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## **An exploratory study of heterotrophic protists of the mesopelagic Mediterranean Sea**

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### **Abstract**

Is there a mesopelagic protist fauna composed of species different from that of the overlying surface community? Does the mesopelagic community show seasonal changes in abundances and species composition? We addressed these questions by considering 3 distinct groups in which species identification is relatively unambiguous: tintinnid ciliates, phaeodarian radiolarians, and amphisolenid dinoflagellates. We sampled weekly at 250 m and 30 m depth from January to June a deep-water coastal site characterized by seasonal changes in water column structure; notably in winter the mixed layer extends down into mesopelagic depths. We found a deep-water community of tintinnid ciliates comprised of forms apparently restricted to deep waters and species also found in the surface layer. This latter group was dominant during the winter mixis period when tintinnid concentrations were highest and subsequently declined with water column stratification. Phaeodarian radiolarians and the amphisolenid dinoflagellates were regularly found in deep samples but were largely absent from surface water samples and showed distinct patterns in the mesopelagic. Phaeodarian radiolarians declined with water column mixing and then increased in concentration with water

column stratification while amphisolenid dinoflagellates concentrations showed no pattern but species composition varied. We conclude that for all three protists groups there appear to be both distinct mesopelagic forms and seasonal patterns.

**Keywords:** amphisoleniaceae, deep sea, microplankton, phaeodarea, tintinnida

## **Introduction**

Recent reviews have stated that there is an urgent need to improve our understanding of the biology of the mesopelagic (e.g. St. John *et al.*, 2016; Costello and Breyer, 2017).

Primarily this is because of questions with regard to the standing stocks of deep-water fish (e.g., Irigoien *et al.*, 2013; Sutton, 2013) and as well uncertainties with regard to fluxes of carbon into the bathypelagic and consequently the possible effects of climate change (e.g. Sanders *et al.*, 2016). The food web of the mesopelagic is often depicted as a simple system (compared to the surface layer) in which the inputs are sedimenting particles (aggregates of marine snow, phytoplankton flocs and zooplankton fecal pellets) exploited and re-packaged by resident or vertically migrating metazoan zooplankton and fish (e.g. Burd *et al.*, 2010; Robinson *et al.*, 2010). Microzooplankton, thought to dominate consumption of primary producers in the surface layer (e.g. Calbet and Landry, 2004), if not entirely absent in depictions of mesopelagic food webs (e.g. Burd *et al.*, 2010), are relegated to a minor role of consumers of free-living prokaryotes (e.g., Giering *et al.*, 2014). This is despite the fact that that microzooplankton in the mesopelagic are known to be a diverse assemblage of both protists and metazoans of distinct ecologies whose aggregate respiration may rival that of the metazoan zooplankton (e.g. Gowing *et al.*, 2003). Microzooplankton in the mesopelagic are thought to be the source of small fecal pellets (minipellets) whose flux may exceed that of

metazoan fecal flux (Gowing and Silver, 1985). A surprising variety of mesopelagic consumers prey upon microzooplankton ranging from copepods (Sano et al. 2013), myctophid fishes (Conley and Hopkins, 2004), large tunicates (Hopcroft and Robinson, 1999) to midwater polychaetes (Uttal and Buck, 1996). Close interactions between metazoan consumers and microzooplankton have been proposed as "gardening by metazoans" through fragmenting detrital particles, promoting microbial populations (Mayor *et al.*, 2014).

In recent years a good deal of data on mesopelagic protists has become available through genomic surveys and studies employing flow cytometric analyses (e.g., reviewed in Edgcomb 2016). These studies suggest that in the mesopelagic protists are diverse, abundant and distinct from the surface layer assemblages. However, studies of temporal variability of different groups of heterotrophic protists in the mesopelagic appear to be quite limited. To our knowledge they concern only the Arabian Sea and the North West Mediterranean Sea. The studies have given contrasting results, likely reflecting differences in the dynamics of the systems examined. Gowing *et al.* (2003) sampled several stations in the Arabian Sea through 4 cruises during different monsoonal seasons in 1995. The upper mesopelagic (200-250 m depth) populations of protists, grouped as 'dinoflagellates', 'ciliates' or 'sarcodines', were relatively consistent in concentration, varying in abundance only within a factor of two over time covering distinct monsoonal seasons (Gowing et al. 2003 supplementary data table). Our study site in the N. W. Mediterranean Sea is clearly distinct in terms of some fauna as well dynamics compared to the Arabian Sea.

Indeed, the Mediterranean mesopelagic may be distinct from most other systems. With regard to the biological component, the taxonomic composition of fish and metazoan fauna has been described as relatively impoverished in terms of species

richness (Sutton *et al.*, 2017). However, mesopelagic fish biomass appears to be comparatively high based on the characteristics of the deep-scattering layer, from which fish biomass has been modeled (Proud *et al.*, 2017). With regard to microbes, the metabolic activity of prokaryotes appears to be higher for mesopelagic Mediterranean populations compared to those found at similar depths in other systems, related possibly to the relatively warm temperature of the deep waters, 13°C (Luna *et al.*, 2012). Also, the abundances of typical bacterivores, heterotrophic nanoflagellates, appear to be low relative to abundances of prokaryotes compared to other systems perhaps because of high ciliate concentrations (Aristegui *et al.*, 2009).

The Mediterranean Sea also differs with regard to water column dynamics. The N.W. Mediterranean is a site of winter deep-water formation. In the winter the surface mixed layer extends below 200 m into the mesopelagic and occasionally down to depths of over 2000 m in the Gulf of Lyons (e.g. Herrmann *et al.*, 2008; Houpert *et al.*, 2016). In contrast to clear seasonal changes in water column structure, vertical export flux to the mesopelagic (measured using sediment traps at 200 m) show highly variable fluxes without any clear seasonal pattern or relationship with mixed layer depth (e.g., Heimbürger *et al.*, 2013). Overall then, it perhaps not surprising that the microbial community of the mesopelagic in the N.W. Mediterranean may be more variable than that of the Arabian Sea. Data are limited to those from only 3 studies but worth reviewing in some detail.

Tanaka and Rassoulzadegan (2002), through bi-monthly sampling, examined changes in the vertical profiles of 'bacteria', 'heterotrophic nanoflagellate's and 'ciliates' in a deep site in the open N.W. Mediterranean Sea. They found large seasonal changes in upper mesopelagic (200-300 m depth) concentrations of 'ciliates' and 'heterotrophic nanoflagellates', ranging well over an order of magnitude between dates while

concentrations of 'bacteria' varied less, within a factor of 3. For all 3 groups, the highest concentrations corresponded with the period of winter water column mixis.

Subsequently, Winter *et al.* (2009a,b) sampling at the same site and depth using bi-monthly sampling found similar seasonal variability in prokaryotic abundances (varying by a factor of 3) and documented seasonal shifts in community composition of prokaryotes. In a more recent study, Weinbauer *et al.* (2013), through monthly sampling at a coastal deep water site in the NW Mediterranean (the site examined here) over one year, documented seasonal changes in the composition of the mesopelagic prokaryotic community and the abundances of heterotrophic nanoflagellates. They found marked changes in the mesopelagic assemblages. The shifts corresponded with seasonal changes in water column structure; in particular winter water column mixis that preceded marked shifts in the relative abundances of archaea and eubacteria as well increases in the abundances of heterotrophic nanoflagellates. However, they reported a pronounced peak in both prokaryotes and heterotrophic flagellate abundance at the beginning of the stratified period. Similar to the findings of Tanaka and Rassoulzadegan, throughout an annual cycle prokaryote abundance varied less, only by a factor of 2, while their presumed predators, heterotrophic nanoflagellates, varied by over an order of magnitude (Weinbauer *et al.*, 20123, Fig. 4). Based on the limited data available protists appear to be a highly dynamic component of the mesopelagic in the N.W. Mediterranean. However, data on species compositions is completely lacking as well as short-term (< month) variability.

Here we build on the study by Weinbauer *et al.* (2013) using a more intensive sampling at the same deep-water coastal site, sampling at weekly intervals both a surface layer depth and a mesopelagic depth. To examine possible changes in species composition, we focused on distinct groups of protists in which species identifications

(based on gross morphology) can be made unambiguously using light microscopy. In this exploratory study we set out to address two questions: Is there a mesopelagic protist fauna distinct in terms of species composition from that of the overlying surface? Is the mesopelagic protist community dynamic, does it show seasonal changes in species compositions and concentrations?

The three groups of protists we investigated are each phylogenetically coherent but are of distinct ecologies. In common are morphologies allowing sampling using fine mesh nets and relatively unambiguous species identifications: tintinnid ciliates, phaeodarian radiolarians, and amphisolenid dinoflagellates. Tintinnid ciliates are grazers on primarily nano-sized prey items (2-20  $\mu\text{m}$  in length) and are characterized by the possession of a (more or less) species-specific lorica or shell into which the ciliate cell may contract. The biology and ecology of tintinnid ciliates is relatively well known (e.g. Dolan *et al.*, 2013). Tintinnids are thought to be largely restricted to the euphotic zone of the oceans although some species are most abundant in the mesopelagic at least in the Adriatic Sea (Krsinic, 1998). Pheodarian radiolarians of the order Phaeogromidae (e.g. Challengarids) are generally considered to be deep-water residents (Anderson 1983; Kling and Boltovskoy, 1999; Nakamura and Suzuki, 2015) largely feeding on sinking organic aggregates (Gowing and Bentham, 1994; Nothing and Gowing, 1991). Heterotrophic dinoflagellates of the family Amphisoleniaceae group species of the enigmatic genera *Amphisolenia* and *Triplosolenia*, species of which lack chloroplasts but host endosymbionts. *Amphisolenia* can harbor both prokaryotic and eukaryotic phototrophs but nothing is known with regard to their role in the nutrition of the host cell (Gaines and Elbrachter, 1987). *Triplosolenia* have been included in lists of "shade species" found in deep, poorly lit waters (Taylor and Pollingerher, 1987). *Triplosolenia* have never been found to contain either chloroplasts or food vacuoles (Gaines and

Elbrachter, 1987) and like *Amphisolenia* may contain endosymbiotic cyanobacteria (Saldarriaga and Taylor, 2017).

## **Material and Methods**

### Study Site

Sampling at 250 m and 30 m depth was conducted at 'Point C', a standard sampling site approximately 1 Km offshore near the entrance of the Bay of Villefranche (43°51' 00"N, 07° 19' 00"E). At the site water column profiles of temperature, salinity conductivity and oxygen of the 300 m depth water column are obtained weekly as part of a French national network of marine sites, SOMLIT (<http://somlit.epoc.u-bordeaux1.fr/fr/>). Our sampling at Point C was conducted on the same day or within one day of the CTD water column profiling. At this site, as in most of the N.W. Mediterranean Sea, there are large seasonal changes in the depth of the surface mixed layer. During winter (Jan. - March) the mixed layer extends down to the mesopelagic depths (> 200 m) with deep water (> 500 m) formation in areas such as the Gulf of Lions (e.g., D'Ortenzio *et al.*, 2005). The deep-water site, Point C, is located about 700 m from shallower (80 m depth) main coastal sampling station "Point B". At this main station, hydrology and biology have been well studied. It is characterized by winter mixing of the water column and annual average water column concentrations of chlorophyll of about 0.3 µg chlorophyll *a* l<sup>-1</sup> with pronounced maxima in spring and autumn contrasting with low concentrations in the summer (e.g. Bustillos-Guzman *et al.*, 1995; Mostajir *et al.*, 1995).

### Sampling and sample processing

Sampling was conducted at weekly intervals from January 9 to June 1 2017. Water samples were obtained using multiple casts of a 30 liter Niskin Bottle. The Niskin bottle water was gently emptied into plankton net (20 µm mesh plankton net with a 250 ml



collector) placed in a 30-liter container. We first sampled at 250 m depth, concentrating material from 120 - 240 l. Water volumes varied as sea conditions did not always permit several casts and volumes sampled were increased during the stratified period when organismal concentrations declined. Elapsed time between bottle casts at 250 m depth was about 5 minutes. Net material was preserved with Lugol's fixative (2% final conc.). The net was then thoroughly rinsed and sampling at 30 m depth, material from 90 to 120 l was collected (time between bottle casts was about 2 minutes). From the first bottle casts water samples were also taken for counts of heterotrophic prokaryotes and eukaryotes beginning in late February. Here only data from samples from 250 m are considered.

#### Prokaryotes and heterotrophic protists cell counts using flow cytometer

Beginning in late February, samples were taken for flow cytometric analysis. Total cell abundances for heterotrophic prokaryotes and eukaryotes were determined by flow cytometer on 4 ml pre-filtered seawater (water passed through the 20  $\mu$ m net plankton net) preserved onboard with a mix of glutaraldehyde (0.25% final concentration, EMS)/Pluronic F68 (0.01% final concentration, Sigma-Aldrich) for 15 min at 4°C, in the dark, then flash frozen in liquid nitrogen and stored at -80°C until analysis (Mari *et al.*, 2014). All samples were analyzed with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a blue laser emitting at 488 nm at a maximum flow rate of 75  $\mu$ l min<sup>-1</sup>. For prokaryotes, 0,5 ml undiluted samples were stained with SYBR Green I (5X final concentration, Invitrogen) and incubated in the dark at room temperature for 10 min. The flow rate varied between 45  $\mu$ l min<sup>-1</sup> (medium) and 75  $\mu$ l min<sup>-1</sup> (high) and acquisition time was 60 sec. Their analysis was based on their signature in a side scatter (SSC, related to cell structure) plot versus green fluorescence (FL1) plot (Gasol and Del Giorgio, 2000). 1  $\mu$ m fluorescent latex beads (Polyscience Inc.,

Europe) were always used as internal standards. For heterotrophic protists, subsamples of 1 ml from the same thawed sample were stained with SYBR Green I (1X final concentration, Invitrogen) and incubated in the dark, at room temperature for 10min. A mix of fluorescent beads of different sizes (0.5, 1.0, 3.0, and 10.0  $\mu\text{m}$ , Polyscience Inc., Europe) was used as internal standards. Heterotrophic protists populations were distinguished from large bacteria on an SSC versus FL1 plot as described by Christaki *et al.*, 2011. In our case, the flow rate was established at about  $75 \mu\text{l min}^{-1}$  (high) and the data acquisition was achieved for 12 min (see Fig. 1 in Supplementary files: cytometric methods). Pure cultures of the heterotrophic flagellate *Amastigomonas* sp. were used to validate the heterotrophic protists gate on the FL1 versus SSC plot. Undiluted, unstained natural seawater samples were used to better visualize and exclude the autotrophs from the FL3 versus SSC gate. Negative controls represented by 0.2  $\mu\text{m}$  daily filtered seawater plus SYBR Green and the mix of beads were systematically run and never showed any event in the gates of interest. All data were analyzed with CellQuest™Pro (BD) software and concentrations expressed as cells  $\text{ml}^{-1}$ .

#### Heterotrophic microplankton cell counts using light microscopy

The plankton net material was concentrated through sedimentation in graduated cylinders with a supernate of about 200 ml removed through gentle siphoning. For each sample, multiple aliquots of (1-3 ml) of the concentrated material were examined using an inverted microscope (Olympus IX71 equipped with DIC optics, a DP71 camera and CellSense image analysis software). Generally material from at least 100 liters from 250 m depth, or 20 liters from 30m depth, was examined for each sample. Species identifications of the three groups considered here were made using a variety of taxonomic works. Those concerning tintinnid ciliates included Abboud-Abi Saab (2008), Balech (1959) Jørgensen (1924) Kofoid and Campbell (1929, 1939) Krsinic (2010). For

the phaeogromida phaeodarian radiolarians (i.e., Challengerids, Medusettids, Lirellids) the works consulted were Kling and Boltovskoy (1999), Nakamura and Suzuki (2015) and Borgert (1906; 1911). For the Amphisolenid dinoflagellates (species of *Amphisolenia* and *Triplosolenia*) works consulted were Kofoid (1906), Kofoid and Skogsberg (1928) and Taylor (1976).

### Categorization of Species

Species were assigned to several (some non-exclusive) categories of occurrence: "rare", "present", "common", "deep water species", "surface water species" and "entire water column species" using the following criteria. Occasional or "rare" species were those found on only one or two dates ( $\leq 10\%$  of sampling dates). These species were not further categorized. Species found on at least 3 dates were categorized as "present" and further subdivided.

Species found on at least 3 dates in deep water samples were categorized as "deep water species" if fulfilling both the conditions 1) not found on more on than 2 dates in surface samples and 2) if found in the surface sample, the concentration did not exceed trace (i.e., more than 1 cell found). Among deep-water species, "common species" were those found on 10 or more dates ( $\geq 50$  of samples.)

Species found on at least 3 dates in surface water samples were categorized as surface water species if fulfilling both the conditions 1) not found on more on than 2 dates in deep water samples and if found in a deep water sample, the concentration did not exceed trace (i.e., more than 1 cell found). Among surface water species, "common species" were those found on 10 or more dates ( $\geq 50$  of samples.)

Species found in both deep-water samples and surface water samples on 3 or more dates, and in greater than trace concentrations, were categorized as "entire water column species". We assumed, but did not regularly take samples to prove, that species

found at both 30 m and 250 m likely occurred throughout the water column. Complete count data are furnished as a supplementary data file.

#### Stratification index and Mixed Layer Depth estimations

A stratification index was used to characterize the structure of the water column. The index was calculated as the difference in potential density between 10 m and 300 m using the salinity and temperature profiles obtained by the Seabird SBE25 CTD following Behrenfeld *et al.*, (2006), Dave and Lozier (2010) Lozier *et al.*, (2011). When the difference in potential density is smaller than  $0.125 \text{ kg m}^3$ , the upper 300 m can be considered as non-stratified (De Boyer Montégut *et al.*, 2004).

The Mixed Layer Depth (MLD) was estimated based on potential density profiles, calculated from pressure, temperature and salinity data. For each potential density profile, the MLD corresponds to the depth where the difference between the potential density at the reference depth (10 dbar) and the measured potential density is higher than the threshold of  $0.03 \text{ kg m}^3$ , which is used in the Mediterranean Sea (D'Ortenzio *et al.*, 2005; de Boyer Montégut, 2004).

## **Results**

### Water Column Structure

Seasonal changes in stratification and the depth of the surface mixed layer (Fig. 1) were typical for the site. In early January the mixed layer reached 150 m depth and subsequently deepened to nearly 250 m by early February. Water column stratification began in late March and by mid-April the water column was strongly stratified. The surface mixed layer was restricted to the top 25 m of the water column from early April on and it remained shallow through the end of the sampling period in late June. Based upon the stratification index, the mixed period (index  $< 0.125$ ) included sampling from

January 30 to March 9 (n = 6 dates) and strongly stratified water column (index > 0.6) encompassed sampling from April 18 to June 1 (n = 7 dates). Simple T-tests were used to test for significant differences in organismal concentrations comparing mixed and stratified periods.

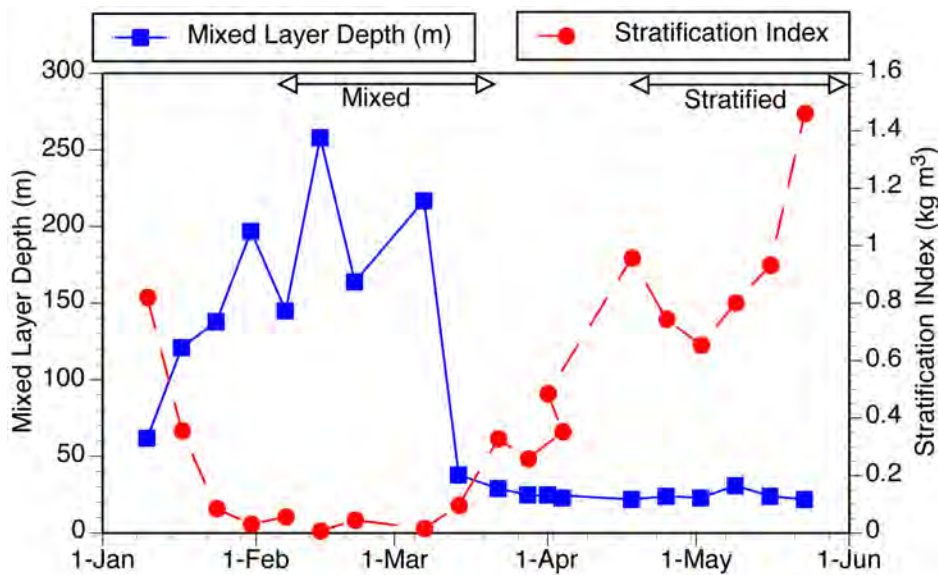


Fig. 1. Temporal changes in water column structure: two contrasting periods of "mixed" and "stratified" are indicated based on large differences in the stratification index.

### Heterotrophic prokaryotes (bacteria) and Eukaryotes (pico and nanoflagellates)

At 250 depth heterotrophic prokaryote (bacteria & archeobacteria) abundance was highest during the mixed period, declining from a peak abundance of  $4.6 \times 10^6$  cells ml<sup>-1</sup> to about  $2 \times 10^5$  cells ml<sup>-1</sup> during the stratified period, thus varying only by about a factor of 2 to 3 (Fig. 2). Irregular shifts in abundance characterized the heterotrophic eukaryote population. During the mixed period abundance was relatively high ranging from 390 - 420 cells ml<sup>-1</sup>. During the transitional period between the stratified and mixed periods concentrations were about 300 cells ml<sup>-1</sup>. Subsequently, the heterotrophic eukaryote population increased during the beginning of the stratified

period to a peak of about 630 cells ml<sup>-1</sup> and subsequently declined to low levels of about 160 cells ml<sup>-1</sup>. Overall, the heterotrophic eukaryotes concentrations varied by a factor of 4, considerably more than the prokaryotes.

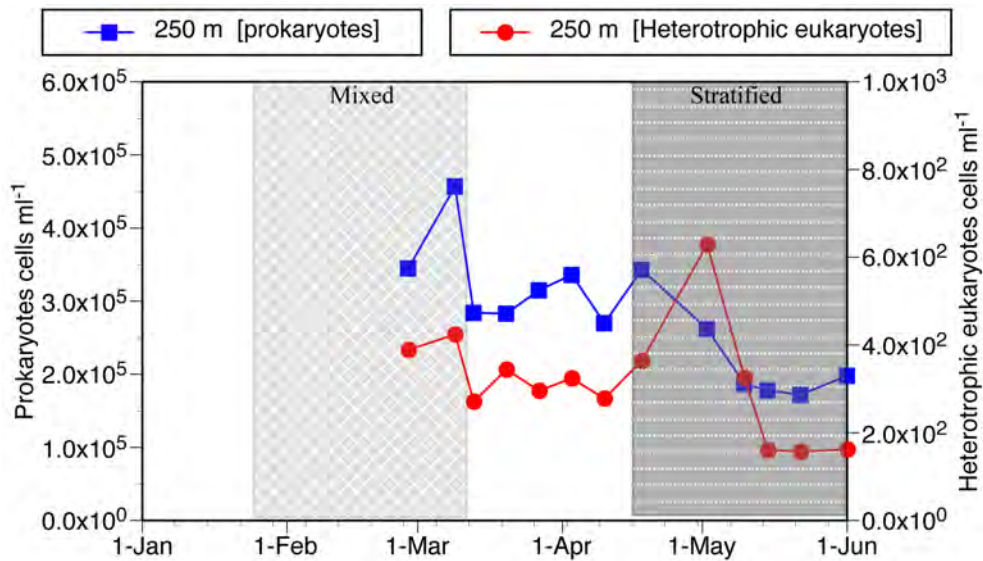


Fig. 2. Temporal changes in concentrations of heterotrophic prokaryotes and eukaryotes at 250 m during the study period. For water column characteristics distinguishing 'mixed' from 'stratified' periods indicated see Fig. 1.

### Tintinnid Ciliates

A large number of tintinnid species were encountered (see supplementary Table 1). Out of a total of 74 species, 53 were found in the deep-water samples (250 m) and 58 in the surface samples (30 m). However, some were found only once or twice in either deep water or surface samples ( $\leq 10\%$  of dates). These numbered 18 out of 58 in the surface samples and 7 out of 53 in the deep-water samples. Leaving aside these rare occurrence species yields then a pool of 46 for the deep-water samples and 40 in the surface samples. The 46 species found in deep waters on at least 3 dates can be divided into 17 deep-water species, and those also found in the surface samples, 29 species. Of the 34

species in surface samples found on at least 3 dates, the surface water species numbered 12. Out of the total species pool, the species found on at least 3 dates were putatively 17 deep-water forms, 22 occurring throughout the water column and 12 surface water species.

Relatively few species occurred commonly, defined here as found on at least 50% of the sampling dates. In the deep water, 20 species were common and in surface layer samples only 10 species were common. In the deep-water samples, the 20 commonly occurring species were composed of two sets: 9 deep-water species and 11 species also found in surface samples. This latter group of forms found both in the deep water and surface samples, was 8 of the 10 common species of the surface layer samples. Thus, common deep-water tintinnids were a mixed assemblage of deep-water species and most of the common surface layer forms. The 9 common deep-water species includes 5 apparently described forms of basically two size-groups (see fig. 3).

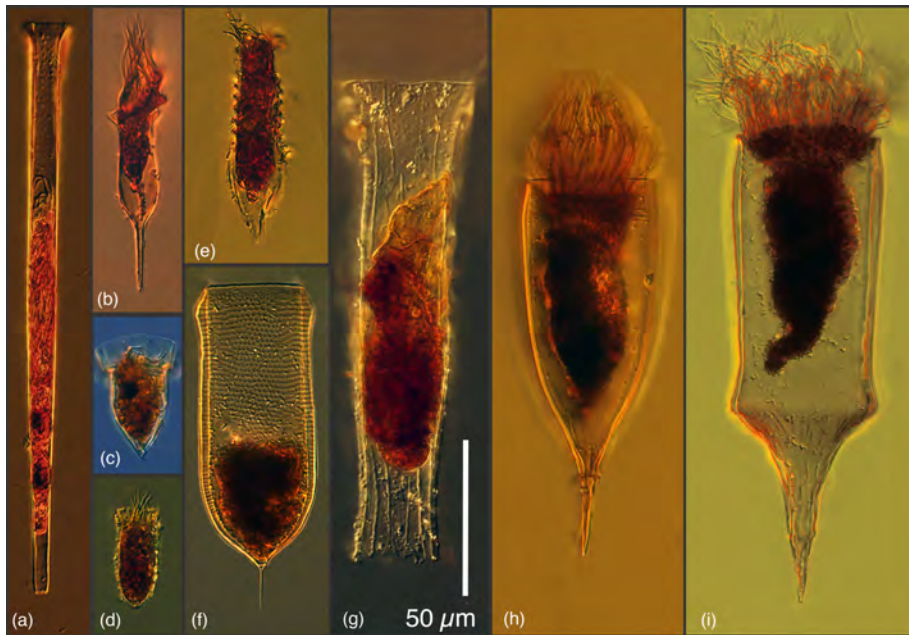


Fig. 3. Common forms of tintinnid ciliates found in deep waters, absent or nearly absent from surface waters: (a) an undescribed *Salpingella* species, (b) an undescribed *Albatrossiella* species, (c) an undescribed *Ormosella* species, (d) a small undescribed *Amphorellopsis* species, (e) an undescribed 'ringed' *Amphorellopsis* species, (f) *Favella aciculifera*, (g) *Daturella striata*, (h) *Xystonellopsis scyphium*, (i) *Parundella messinensis*. Note that there are two basic groups presumably feeding on different sized prey items: forms with small oral opening diameters of 10-20  $\mu\text{m}$  (a-e) and forms with markedly larger oral opening diameters of 40-50  $\mu\text{m}$  (f-g).

Temporal changes in concentrations differed considerably between surface and deep layer populations (fig. 4). The surface population showed much higher variability compared to the deep-water population. Partly this reflected a bloom of salps that corresponded with the near disappearance of tintinnids in the surface water samples in March. Within the deep water population, changes in concentrations largely reflected concentrations of surface and entire water column species in deep waters, which increased markedly with the depth of the mixed layer, compared to shifts in the concentrations of deep water species (fig. 5). Overall concentrations of tintinnids were



significantly ( $p < 0.006$ ) higher (3.3 cells  $l^{-1}$ ) during the period of mixis compared to the stratified period (1.4 cells  $l^{-1}$ ). The species richness of deep-water forms was relatively constant with several forms present regardless of cell abundance while the numbers of entire water column and surface species showed a distinct peak during the mixed period (Fig. 6). Unlike the Phaeogromid radiolarians and Amphisolenid dinoflagellates (see below), there was no clear pattern among any deep-water tintinnid species of presence vs. absence during the mixed compared to stratified periods.

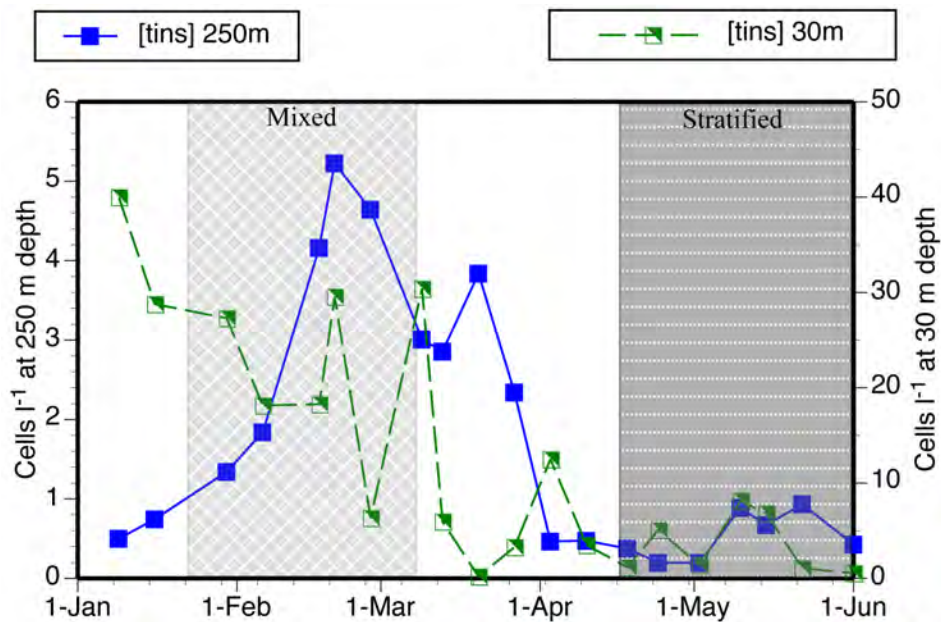


Fig. 4. Temporal changes in the concentrations of tintinnids at 250 m and 30 m during the study period. Note the different scales for populations at 250 m and those found at 30m. For water column characteristics distinguishing 'mixed' from 'stratified' periods indicated see Fig. 1.

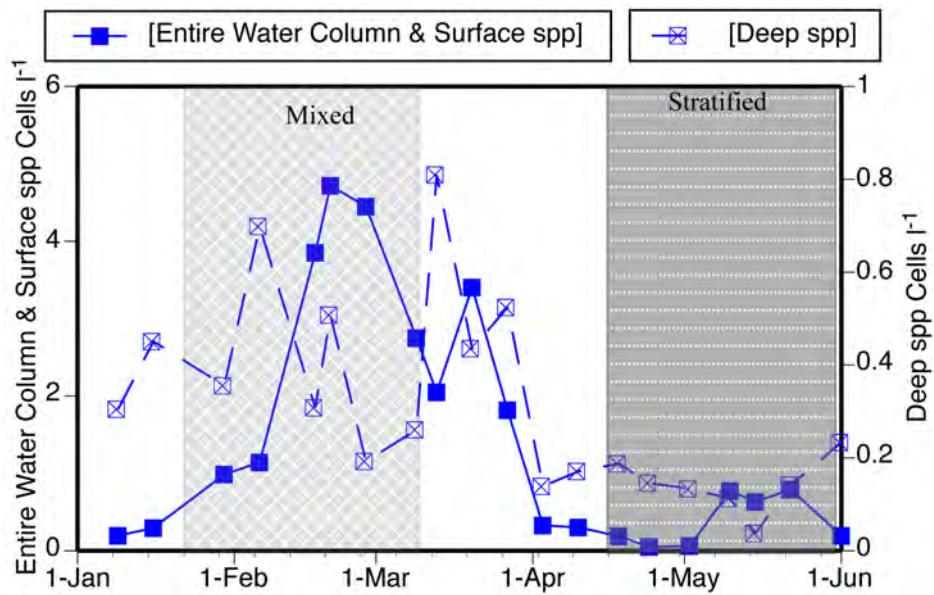


Fig. 5. Temporal changes in the concentrations of tintinnids at 250 m distinguishing two groups- those found only (or nearly only) in the deep water samples, 'Deep spp' from other forms, found mainly or exclusively in the surface water samples, "Entire Water Column & Surface spp'. Note the different scales for the two groups of species. For water column characteristics distinguishing 'mixed' from 'stratified' periods indicated see Fig. 1.

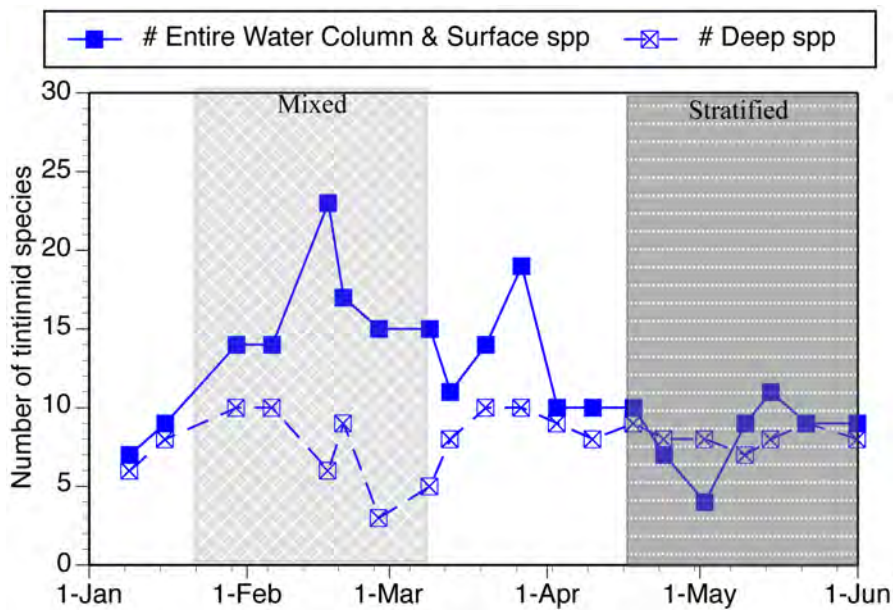


Fig. 6. Species richness of the deep-water tintinnid assemblage. Temporal changes in the numbers of tintinnid species at 250 m distinguishing two groups- those found only (or nearly only) in the deep water samples, 'Deep spp' from other forms, found mainly or exclusively in the surface water samples, "Entire Water Column & Surface spp'. Note the relatively invariant number of deep water species.

### Phaeogromid Radiolarians

A total of 9 phaeogromid species were found in the samples from 250 m, and none were encountered in the samples from 30 m (see supplemental table and data file for details).

In the samples from 250 m, 7 species were found on at least 3 dates: and 4 occurred commonly (Fig. 7). The lowest concentrations were recorded from the beginning of the mixed period and increased over time to peak at the start of the stratified period (Fig. 8). Consequently, the average concentration in the mixed period ( $0.05 \text{ cells l}^{-1}$ ) compared to the average during the stratified period ( $0.14 \text{ cells l}^{-1}$ ) was significantly ( $p < 0.015$ ) lower. The higher concentrations of the stratified period reflected mainly the concentrations of species which were not encountered during the mixed period or present in trace concentrations, but consistently found in the stratified period:

*Challengeranium diodon*, *Challengeron willemoseii*, *Euphysetta lucani* and *Euphysetta pusilla*. Species richness was highest during the stratified period (Fig. 8).

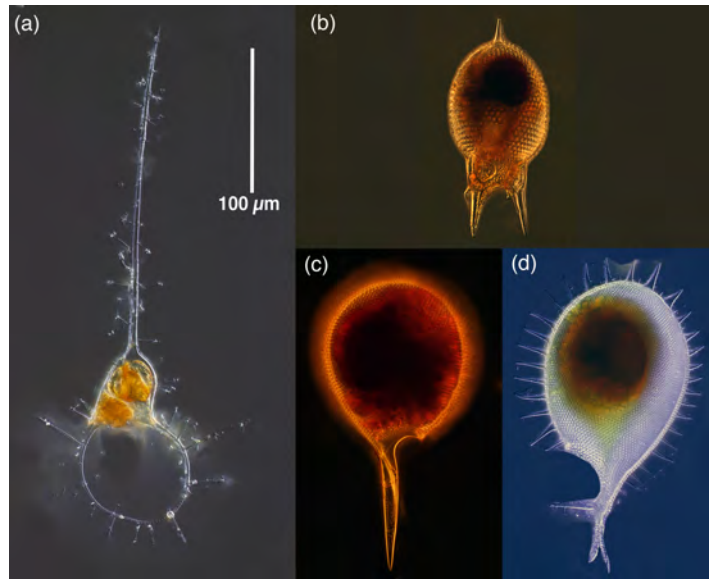


Fig. 7. Common forms of phaeogromid radiolarians found in deep waters: (a) *Medusetta parthenopaea*, (b) *Challengeranium diodon*, (c) *Challengeria xiphodon*, (d) *Challengeron willemoesii*.

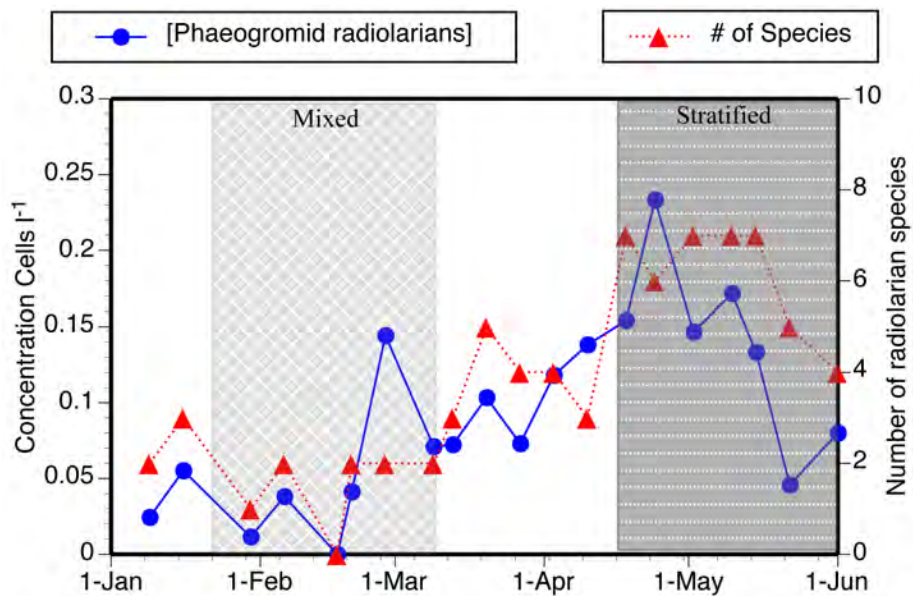


Fig. 8. Temporal changes in the concentrations and species richness (numbers of species) of phaeogromid radiolarians at 250 m. Note that changes in concentrations and species richness are similar. For water column characteristics distinguishing 'mixed' from 'stratified' periods indicated see Fig. 1.

### Amphisolenid Dinoflagellates

Only 4 amphisolenid species were encountered, 3 species of *Amphisolenia* (*A. bidentata*, *A. extensa*, *A. globulosa*) and *Triplosolenia bicornis*. They were occasionally encountered in surface water samples (4 of 20 dates) in trace concentrations but with exception of the late January sampling, consistently present at 250 m. Two species were found commonly, *Amphisolenia globulosa* and *Triplosolenia bicornis* (Fig. 9). There was no significant difference between concentrations in the mixed period vs. the stratified period, both averaged 0.06 cells l<sup>-1</sup>. However, there was a marked shift in the composition of the assemblage from the mixed period with relatively abundant *Amphisolenia* and an absence of *Triplosolenia* to the stratified period dominated by *Triplosolenia bicornis* (Fig 10).



Fig. 9. Common species of amphisolenid dinoflagellates found in deep waters: (a) *Amphisolenia globulosa*, and (b) *Triplosolenia bicornis*.

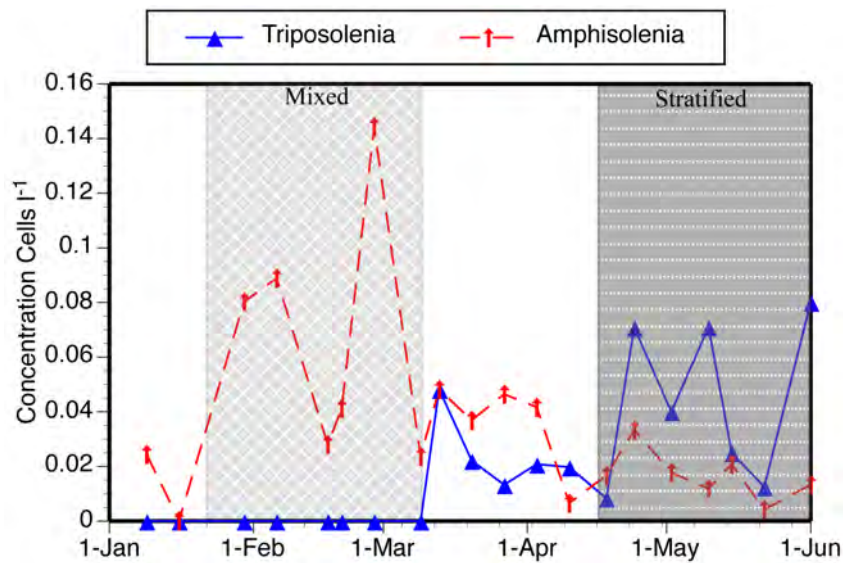


Fig. 10. Temporal changes in the concentrations of species of *Amphisolenia* and the concentration of *Triposolenia bicornis* at 250 m. Note the distinct temporal trends of the two genera. For water column characteristics distinguishing 'mixed' from 'stratified' periods indicated see Fig. 1.

## Discussion

We found clear, consistent and large differences between the surface layer and mesopelagic layer populations both in terms of abundances and species compositions. The differences are unlikely to be due to sampling variability. A previous study of sampling variability, with regard to tintinnids conducted at a nearby site, using sample sizes considerably smaller than those employed in the present study (10 l) found the major differences among replicate samplings to be the number and identity of rare species (Dolan & Stoeck, 2011).

The seasonal changes we documented in water column structure and mesopelagic abundances of heterotrophic prokaryotes and eukaryotes largely conform to previous studies in terms of magnitudes. Both Tanaka & Rassoulzadegan (2002) and Winter *et al.* (2009a,b) investigated populations at the open water Dyfamed site in the

NW Mediterranean while Weinbauer *et al.* (2013) reported on samples from our study site. We found prokaryote abundances to range between  $1.7 - 4.6 \times 10^5$  cells ml<sup>-1</sup> and heterotrophic eukaryotes concentrations ranging from 157 - 629 cells ml<sup>-1</sup> (Fig. 2). Tanaka & Rassoulzadegan found prokaryote abundances at 300 m to vary between  $1 - 4 \times 10^5$  cells ml<sup>-1</sup> from January to May while heterotrophic nanoflagellate concentrations varied between 40 and 200 cells ml<sup>-1</sup>. Winter *et al.* (2009b) reported mesopelagic prokaryote abundances of  $1 - 2.1 \times 10^5$  cells ml<sup>-1</sup> between February and June. For the period between March and June, Weinbauer *et al.* (2013) reported prokaryote abundance of  $2.5 - 3.2 \times 10^5$  cells ml<sup>-1</sup> and the heterotrophic nanoflagellate concentration from 120 to 250 cells ml<sup>-1</sup>. Despite largely identical seasonal changes in water column structure, the periods of peak abundance varied among the previous studies and compared to our findings. For example Tanaka & Rassoulzadegan (2002) and Winter *et al.* (2009b), both reporting on samples from the open water Dyfamed site, reported peak prokaryote abundances in February and May respectively. Weinbauer *et al.* (2013), sampling at the same site as the present report, found peak prokaryote and heterotrophic nanoflagellate abundance in May. We found peak prokaryote and eukaryote abundances in March and May, respectively (Fig. 2). However, these apparent differences may be due to the relatively coarse sampling of bi-monthly to monthly previously employed.

To our knowledge, our study is the first to address weekly temporal changes among mesopelagic protists and document changes in the abundances of individual species. All three groups we examined contained species absent from surface water samples. All three groups showed marked changes in either species compositions, or concentrations, or both over the study period that covered contrasting water column conditions. In the assemblage of tintinnid ciliates 9 species were found commonly in the

deep-water samples. The deep-sea tintinnids were a minority of the tintinnid assemblage during the period of water column mixing when surface water species were found at depth but dominated the assemblage in the stratified period. Peak abundance occurred at the beginning of the mixed period. The phaeogromid radiolarians, completely absent from the surface samples, showed increased abundances and changes in species richness comparing the mixed and stratified periods. They showed peak abundance at the beginning of the stratified period. Among the amphisolenid dinoflagellates peak abundances were recorded in the middle of the mixed period. Species of *Amphisolenia* were found during the mixed period and *Triplosolenia bicornata*, absent during the mixed period, dominated numbers during the stratified period.

The protist species we found that appear to be mainly mesopelagic residents includes both forms suspected to be deep-water species and those known to inhabit deep-water layer. For example, among the Amphisolenid dinoflagellates, *Triplosolenia bicornis* was originally described from plankton net tow material gathered from 250 -to 50 depth, "rarely from surface hauls" (Kofoid 1906) and Jorgensen (1923) described it as almost exclusively from deep water in the Mediterranean and missing from winter samples, similar to our findings. *Amphisolenia* species, while known from surface water samples (e.g. Gomez et al. 2011), are most abundant in sub-surface waters (100-200) but occur in down to 400 m depth (Tarangkoon *et al.*, 2010). With regard to phaeogromid radiolarians, the species we denoted as common in the mesopelagic are also known from the mesopelagic of the Adriatic Sea (Krsinic & Krsinic, 2012) and the western and central Pacific Ocean (Yamashita *et al.*, 2002). Similar to our findings in the NW Mediterranean, in the Adriatic the radiolarians are very rarely found in the surface layer (Krsinic & Krsinic, 2010).



On the other hand the deep-water tintinnid fauna we encountered may be unique to our study site. The 17 tintinnid species we found to be common or present at 250 m (thus absent or rare in surface samples) differed not only in number (8 species) but also identity of those described as deep water forms in the Adriatic Sea (Krsinic, 1998). In common with the Adriatic list of Krsinic (1998), we found *Parundella lohmanni*, *P. messinensis*, *Xystonellopsis scyphium* and *Favella aciculifera*. However of the 5 other species reported as Adriatic deep-water forms we found 1 in both surface and deep water samples (*Ormosella trachleum*), 2 are common in in the Bay of Villefranche, *Amphorides teragona* and *Cyttarocylis ampulla* (Dolan, 2017), and 1 is not known to date from the study area (*Xystonellopsis cymatica*). However, it should be noted that the studies in the Adriatic employed a different sampling method, vertical tows of a 53  $\mu\text{m}$  mesh net thus sampling large volumes of water (several  $\text{m}^3$ ) but with a net size unlikely to catch the small forms we commonly found (Fig. 3).

Concentrations of all three groups varied by over an order of magnitude but quite asynchronously. Tintinnid ciliates, phaeogromid radiolarians and amphisolenid dinoflagellates, are of overall similar sizes and were found in similar concentrations. They would appear to represent roughly similar food items for consumers of microplankton so their distinct abundance patterns argues against strong top-down control by grazers since this would presumably have yielded similar abundance patterns for the three groups. By default the distinct abundance patterns support a hypothesis of variability in group-specific resources and/or variability in group-specific mortality. While we know little about sources of mortality, the three groups in principle exploit very different food resources.

Tintinnid ciliates graze on suspended food items with optimal prey size related to the lorica opening diameter. The average optimal prey size is about 0.25 lorica oral

diameter (Dolan, 2010). The tintinnids common in the mesopelagic were of two groups in terms of lorica oral diameter (fig. 3) those with diameters of about 10-20  $\mu\text{m}$  and forms with diameters of about 40-50  $\mu\text{m}$ . The mesopelagic tintinnid ciliates presumably relied on prey of about 4 and 11  $\mu\text{m}$  diameter respectively, thus nano-plankton-size prey such as heterotrophic nanoflagellates. The phaeogromid radiolarians are thought to feed on sinking organic aggregates (Gowing, 1986). A study of the food vacuole content of several species including species recorded in our study (*Challengeria xiphodon*, *Challengeron willemoesii*, *Euphysetta pusilla*), found the major food items to be bacteria and eukaryotic algae presumably from ingesting suspended organic aggregates (Gowing and Bentham, 1994). Simple correlation analysis revealed no significant relationship with prokaryotic or eukaryotic heterotrophs enumerated using flow cytometry with tintinnid or radiolarian abundances. However, it should be noted that we have a relatively small number of data points for the flow cytometry counts ( $n = 13$ ). With regard to amphosolenid dinoflagellate nutrition, virtually nothing is known. They are known to harbor endosymbiotic phototrophs, some with both prokaryotic and eukaryotic endosymbionts; presumably the dinoflagellate host cell profit from the presence of endosymbionts but their role in the nutrition of the host cell has not been examined (Daugbjerg *et al.*, 2013). However, based on the Secchi disk depths, which varied between 8 and 21 m (data not shown), the euphotic zone reached a maximum depth of about 40 m arguing against a role for endosymbiont photosynthesis in the populations at 250 m depth. The possibility that amphisolenid dinoflagellates below the photic zone digest their symbionts for nutrition can not be excluded. Overall, our study admittedly raises many more questions than the two we set out to answer. For example, the factors controlling the abundance of the mesopelagic protists considered here

clearly remain to be revealed and the possibility that the morpho-species considered may or may not be genetically distinct merits attention.

## **Conclusions**

We examined short-term temporal changes in the species composition and abundance of 3 distinct groups of mesopelagic protists in the N.W Mediterranean Sea over the period corresponding to large changes in water column structure. All three groups examined contained species absent from surface water samples. All three groups showed marked changes in either species compositions, or concentrations, or both over the study period. We established for all three protists groups (tintinnid ciliates, phaeogromid radiolarians, and amphisolenid dinoflagellates) that there appear to be specific mesopelagic forms and the three groups display distinct seasonal patterns of abundance and species composition. We conclude that the mesopelagic protist assemblage is clearly diverse and dynamic at least with regard to groups in which species can be identified using light microscopy.

## **Supplementary data**

Supplementary materials, tables summarizing species occurrences (Supplementary Tables File) and count data for each date (Supplementary Data File) are available at the ICESJMS online version of the article.

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