [Spatharis et al., 2019].

In this study we tried to define taxa that are important for differences detected in the marine microbial network across the different gulfs studied as well as their characteristics in terms of trophic level (autotrophs, mixotrophs, heterotrophs, parasites), niche breadth (generalists, specialists) and abundance (abundant, rare). Our target was to elucidate whether these taxa and their trophic and ecological characteristics differentiate between different depths, as well as their associations. Although we expected that phytoplankton species along with their grazers would have key role in the upper layers, and that parasites would be more important in higher depths, it was shown that the latter were important in all depths across different gulfs

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exhibiting significant interactions with all trophic groups and representing key species for the explanation of spatial variation.

Materials & Methods

Collection of samples and DNA extraction

Samples were collected from seven gulfs in the Aegean Sea, Greece, set within a polygonal area of 72 600 km2 (Table 1; Fig. S1), characterized by a range of environmental conditions due to differences in hydrology, geomorphology, substrate, terrestrial runoff and local anthropogenic pressures [Spatharis et al., 2019]. Within each site, five stations were sampled at 1 m (surface), at the Secchi disc depth (Secchi) and at the two times Secchi disc depth (2xSecchi) or maximum depth, depending on site depth (Table S1), collecting 1 l seawater with a Niskin type sampler. In some cases duplicates or triplicates were collected in order to check sampling and sequencing consistency (Table S1).

Sampling took place during July 2014 within 19 days (5–24 July) for the same reasons previously described in Spatharis et al. (2019). July was selected for sampling based on previous studies suggesting physico-chemical variables and phytoplankton composition relative stability during this summer period, at least for a subset of the coastal areas included in the present study [Spatharis et al., 2007; Naselli-Flores et al., 2003]; this is in contrast with winter months, during which episodic rainfall events, and strong wind mixing of the water column and sediment resuspension —which at this large scale may not simultaneously affect all sites—add noise that could distort food web dynamics.

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Water samples were filtered using low vacuum filtration (<150 mmHg) on 0.2 µm isopore filters (Sartorius Stedim Biotech, Germany).

Environmental Parameters

At each station and depth, several environmental parameters were recorded. Specifically, salinity and temperature were recorded onsite, while 3 l seawater samples were collected with a Niskin type sampler for later nutrient measurement (nitrate, nitrite phosphate, silicate, organic nitrogen, organic phosphorus). Organic nitrogen and phosphorus were strongly correlated with dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) and were therefore excluded from further analysis. The covariates used in the analysis were thus salinity, temperature, DIN, DIP and silicate.

DNA extraction and sequencing

DNA extraction from filters was performed with the MoBio Power Soil kit (MoBio Inc. Carlsbad, CA, USA) following its standard protocol with minor modifications for filters processing.

Sequencing of the V2-V3 region of the 18S rRNA gene was performed upon amplification using the primer pair 18S-82F (5′-GAAACTGCGAATGGCTC-3′) [López-García et al., 2003] and Euk-516r (5′-ACCAGACTTGCCCTCC-3′) for Eukaryotes [Amann et al., 1990]. Construction of libraries was performed by 'Genes Diffusion' company (Lille, France) and amplicons were finally sequenced with Illumina MiSeq PE 2x300 (CNRS-UMR8199, Lille).

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18S rRNA gene amplicon analysis

Processing of the resulting sequences, i.e. sequence assembly and quality control, was performed with the MOTHUR software (v 1.35) [Schloss et al., 2009]. Only sequences with ≥480 bp, no ambiguous bases and homopolymers shorter than 8 bp were considered for further analysis. These sequences were aligned using the SILVA SSU database (release 119) [Pruesse et al., 2007]. Chimeras were removed using the Uchime Software [Edgar et al., 2011]. All sequences were binned into Operational Taxonomic Units (OTUs) and were clustered (average neighbor algorithm) at 97% sequence similarity. Single singletons, that appeared only once in the whole dataset, were removed using MOTHUR (v 1.35). Coverage values were calculated with MOTHUR (v 1.35) as well as diversity indices. The batch of sequences from this study has been submitted in NCBI Short Read Archive under accession code PRJNA515026. Taxonomic classification was assigned using BLAST [Altschul et al., 1990] on the Protist Ribosomal Reference (PR2 version 4.14.0) curated Database (built on Genbank; June 2021), containing 197,602 sequences [Guillou et al., 2013].

Similarity profiles, multivariate analysis and differentially abundant features

The Bray–Curtis similarities coefficients were calculated, based on non-transformed number of reads in order to identify relationships between the samples, using R (package stats; R core team and contributors worldwide). NMDS ordination plots were prepared in R (package vegan) using Bray-Curtis similarities. Differentially abundant OTUs between gulfs, were identified with DESeq2 package

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version 3.0.2 [Anders & Huber, 2010; Love, Huber & Anders, 2014] which performs differential analysis of count data, estimating dispersions and fold changes, thus enabling a more quantitative analysis focused on the strength rather than the mere presence of differential expression.

Abundant and rare OTUs, generalists and specialists and trophic groups

OTUs were classified as abundant or rare based on total relative abundances of non-transformed number of reads and on the thresholds used in previous studies [Logares et al., 2014] using >1% for abundant OTUs and <0.2% for rare OTUs. Habitat specialization was calculated as described in [Székely & Langenheder, 2014] using Levins' niche width index (B) [Levins, 1968], where $B = 1/\sum_{i=1}^{N} p_{ij}^2$ (pij: the proportion of OTU j in sample I; N: the total number of samples). Thus B, although is not taking the environmental conditions in a local community into account, is describing the extent of niche specialization based on the distribution of OTUs abundances.

Specialization categories were set along arbitrary cut-off values (B >15, B:10-15, B:1-10 B=1) with B =1 corresponding to extreme specialists (one sample) and B > 15 corresponding to top generalists (>66% of available habitats). Niche width was calculated based on the presence of each OTU at all sites and depths (112 samples) as each sample was considered to be a different habitat in terms of physico-chemical conditions. OTUs were sorted into major trophic groups, such as autotrophs, nanoheterotrophs (picograzers), nanoplankton grazers (nanograzers), microplankton grazers (micrograzers), mixotrophs and parasites following the classification shown in Genitsaris et al., 2016.

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Correlation and Network analysis

For the network analysis, we focused on the OTUs indicated by DeSeq as they were considered 'key' species for community structure regulation. The relationships/interactions amongst these OTUs were characterized by computing the maximal information coefficient (MIC) between each OTU pair [Reshef et al., 2016], calculated using MICtools v1.1.4 [Albanese et al., 2018]. MIC values >0.5, corresponding to a p-value <0.05, were used for the networks. For networks visualization Cytoscape 3.0 [Smoot et al., 2011] was used.

Results

Protist community structure

Overall 4,389,394 sequences were obtained resulting in 5,005 operational taxonomic units (OTUs) in all 112 samples. Diversity indices indicated that the highest richness as well as Shannon diversity were observed in LK, while in S the respective values were the lowest ones followed by T and KV (Table S2). Shannon diversity index was stable over depth in most gulfs apart from S, T and KV where diversity increased in deeper layers (Table S2).

Protistan community composition (PCC) similarities **within gulfs** decreased over depth apart from K, S and KV (Fig. S2a). Regarding between gulf similarities ANOVA results on Hellinger transformed OTUs abundances indicated several significant differences ($p<0.05$; Table S3a) at the surface level while at Secchi depth the only difference was

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observed between T and K (p=0.041) and at 2xSecchi no differences were observed (Table S3a). Bray-Curtis similarities between gulfs ranged from 0.25 to 0.30 with the highest values being observed in Surface (Fig. S2b). This range was very low, although differences were significant between Surface and the other depths (t-test Monte-Carlo permutation; p=0.0001 for both Secchi and 2xSecchi). Overall these results implied more distinct between gulfs, but homogenous within gulfs, communities at the surface level while communities between gulfs were less distinguishable at higher depths.

Similar results were observed after analysis with NMDS showing spatial grouping, mostly for the surface and Secchi level samples (Fig. 1). Ordination stress was quite high (0.12, 0.14 and 0.18 for different depths respectively; Fig. 1). The only environmental parameters that were important for these ordinations (p<0.001) were silicate and ammonia (p=0.00099) for both surface and Secchi samples, NO2 only for surface (p=0.00099) and phosphates and Chla (p=0.00099) for Secchi depth. Finally, at 2xSecchi depth only temperature appeared as significant for the ordination (Fig. 1).

Taxonomic classification for each gulf and depth revealed different patterns. Overall nine major taxonomic groups comprised ~90% of taxonomic diversity in all samples (Fig. 2). Bacillariophyta, Dinophyceae and Syndiniales were the most abundant groups in most cases, exhibiting fluctuating abundances across gulfs and depths. However Chrysophyceae prevailed in S samples where Bacillariophyta decreased and Haptophyta exhibited their highest relative abundances in K (Fig. 2).

Effect of depth in trophic groups; abundant OTUs and niche breadth

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Overall autotrophic algae (shown in green colors in Fig. 2) abundance dropped from surface to deeper layers. It has to be noted though that in KV, M and LK samples 2xSecchi depth coincides with maximum depth, thus these samples were collected from bottom water (Table S1). Overall parasites (mainly Syndiniales) relative abundances were constantly >10% in all gulfs (Fig. 2). Correlation analysis and the calculation of MIC scores for each depth separately, showed that in surface strong correlations were observed between all trophic groups apart from autotrophs, while in Secchi depth correlations were observed only between grazers and parasites and no correlations were observed in 2xSecchi (Table S4).

The whole dataset included 25 abundant (>1%) and 60 common (>0.2%) OTUs, accounting for 0.5% and 1.19% of the total OTUs number respectively (Table S5). However, in terms of relative abundances these OTUs accounted for >60% of protistan community composition. These abundant species belonged to all trophic groups, exhibiting different abundance patterns amongst gulfs and depths (i.e. prevalence of *Chrysophyceae* in S samples) (Fig. 3).

Amongst the 5,005 OTUs detected, 2,314 represented extreme specialists (present only in one site and depth), 2,038 represented specialists (present in <30% of all sites and depths; 1<B<10), 227 represented generalists (10<B<15) and 426 were top generalists (B>15) reaching ~50% of OTUs number/ sample (Fig. S3a; Table S5). In most samples top generalists and generalists accounted for >90% of OTUs relative abundances (Fig. S3b), apart from Secchi and 2xSecchi samples in KV, S and T and 2xSecchi in G. Overall the number of extreme specialists and specialists increased with depth with differences being significant between surface and 2xSecchi (p=0.0126; F=5.06).

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364 The vast majority of abundant OTUs consisted of top generalists (17/25) and generalists
365 (5/25), while a similar pattern was observed for common OTUs (50/60; 6/60)(Table S5).
366 Only 17/426 top-generalists were abundant and 47/426 were common, while the rest
367 were rare species. Similarly for generalists 216/227 species were rare (Table S5).
368
369 **Differentially abundant OTUs across depths exhibit different trophic and niche**
370 **breadth patterns**
371 The number of differentially abundant OTUs across the seascape (using the binomial
372 test and false discovery rate <0.05), varied between depths, with 44, 23 and 51 OTUs at
373 surface, Secchi, and 2XSecchi depths respectively (Table S6). For surface samples
374 these OTUs belonged to all trophic groups (Table S6) and more specifically included
375 increased relative abundances of phototrophic algae in T, KV and G, of nanograzers in
376 G and S, of mixotrophs in S and of parasites in KV and M (Fig. 4). For Secchi depth,
377 differences between sites were attributed to fluctuations of OTUs, belonging to
378 mixotrophic and parasitic groups (Fig. 4) that both peaked in KV followed by S, while
379 fluctuations of nanograzers were also important. Finally, for 2xSecchi the majority of
380 differences were attributed to OTUs that mostly belonged to heterotrophic and
381 mixotrophic groups, although in specific sites such as LK, M, S and T increases of algae
382 (*Chrysophyceae*) were responsible for gulf separation, while parasites peaked in KV
383 (Fig. 4, Table S6). We have to highlight that in all depths >70% of differentially abundant
384 OTUs belonged to rare OTUs (Table S6) showing the importance of rare members of
385 the total protistan community for spatial differentiations in PCC.

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Regarding these OTUs niche breadth, at surface 28/44 OTUs were top-generalists and generalists, while at Secchi and 2xSecchi depths the respective ratios were only 7/23 and 9/51, (Table S6), corroborating with the total number of specialists increasing with depth (Fig. S3a). Amongst these generalists and top-generalists differentially abundant species only 4/28, 1/7 and 3/9 species were also abundant at surface, Secchi and 2xSecchi, respectively (Table S6, Fig. 3). These OTUs were related to *Leptocylindrus* (OTU00031) and to *Chaetoceros* (OTU00036) in surface, to *Chrysophyceae* (OTU00022) in 2xSecchi, while the uncultured *Syndiniales* (OTU00056) was important for all depths and an uncultured *Dinophyceae* (OTU00004) was important in surface and 2xSecchi (Table S6). Apart from OTU00056, two more species, OTU00288 and OTU00737 were important for differences between gulfs in all depths. OTU00288 was a generalist although rare mixotrophic haptophyte, and OTU00737 was a rare specialist nanograzer belonging to *Spirotrichea.*

Correlations between differentially abundant OTUs; network analysis for different depths

Network analysis for the 44 surface species that were responsible for between gulfs differences indicated 155 significant relationships between 42 species while two species indicated no correlation (Table S7). Amongst these species 11 species exhibited 105 significant interactions amongst them as well as with 19 other species and were responsible for the majority of interactions observed (Fig. 5a; Table 2; S7).

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Moved down [1]: The highest number of generalists explaining the differences between gulfs at surface could be associated with a highest number of different niches as also suggested from the distinct communities (Fig. 1; S2). ¶

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These eleven species belonged to algae (*Chlorophyta, Bacillariophyta*) that mostly interacted with parasites followed by other algae and mixotrophs, parasites (*Syndiniales*) apart from algae also interacted with mixotrophs, and mixotrophs (*Haptophyta, Dinophyceae*), nanoheterotrophs and nanograzers that interacted almost equally with all groups (Table S6). The highest MIC scores were observed between algae, mixotrophs and some parasites while the correlations with other heterotrophs were minor (Table 2; S7). Most importantly these 11 OTUs belonged to generalists and top-generalists and only the nanoheterotroph OTU00263 and the parasitic OTU00177 (*Syndiniales)* were specialists (Table S6). On the other hand Secchi network had much lower centrality (that is few central nodes that are close to all the other nodes; Table S8) with only 15 species (out of 23) exhibiting 23 relationships with MIC scores >0.05, thus indicating weaker interactions between species. However species exhibiting the higher number of interactions included mainly parasites, nano-grazers and the rare generalist mixotrophic haptophyte (OTU00288; 100% identity to AB058358 *Haptolina*) that was present in all three networks. Phototrophs (*Bacillariophyta*) had low centralities interacting mostly between them and with nanograzers (*Ciliophora*) (Table S7). The top generalist but rare nanograzer OTU00409 (*Spirotrichea*) strongly correlated with both *Syndiniales* and mixotrophs, while the parasitic *Blastodinium* (OTU00052) interacted with mixotrophic *Dinophyceae* and connected to the rest of the network through OTU00056. Finally, at 2xSecchi two networks occurred with the first including only six species (mainly parasites; *Syndiniales* and *Blastodinium*) and the second including only 10 interactions between nine species and being built on the associations between

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parasites and algae (*Chrysophyceae; Bacillariophyta*) that were generalists or top generalist but not abundant (Fig. 5c; Table 2; S7).

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Discussion

Our study investigated protistan community composition (PCC) in different coastal sites and depths, from seven different gulfs in the Aegean Sea (regional Mediterranean Sea). Different depths were selected based on Secchi depth and light penetration, resulting in bottom depths for 2xSecchi in shallow sites and thus not allowing for robust conclusion regarding the effect of light penetration in protistan communities. However this was the first time that a similar study was performed to that extent (area of 72 600 km2) in the specific area (East-Mediterranean), with samples collected within a time range of 19 days, significantly minimizing the effect of temporal variation on assemblage composition, as also explained previously. Thus we believe that the results provided here will be valuable for future efforts for further disentangling protist diversity interactions in this area specifically, but also in other temperate Gulfs.

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We focused on the taxonomic affiliation and ubiquitousness of different species in order to investigate interactions amongst species in different sites as well as their impact in the dynamics of protistan communities. Although ordinations using non-parametric methods provided evidence for site separation in all depths, these results were weakly supported statistically apart from the surface level (Table S3a), implying better separation in surface compared to deeper layers. However when similarities between gulfs were calculated, they were higher in surface samples compared to Secchi depth (Fig. S2), suggesting that although surface samples might be more influenced by local factors enhancing growth of specific species, differentiating

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community composition, they show higher dispersal. The hypothesis that epipelagic communities are more homogenous due to faster currents [Villarino et al., 2018] in surface compared to deeper layers could not be thoroughly examined in our study due to low maximum depths that in many cases corresponded to bottom water.

As suggested from previous studies relying on a synthesis of variables from hydrological, climatological and satellite data [Reygondeau et al., 2017; Ayata et al., 2018], the Aegean Sea is separated in East and West compartment fed by the Atlantic water and Black Sea outflow waters respectively. Thus Kavala, Thermaikos and Saronikos are categorized in the west compartment, whereas sites Gera, Kalloni Kodias and Moudros are categorized in the east compartment, This partially explains the higher Shannon indices observed in the east compartment (G,K,LK,M), compared to indices observed in the West compartment (KV,T,S), (Table S2), based on the biogeography principle that larger geographical areas or water masses (Atlantic compared to Black Sea) are more species diverse as they provide the scope for more niches [Costello & Chaudary, 2017]. The potentially higher number of niches in the east compartment could also explain the higher number of top-generalists and generalists observed compared to the west part (Fig. S3). Also the highest number of generalists explaining the differences between gulfs at surface (Table S6) could be associated with a highest number of different niches as also suggested from the distinct communities (Fig. 1; S2).

A clear top-down phytoplankton control from grazers (mixotrophs, nanograzers), was not confirmed for any depth, as shown from correlation analysis between trophic groups, for the whole community. However, regarding specific species that were

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differentially abundant between gulfs some significant interactions between algae and grazers were observed for all depths (Table S7; Fig. 5), although they were outcompeted by the strongest and more abundant interactions between algae and parasites (mainly Syndiniales). Overall parasites emerged as important contributors in protistan community composition (PCC) shaping at all depths, exhibiting strong interactions with all trophic groups (Table S7; Fig.5).

Syndiniales are known to form parasitic relationships with other protists (dinoflagellates, ciliates, cercozoans, radiolarians) and metazoans (copepods, fish eggs), with free-living dinospores released in very high numbers following host death [Burki, Sandin & Jamy, 2021, Clarke et al., 2019], while their importance in marine trophic webs has been confirmed on a global scale after the Tara ocean [De Vargas et al., 2015] and the Malaspina-2010 expeditions [Pernice et al., 2016] that both studied 18S rRNA diversity and exhibited the prevalence of Syndiniales sequences amongst protists. However it is worth mentioning, that especially for Syndiniales high numbers might be due to their overrepresentation in DNA surveys compared to RNA surveys probably because of their high abundance in picoplankton in inactive stages with few ribosomes [Massana et al., 2015].

Although syndinian protists parasitic lifestyle is well-known [Guillou et al., 2008] their exact ecological role remains a ‘black-box’ for marine food webs modeling. Previous studies have shown co-occurrence patterns of Syndiniales with Spirotrichea and Dinophyceae exhibiting both exclusion and copresence patterns [Anderson & Harvey, 2020]. Other studies, working on high-resolution time-series in a productive coastal pond [Sehein et al., 2022], indicated seasonal shifts in protist populations,

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exhibiting elevated concentrations of free-living parasitic Syndiniales along with their infected dinoflagellate hosts during high productivity periods, while others [Long et al., 2021] have proved the existence of Dinophyceae 'weapons' against syndinians contributing to avoid the total collapse of Dinophyceae blooms. A recent seasonal study in a meso-eutrophic coastal system, focusing on Syndiniales Group II has shown that syndinians peaked in summer along with *Prorocentrum minutum,* while in autumn had diverse host [Christaki, Skouroliakou & Jardillier, 2023].

These previous findings corroborate with the strong interactions detected at Secchi depth between Dinophyceae and Syndiniales (Fig. 5b) as well as with the significant interactions between mixotrophs and parasites in total (Table S4) and with the co-presence of abundant populations of Group II Syndiniales with their potential hosts, such as *Gyrodinium, Heterocapsa, Scrippsiella* and *Prorocentrum* [Coats et al., 1996; Chambouvet et al., 2008; Salomon et al., 2009; Christaki, Skouroliakou & Jardillier, 2023]. These findings and interactions potentially support previous studies suggesting the importance of parasites in controlling mortality in marine systems, influencing Dinophyceae bloom dynamics and species succession, [Chambouvet et al., 2008, Skovgaard et al., 2005; Jephcott et al., 2016].

However Syndiniales associations with autotrophs are poorly studied. Investigations performed for Bacillariophyta and Mamiellophyceae mostly indicated exclusion (Anderson & Harvey, 2020) with authors suggesting resource competition (either direct or indirect) between taxa, as nutrients such as silicates were important in explaining patterns in Syndiniales composition. Another study [Sassenhagen et al., 2020] also indicated direct and indirect interactions between diatoms and syndinians by

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584 studying the infection of diatoms by Syndiniales also exhibiting host specificity despite
585 their large host range. Thus, although the nature of the strong interactions observed in
586 surface and 2xSecchi (Syndiniales vs Chlorophyta, Bacillariophyta, Chrysophyceae)
587 cannot be easily clarified, direct (resources competition) or indirect (effect on
588 phytoplankton grazers) interactions could be suggested.
589 *Blastodinium* is a well-known copepod parasite [Skovgaard, 2005; Alves de
590 Souza et al., 2011] and interactions detected in our study with Dinophyceae probably do
591 not reveal trophic relationship but co-occurrence inside the host (i.e. copepod feed). The
592 conspicuous role of the generalist but very rare OTU00288 (Prymnesiales) interacting
593 with members from different trophic groups at each depth further confirmed the potential
594 versatility of haptophyte species exhibiting different lifestyles swiping from autotrophy to
595 heterotrophy, but also being able for toxic blooms formation [Johanessen et al., 2015]
596 However interactions mainly involving exclusions have been detected between
597 Syndiniales and Prymnesiophyceae [Anderson & Harvey].
598 Previous studies have suggested that rare microbial community members might
599 assist in community stabilization due to their ability to rapidly respond to environmental
600 changes [Shade et al., 2014]. This was further confirmed from our results since most of
601 the species exhibiting significant interactions were generalists but not abundant
602 members of the total population highlighting the importance of the rare biosphere.
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605 **Conclusions**
606 We studied the spatial structure of the entire protistan community in seven different
607 gulfs and three different depths in a regional Mediterranean Sea. Our aim was to define

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taxa that are important for explaining microbial network differences observed across the different gulfs studied as well as their trophic interactions. Although we hypothesized that clear interactions between phytoplankton species and their grazers would be important for explaining these differences, we found that parasites (especially syndinians) were 'key species' in all depths across different gulfs. Distinct associations between parasites (mainly Syndiniales), with phototrophs and mixotrophs appeared as important, for spatial differentiation, in surface/2xSecchi and Secchi depths, respectively, suggesting novel direct or indirect trophic relationships. Nevertheless, due to the high number of potential hosts that could vary from algae to metazoans (crustaceans, fish) it was not feasible to disentangle whether parasites interactions with the other protists were direct or indirect. Thus the dynamics of this group that appeared as an important player in shaping protist variation could be influenced by factors (such as fish stock) far beyond the traditional factors measured in traditional ecological studies. Although our findings agreed with previous studies suggesting the importance of parasites in controlling Dinophyceae bloom dynamics, species succession and mortality in marine systems, influencing , this was the first time, to our knowledge, that phototrophs-syndinians associations emerged as an important factor for shaping protist community composition in a coastal environment, presenting thus a novel perspective regarding parasites associations.

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