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Distribution and host diversity of Amoebophryidae parasites across oligotrophic waters of the Mediterranean Sea

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Abstract. Sequences affiliated to Syndiniales (Marine alveolate, MALV) regularly dominate 18S rDNA genetic libraries of nearly all marine ecosystems investigated so far. Among them, Amoebophryidae (MALV group II) is composed of numerous and genetically distant environmental sequences, where *Amoebophrya* is the only known and formally described genus. *Amoebophrya* species include virulent pathogens for a wide range of dinoflagellate species. Beside their regular occurrence in marine ecosystems, their quantitative distribution and the environmental factors triggering host infection have barely been studied in open oligotrophic waters. In order to understand the functional role of these parasites in natural environments, we studied the distribution and contribution to the eukaryotic community of the small free-living stage of Amoebophryidae (the dinospores) along a transect in the Mediterranean Sea, as well as their host diversity at three oligotrophic stations. Dinospores were more abundant at a coastal station (max. 1.5×10^3 cells ml^{-1}) than in oligotrophic waters (max. 51 ± 16.3 cells ml^{-1}), where they represented 10.3 to 34.9% of the total eukaryotic community at 40 and 30 m depth, respectively and 21.2% on average along the water column. Positive correlation was found between dinospore occurrence and higher concentration of $NO_3 + NO_2$ at the coastal station. At selected stations, out of 38 different dinoflagellates taxa identified, 15 were infected, among which a majority were not recognized as Amoebophryidae host so far. Prevalences (percentage of infected cells) generally varied between 1% and 10%, with a notable exception for *Blepharocysta paulsenii* for which 25% of cells were infected at the most oligotrophic station. The present study shows that dinospores are able to thrive and infect dinoflagellates both in coastal and ultra-oligotrophic open waters. Our results emphasize the role of parasitism in microbial food web dynamics and ultimately on biogeochemical cycles.

1 Introduction

Unicellular eukaryotes are responsible for a significant share of primary production on earth and constitute key functional groups driving major biogeochemical cycles on a global scale. Yet their diversity is poorly known especially for cells of the picoplanctonic size fraction (<3 µm), whose distinctive morphological features are not easily perceptible. Environmental surveys based on culture-independent techniques, such as environmental 18S rDNA clone libraries, have revealed a tremendous diversity within this size class (Massana and Pedrós-Alió, 2008; Not et al., 2009). All investigations performed so far pointed out the overwhelming occurrences of sequences affiliated to putative parasites belonging to the alveolates (MALV) (Massana and Pedrós-Alió, 2008). It is now generally considered that MALV sequences correspond to Syndiniales, a group of marine parasitoid dinoflagellates. Within Syndiniales, the family Amoebophryidae (also known as MALV group II) is the most diverse, represented by 44 clades (Guillou et al., 2008). They consistently represent 10 to 50% of the sequences retrieved in clone libraries established from a distinct range of marine ecosystems (Romari and Vaulot, 2004; Medlin et al., 2006; Guillou et al., 2008).

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Currently, the Amoebophryidae family is represented only by a single formally described genus, *Amoebophrya*, including seven species infecting a wide range of organisms such as dinoflagellates, ciliates and radiolarians (Cachon, 1964; Cachon and Cachon, 1987; Coats, 1999; Park et al., 2004). Prevalences (i.e. percentage of infected hosts) estimated on coastal systems ranged from <1 to 80% (Coats, 1999; Park et al., 2004). Most of the available information concerns *Amoebophrya ceratii*, the most studied species within this genus, which was found to infect several marine dinoflagellate species (Park et al., 2004). Yet a combination of phylogenetic and culture studies revealed that *A. ceratii* corresponds to a "species complex" including several more or less host-specific species (Janson et al., 2000; Coats and Park, 2002; Park et al., 2007).

The life cycle of the genus *Amoebophrya* is fairly well understood since its original description by Cachon in 1964. The vegetative life-cycle of *A. ceratii* starts when a small (2–10 µm) biflagellate zoospore (the dinospore) invades the nucleus and/or the cytoplasm of a host cell (Coats, 1999; Park et al., 2004). Then, the endoparasitic stage (the trophont) grows and expands to fill up the host cell volume. At this stage, the parasite acquires a typical beehive-shape characterized by numerous nuclei and a mastigocoel cavity (Fritz and Nass, 1992). In optimal culture conditions, the life-cycle is completed within 2–3 days with the death of the host cell. The mature trophont breaks the host cell wall, eventually becomes vermiform and fragments within few hours into 60 to 400 dinospores able to infect new hosts (Cachon, 1964; Coats and Boackstahler, 1994; Coats and Park, 2002). Parasites are normally lethal to their hosts rendering them reproductively incompetent or photophysiologically deficient (Park et al., 2002b), but can also affect mobility and phototactic behavior of the host (Park et al., 2002a). MALV II environmental sequences retrieved from the smallest size fraction of marine plankton likely result from such dinospores (Guillou et al., 2008). Amoebophryidae-host dynamics are determined by the alternation between the different phases of the life-cycles being also affected by several factors such as parasite generation times, dinospore longevity and dinospores:hosts ratio (Coats and Boackstahler, 1994; Coats and Park, 2002; Park et al., 2007).

Most studies conducted so far on this group of parasitic protists intended to assess their molecular diversity and infectivity (e.g., Janson et al., 2000; Gunderson et al., 2002; Salomon et al., 2003; Guillou et al., 2008; Kim et al., 2008). Little information is available on their abundance, distribution and impact on populations in the natural environment. The importance of parasitism in the host dinoflagellate population dynamics is still debated. Based on field studies, it has been suggested that *Amoebophrya* parasites played an important role in causing the decline or preventing the formation of dinoflagellate blooms. *Amoebophrya ceratii* was able to remove daily 54% of the dominant bloom forming dinoflagellates in a sub-estuary of the Chesapeake Bay (Coats et al., 1996). In the Mediterranean Sea and along the western coast of North America the highest infection level of *A. ceratii* matched closely with the decline of the dinoflagellate bloom of the studied areas (Cachon, 1964; Taylor, 1968; Nishitani et al., 1985). Along three consecutive years in the Penzé Bay (Brittany, France) *Amoebophrya* spp. were found to infect up to 46% of dinoflagellate host cells, particularly the toxic species *Alexandrium minutum* (Chambouvet et al., 2008). Models based on these results showed that the parasite was able to eliminate the host population over a 10 days period (Montagnes et al., 2008). Alternatively, other studies considered *Amoebophrya* parasitism as a minor factor causing host mortality. Only 0.5–2% of the population of *Dinophysis norvegica* was removed by parasitism in the Baltic Sea (Gisselson et al., 2002), and during the decline of the bloom of *Neoceratium falcatiforme* (ex. *Ceratium facatiforme*) ca. 11% of the host cells were killed by *Amoebophrya* (Salomon et al., 2009). Overall, high specificity and virulence of particular Amoebophryidae taxa highlight their potential in controlling host populations. However, their distribution in the marine environment has barely been studied and their occurrence in the open ocean, in particular, has not been documented yet.

In the present study we used fluorescent in situ hybridization (FISH), with an oligonucleotide probe specific for Amoebophryidae (MALV II), to investigate the distribution of dinospores along a transect conducted in the Mediterranean Sea, from coastal to open ocean oligotrophic locations, in the frame of the BOUM cruise. We were able to estimate dinospores' abundances and their contributions to the total eukaryotic cells. Moreover we studied the relationship between dinospores' abundances and abiotic parameters in the natural environment, as well as the potential host spectrum and the prevalences in open water settings.

2 Material and methods

2.1 Sampling strategy

The BOUM cruise (Biogeochemistry from the Oligotrophic to the Ultra-oligotrophic Mediterranean) took place in the Mediterranean Sea during June–July 2008. The cruise track included two transects (north-south and west-east) from the coastal waters off Marseille (France, West Mediterranean) to the open sea off Israel (East Mediterranean) (Fig. 1).

For our analysis, a total of 10 stations were sampled along the cruise track. Three sampling stations (27, 24 and A) were located in the north-south part of the sampling transect, while the remaining stations (19, 15, B, 3, 7, 11, C) were located in the west-east part (Fig. 1). At each station, samples were taken at 5 or 6 distinct depths (from 5 to 160 m) with 24 12 L Niskin bottles rosette equipped with a conductivity-temperature-depth (CTD) and fluorescence sensors. Nutrient concentrations were measured on board with an autoanalyser

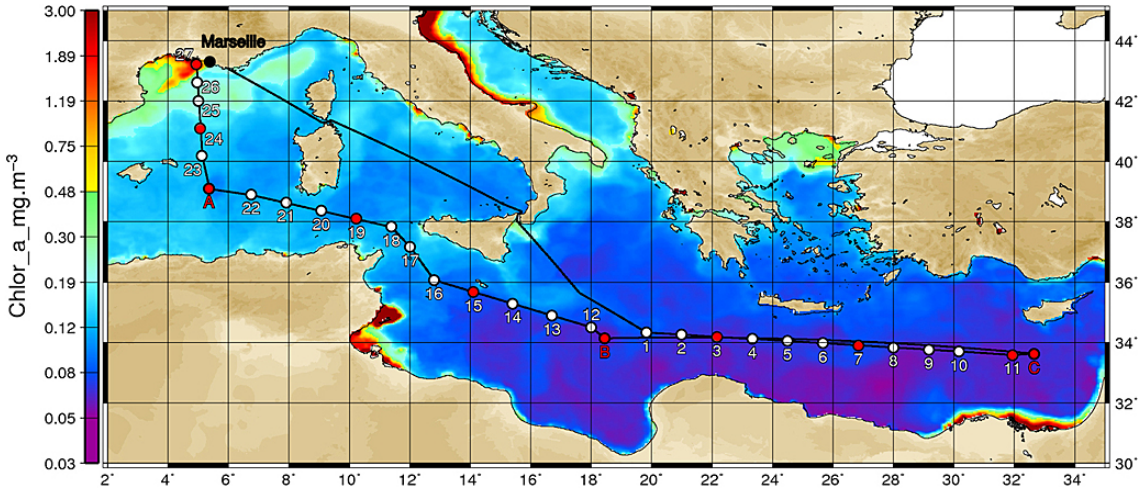

Fig. 1. Map of the BOUM cruise superimposed on a SeaWiFS ocean color composite indicating values of total chl-*a*. Location of stations analyzed in the present study is indicated by red dots. The track of the cruise is indicated, with all sampled stations.

(Bran+Luebbe autoanalyser II) as described in Pujo-Pay et al. (2010).

At each sampling station and depth, seawater was taken directly from Niskin bottles for enumeration of total eukaryotic cells and Amoebophryidae dinospores. Samples were fixed on board with paraformaldehyde (1% final concentration) and stored for 1 h in the dark at 4 °C. Fifty to 200 ml of fixed seawater were filtered onto 0.22 µm Anodisc filters (Whatman) with a vacuum pump (∼200 mm Hg). Filters were then dehydrated through an ethanol series (50%, 80%, 100%, 3 min each), briefly dried at room temperature, and stored at −80 °C.

For the estimation of the host range and prevalences, vertical net samples were collected from 150 m to surface at stations A, B, C using a 60 µm-mesh size net-tow. Samples were fixed on board with paraformaldehyde (1% final concentration) for 1 h, washed and stored in ethanol:PBS (1:1 *v*/*v*) at −80 °C until further analysis. In the laboratory, 1 ml of net samples were diluted in 20 ml of sterile sea water and filtered onto black polycarbonate filters (5 µm; 25 mm diameter) using a vacuum pump (∼200 mm Hg). Filters were then dehydrated by an ethanol series (50%, 80%, 100%, 3 min each), briefly dried at room temperature, and stored at −20 °C.

2.2 FISH-TSA

Fluorescent in situ hybridization coupled with tyramide signal amplification (FISH-TSA) was used to enumerate (1) total eukaryotic cells, (2) Amoebophryidae dinospores and (3) infected hosts and prevalences. The combination of oligonucleotide probes EUK1209R, NCHLO01 and CHLO01 was used to enumerate total eukaryotic cells (Not et al., 2002) whereas the oligonucleotide probe ALV01 was used to target Amoebophryidae (Syndiniales, MALV Group II), dinospores and prevalences (Chambouvet et al., 2008). Oligonucleotide probes were purchased with a 5′ aminolink (C6; MWGBiotech AG) and labeled with horseradish peroxidase (HRP) according to Urdea et al. (1988) and Amann et al. (1992).

Anodisc filters (used to enumerate eukaryotic cells and dinospores) were thawed and cut into pieces (ca. 1/12). For each piece of filter, the face supporting the cells was marked with a pen. For eukaryotic cells, filters were covered with 9 µl of 40% formamide hybridization buffer (40% deionized formamide, 0.9 M NaCl, 20 mM Tris-HCl pH 7.5, 0.01% sodium dodecylsufate (SDS), 10% Blocking agent (Boehringer Mannheim)) and 1 µl of oligonucleotide probe (50 ng µl^{-1} final concentration). Filters were incubated for 3 h at 35 °C for hybridization and subsequently washed twice at 37 °C during 20 min with 3 ml freshly made washing buffer (56 mM NaCl, 5mM EDTA, 0.01% SDS, 20 mM Tris-HCl pH 7.5). Filters were then equilibrated in 3 ml TNT buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 15 min at room temperature in the dark. Each piece of filter was transferred onto a slide for TSA reaction (Kit NEN Life Science Products); 10 µl of freshly made TSA mix (1:1 dextran sulfate and amplification diluent, 1:50 fluorescein tyramide and the mixture of dextran sulfate) were put on the top of each filter piece and slides were incubated for 30 min in the dark. In order to stop the enzymatic reaction and wash the filters, they were transferred in two successive, 5 ml 55 °C pre-warmed, TNT buffer baths for 20 min each. Filters were then rinsed in water, dried at 55 °C, counterstained with DAPI (1 mg ml^{-1}) during few minutes for visualization of nucleus, and mounted in antifading reagent (Citifluor AF1). Filters were finally covered with a cover glass and sealed with nail varnish. Slides were stored at 4 °C in the dark for two days before analysis.

For Amoebophryidae dinospores the FISH-TSA protocol differed slightly. Hybridizations were conducted overnight (16–17 h) at 42 °C. Filters were then washed 3 times at 46 °C, for 30 min each. After TSA reaction, filters were washed 3 times in 55 °C pre-warmed TNT buffer baths for 30 min. Cell nuclei were counterstained with Propidium Iodide (PI) (10 µg ml^{-1}).

For identification of infected dinoflagellate cells and estimation of Amoebophryidae prevalence 1/4 pieces of the 5 µm filters were covered with 27 µl of 40% formamide hybridization buffer and 3 µl of oligonucleotide probes (50 ng µl^{-1} final concentration). Filters were incubated for 12 h at 42 °C for hybridization, and subsequently washed twice during 30 min at 46 °C. After equilibration in 5 ml TNT buffer for 15 min, filters were then covered with 30 µl of freshly made TSA mix and incubated for 30 min in the dark. They were transferred in two successive 5 ml 55 °C pre-warmed TNT buffer baths for 30 min each. Cells were counterstained with calcofluor (100 ng ml^{-1}) for visualization of dinoflagellate theca. Slides were covered with a cover glass, together with a mix of Citifluor AF1 and Propidium Iodide (10 µg ml^{-1}), sealed with nail varnish and stocked at 4 °C in the dark.

2.3 Epifluorescence microscopy

All hybridized and stained filters were observed with an Olympus BX-51 epifluorescence microscope (Olympus Optical) equipped with a mercury light source, a 11012v2-Wide Blue filters set (Chroma Technology, VT, USA) and a CCD camera (Spot-RT, Diagnostic Instrument, Sterling Heights, MI, USA). Cells were observed with fluorescence filter sets for DAPI (excitation: 345 nm; emission: 455 nm), PI (excitation: 536 nm; emission: 617 nm) and FITC (excitation: 495 nm; emission: 520 nm). A total number of 52 and 54 samples have been counted, respectively for total eukaryotic cells (EUK1209R, NCHLO01 and CHLO01 probes) and Amoebophryidae dinospores (ALV01 probe). Total eukaryotic cells were counted with the 100× objective in 20 randomly chosen microscopic fields; numbers of counted cells varied among filters (4–21 cells per field). Amoebophryidae dinospores were counted with the 100× objective in 3 randomly chosen transects along the total length of the analyzed piece of filter. Variable numbers of dinospores were counted for the different filters (0–88 per transect). Dinoflagellate infected cells were observed and counted with 20× or 40× objectives on the whole surface of the piece of filter processed. A specimen was considered infected when the nucleus of the parasite together with the probe signal was clearly identifiable in the host cell. Prevalences were considered reliable when at least 50 specimens (n) of dinoflagellate species were observed. Below this value, prevalences were calculated, but considered not reliable.

2.4 Statistics and data representation

In order of evaluate any relationship between eukaryotic cells and Amoebophryidae dinospores, a Spearman correlation analyses ($N = 52$) was performed using Statistica 6.0 (StatSoft). The relationship between these variables and some oceanographic variables (i.e. temperature, salinity, fluorescence, $NO_3 + NO_2$, PO_4 and $Si(OH)_4$) was also checked with the same statistic procedure. Prior to the analyses, all data were transformed logarithmically [$\ln(x+1)$]. All maps of vertical distribution provided in this study were drawn using Ocean Data View software (Schlitzer, 2003).

3 Results

3.1 Oceanographic context

The middle-eastern part of the cruise was characterized by surface water masses of higher temperature and salinity than in the western part (Moutin et al., 2011). All along the transect $NO_3 + NO_2$, PO_4 and $Si(OH)_4$ showed concentrations lower than 1, 0.02, and 2 µmol l^{-1}, respectively, characterizing extremely oligotrophic waters. Nevertheless, one could notice that nutrient values gradually increased from 75 m depth towards deeper water at all stations (Pujo-Pay et al., 2010). Integrated concentrations of $NO_3 + NO_2$ along the first 50 m of the water column were higher at station 27 than at other stations (Fig. 3). This pattern was not observed for phosphates and silicates. Fluorescence values were also very low, with the highest values observed at surface on station 27 (for details see Crombet et al., 2010).

3.2 Total eukaryotic cells and Amoebophryidae dinospore abundances

A strong gradient of decreasing abundances of total eukaryotes (including pico- nano and microplanktonic cells) was observed from the coast to the open ocean, from the west to the eastern part of the studied area (Fig. 2a). Higher abundances were observed at the surface layer (5 m) on stations 27 (7.8×10^3 cells ml^{-1}) and 24 (6.6×10^3 cells ml^{-1}), in the western part of the studied area. Eukaryotic cell densities gradually decreased from stations 27 and 24 going eastwards to stations A, 19 and 15 (0.4–5.1×10^3 cells ml^{-1}), and from the surface to deeper layers. The lowest eukaryotic cell abundances (0.1–1.9×10^3 cells ml^{-1}) were observed at the eastern part of the transect (stations B to C), with a homogeneous vertical distribution.

Dinospores targeted by the Amoebophryidae specific probe have been detected at all studied stations but not at all depth of each station. They appeared rather homogeneous morphologically along the transect (Fig. 4a and b), 4–8 µm in size, naked, and presenting a dense nucleus occupying half of the cellular volume. No bacteria was observed inside the dinospore cytoplasm (easily visible after IP staining).

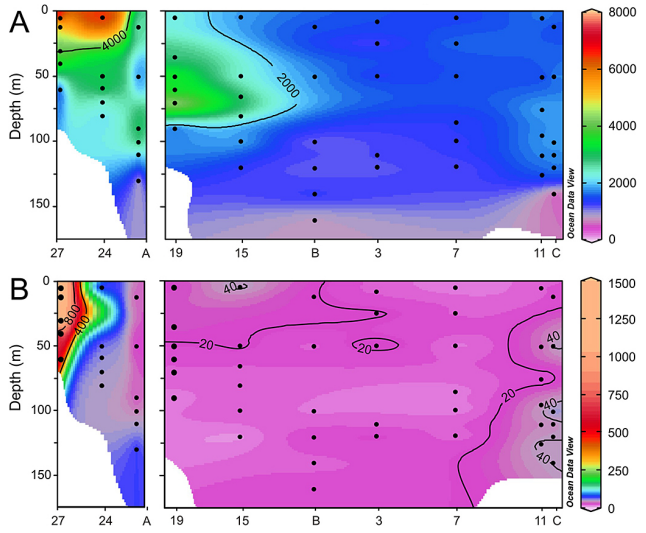

Fig. 2. Abundances (cells ml^{-1}) of total eukaryotes **(A)** and Amoebophryidae dinospores **(B)** obtained by fluorescent in situ hybridization (FISH) analyses. The left and right parts of the figures correspond respectively to the north-south and west-east parts of the cruise transect.

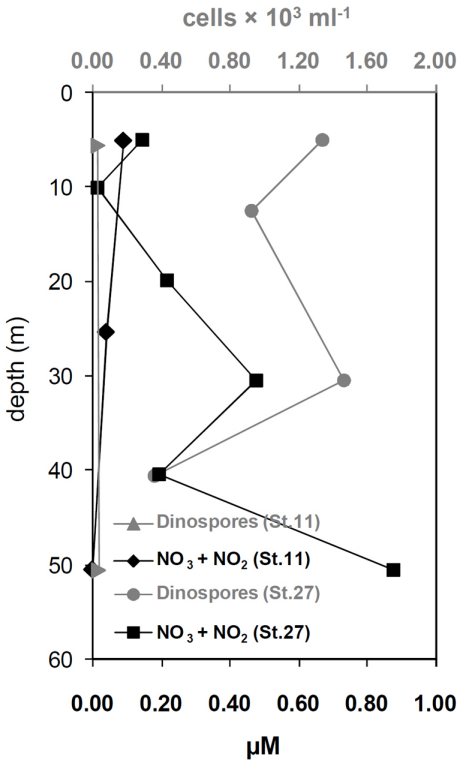

Fig. 3. Dinospore abundances and $NO_3 + NO_2$ concentrations along the first 50 m of the water column at the coastal station 27 at the station 11, chosen as representative of the ultraoligotrophic conditions of the sampling area.

Significant differences of abundance were observed between the north-south and west-east parts of the transect (Fig. 2b). The highest dinospore abundance (1.5×10^3 cells ml^{-1}) was observed at 30 m depth of station 27 in the north-south part of the transect. At this station dinospores represented from the 10.3 (40 m) to the 34.9% (30 m) (average calculated along the water column 21.2%) of the total eukaryotic community. Dinospores abundances were at least 10 times lower at all other stations (Fig. 3). For instance, densities reached 39.5 ± 4.5 cells ml^{-1} and 51 ± 16.3 cells ml^{-1} on average along the water column at stations 24 and C, respectively. The lowest abundances were observed at station 7 (4.2 ± 4.6 cells ml^{-1} on average along the water column). At all but one station (station 27), dinospores accounted for a very small proportion of the total eukaryotic cells, ranging from 0.4 to 3.1%. No significant statistical correlation was found ($R = 0.24$; $p = 0.067$) between eukaryotic cells (including diatoms, heterotrophic and autotrophic nanoflagellates) and dinospore abundance. Similarly, no significant correlations were detected between eukaryotic cells or dinospore density with temperature, salinity, fluorescence, and nutrients. Nevertheless a significant correlation between dinospores and $NO_3 + NO_2$ ($R = 0.73$; $p < 0.05$) was obtained when considering only values restricted to the first 50 m depth (Fig. 3).

3.3 Infected species

Infected hosts have been investigated by FISH using the Amoebophryidae probe at three selected stations (A, B and C). Different stages of the trophont maturation were observed, from the initiation of the infection (Fig. 4c, d) to the intermediate growth (Fig. 4e, f, g, i, j, l, m) and the mature beehive stages, typical of the *Amoebophrya* genus (Fig. 4h and k). If only one growing trophont is usually observed during late stages of infections, several co-infecting dinospores have been repeatedly observed at the beginning of infections (Fig. 4d).

From a total of 38 dinoflagellates taxa identified along these stations, 15 dinoflagellate taxa were infected by Amoebophryidae (Table 1). Infected species belong to three different orders of thecate dinoflagellates (i.e. Dinophysiales, Gonyaulacales and Peridiniales), with the genus *Neoceratium* particularly well represented with 8 infected species. Both the highest dinoflagellate species number and the highest number of infected species were found at station A, with 12 species infected out of the 30 taxa identified at this station. Lower numbers of infected hosts have been observed at stations B and C, with 3 and 8 over 19 and 27 dinoflagellate species identified, respectively.

Infections were more frequent in some species compared to others (Table 1). *Blepharocysta paulsenii* was the only species to be infected at all stations. *Gonyaulax polygramma*, *G. fragilis*, *Neoceratium pentagonum* and *Ornithocercus carolinae* were always infected when detected (prevalences

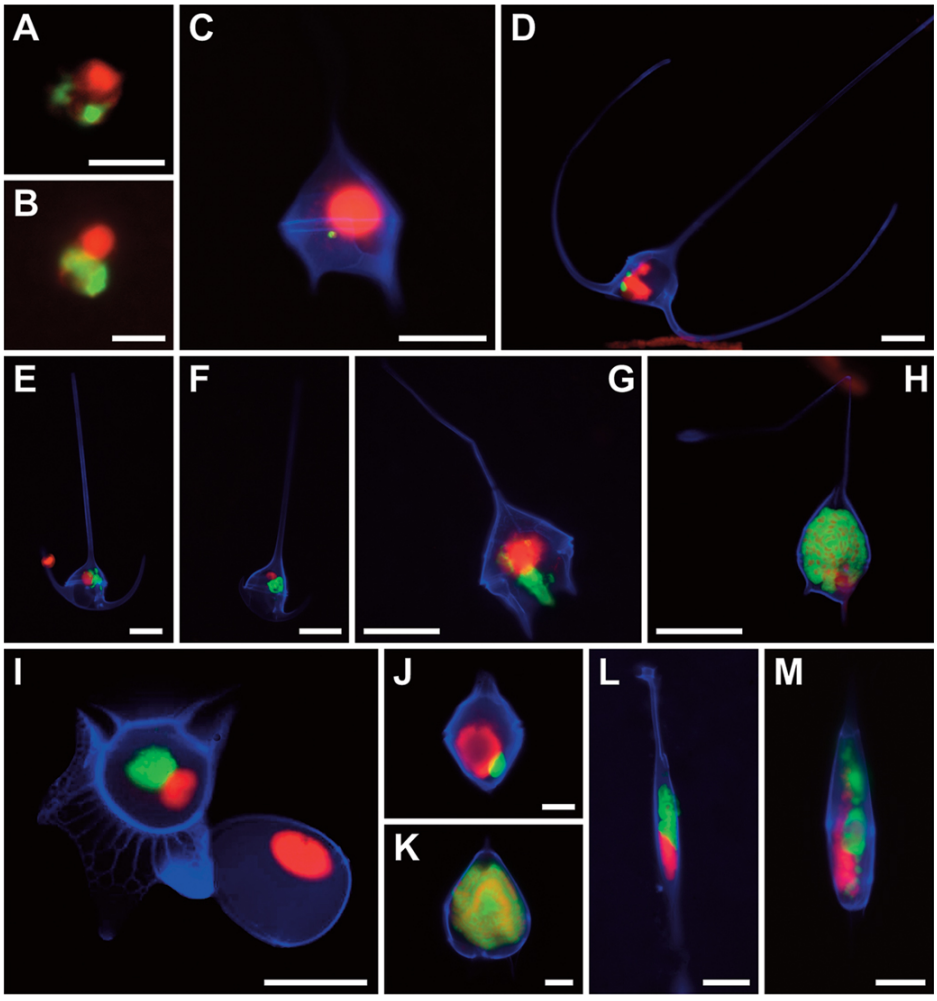

Fig. 4. Micrographs of dinospores **(A, B)** and infected dinoflagellates **(C–M)** observed under UV excitation. The cell nucleus (red), the theca (blue) of the dinoflagellates and the fluorescence of the probe targeting the cytoplasm of Amoebophryidae (green) are shown. Different maturation stages have been observed. **(C)** *Neoceratium pentagonum* with a dinospore inside; **(D)** *Neoceratium trichoceros*, the green area attached to the nucleus indicates that the infection is started; **(E)** *Neoceratium tripos*, **(F)** *Neoceratium pulchellum* and **(G)** *Neoceratium pentagonum* as an illustration of different progressive infection stages; **(H)** *Neoceratium minutum* with a mature trophont having the typical beehive-shape of *Amoebophrya* parasite, small red spots are the multiple nuclei of this stage; **(I)** infected *Ornithocercus carolinae* together with non-infected *Prorocentrum* sp.; infected cells of **(J)** *Gonyaulax polygramma*, **(K)** *Podolampas bipes* with a beehive-shape of the trophont presenting an internal cavity clearly visible, **(L)** *Amphisolenia globifera* and **(M)** *Podolampas spinifera* with a atypical trophont feature. Scale bars **(A)**, **(B)** = 5 µm; **(C)**, **(D)**, **(E)**, **(F)**, **(G)**, **(H)** = 50 µm; **(I)** = 10 µm; **(J)**, **(K)**, **(L)**, **(M)** = 20 µm.

ranking from 4 to 12.5%). Other species were detected at all stations, but not always infected (*Neoceratium fusus*, *N. tripos*, *Podolampas spinifera*). Finally some taxa were present, but never occurred to be infected (*Cladopyxis brachiolata*, *Dinophysis caudata*, *Ornithocercus quadratus*, *Oxytoxum milneri*, *O. scolopax*, *Protoperidinium* spp.) even when present in the samples at high densities (e.g. *Cladopyxis brachiolata*). When a significant number of cells was observed ($n > 50$), prevalences were usually less than 10% (from 2 to 10%), with the notable exception of *Blepharocysta paulsenii*, for which 25% of cells were infected at station C (average on the three stations 10.3%). Noteworthy, some tintinnid ciliates were also observed to contain green dots, however it was not clear if the parasites were growing inside or just ingested by the ciliate.

4 Discussion

4.1 Distribution of the free-living stage of Amoebophryidae

The relative importance of MALV sequences in rDNA genetic libraries may have been underestimated because of inherent biases produced during the DNA extraction and

Table 1. List of infected and non-infected dinoflagellate species recorded at stations A, B and C. Species underlined are new potential hosts of Amoebophryidae. Number of examined specimens for the determination of prevalence (%) of infected species is showed in parenthesis. Significant prevalence values ($n > 50$) are in bold. NI = dinoflagellate species present but non-infected; ND = dinoflagellate species non-detected.

	A	B	C
Dinophysiales			
Amphisolenia globifera Stein	**2% (**$n = 61$**)**	ND	NI
Dinophysis caudata Saville-Kent	NI	NI	NI
Dinophysis odiosa (Pavillard) Tai and Skogsberg	NI	ND	NI
Dinophysis schuettii Murray and Whitting	NI	ND	ND
Dinophysis tripos Gourret	NI	NI	NI
Histioneis remora Stein	ND	ND	NI
Ornithocercus carolinae Kofoid	ND	**4% (**$n = 52$**)**	ND
Ornithocercus magnificus Stein	NI	ND	ND
Ornithocercus quadratus Schütt	NI	NI	NI
Phalacroma cuneus Schütt	ND	NI	ND
Phalacroma doryphorum Stein	NI	ND	ND
Phalacroma favus Kofoid and Michener	NI	ND	ND
Phalacroma mitra Schütt	NI	ND	ND
Phalacroma rotundatum (Claparède and Lachmann) Kofoid and Michener	NI	ND	NI
Gonyaulacales			
Neoceratium contrarium (Gourret) Gómez, Moreira and López-Garcia	2% ($n = 10$)	NI	**2% (**$n = 57$**)**
Neoceratium fusum (Ehrenberg) Gómez, Moreira and López-Garcia	**6% (**$n = 52$**)**	NI	**6% (**$n = 88$**)**
Neoceratium horridum (Gran) Gómez, Moreira and López-Garcia	NI	NI	**2% (**$n = 57$**)**
Neoceratium longirostrum (Gourret) Gómez, Moreira and López-Garcia	NI	ND	ND
Neoceratium minutum (Jørgensen) Gómez, Moreira and López-Garcia	2% ($n = 10$)	NI	**6% (**$n = 52$**)**
Neoceratium pentagonum (Gourret) Gómez and López-Garcia	**4% (**$n = 53$**)**	ND	ND
Neoceratium pulchellum (Schröder) Gómez, Moreira and López-Garcia	3% ($n = 40$)	ND	NI
Neoceratium trichoceros (Ehrenberg) Gómez, Moreira and López-Garcia	1% ($n = 10$)	ND	NI
Neoceratium tripos (Müller) Gómez, Moreira and López-Garcia	20% ($n = 15$)	NI	12% ($n = 20$)
Gonyaulax fragilis (Schütt) Kofoid	9% ($n = 20$)	ND	**10% (**$n = 51$**)**
Gonyaulax polygramma Stein	ND	ND	12% ($n = 8$)
Protoceratium cf. *areolatum* Kofoid	ND	NI	NI
Peridiniales	ND	ND	ND
Ceratocorys gourreti Paulsen	NI	NI	ND
Cladopyxis brachiolata Stein	NI	NI	NI
Blepharocysta paulseni Schiller	2% ($n = 10$)	20% ($n = 10$)	**25% (**$n = 54$**)**
Oxytoxum milneri Murray and Whitting	NI	NI	NI
Oxytoxum scolopax Stein	NI	NI	NI
Oxytoxum tesselatum (Stein) Schütt	ND	ND	NI
Protoperidinium spp.	NI	NI	NI
Podolampas bipes Stein	1% ($n = 10$)	1% ($n = 10$)	NI
Podolampas spinifera Okamura	2% ($n = 15$)	NI	10% ($n = 20$)
Pyrophacus steinii (Schiller) Wall and Dale	ND	ND	NI
Prorocentrales			
Prorocentrum micans Ehrenberg	ND	NI	ND
Prorocentrum spp.	NI	ND	NI

PCR amplification. It was recently suggested that the dominance of MALV environmental sequences from size fractions $<0.8\,\mu m$ could correspond to extracellular material rather than living cells (Not et al., 2009). Our quantitative estimation of MALV II free-living cells distribution in oligotrophic waters demonstrated their occurrence in both coastal and open ocean areas as they were detectable at all sampling location across the transect performed. This confirms the possibility that MALV environmental sequences from oceanic regions were actually retrieved from active parasites. However, dinospores observed were relatively large, which is congruent with previous observations and formal taxonomic descriptions of Syndiniales (from 4 to 8 µm), arguing in favor of the hypothesis suggesting that environmental sequences retrieved from samples passing through less than 0.8 µm are derived from larger cells.

Abundances of Amoebophryidae may be underestimated because of the specificity of the ALV01 probe. None of the oligonucleotide probe designed for FISH analyses covers the entire genetic diversity of MALV II. The ALV01 probe used in this study targets 33 clades over the 44 described, whereas the probe used by Salomon et al. (2009) cover 24 clades, as revealed by a screening of a complete eukaryotic sequence database (KeyDNAtools, http://keydnatools.com/). When considering all environmental sequences belonging to MALV II found in previous studies performed in the Mediterranean Sea (Díez et al., 2001; Massana et al., 2004, 2006; Viprey et al., 2008), the ALV01 probe targets 400 sequences out of the 612 retrieved, which correspond to 22 clades over the 30 detected so far in the Mediterranean Sea. As revealed by the ALV01 probe, abundances of Amoebophryidae were very variable across stations. In the NW areas (stations 27, 24, A, 19, 15) dinospores reached the maximum concentration at station 27 (average: $1.0 \times 10^3 \pm 0.5$ cells ml^{-1}, max. at 30 m: 1.5×10^3 cells ml^{-1}) and contributed up to 34.9% of the total eukaryotes. In the eastern part of the studied area (stations B, 3, 7, 11, C) lower abundances were detected (min. 4.2 ± 4.6 cells ml^{-1}) corresponding to 0.4 to 3.1% of the total eukaryotic cells.

The comparatively high density of dinospores at station 27 cannot be associated to a higher abundance of a particular dinoflagellate species larger than 20 µm, since microdinoflagellate species numbers were relatively comparable across the stations (F. Gómez, personal communication, 2010). Actually, at station 27, dinoflagellates larger than 20 µm in size represented less than 1% of total eukaryotes. The dominant organisms were heterotrophic nanoflagellates and pico- nano-phytoplankton which represented 52% and 30% of the total community, respectively (Christaki et al., 2011). No correlation was detected between dinospores and heterotrophic or autotrophic flagellates (different from dinoflagellates). Indeed, these two components of the phytoplankton community have never been shown to be potentially hosts of Amoebophryidae.

Three possible explanations could be suggested to explain the higher dinospore abundances at stations 27: (i) the occurrence of microdinoflagellate infections before the sampling; (ii) the presence of other potential hosts for dinospores overlooked in this study (including small dinoflagellates); (iii) other abiotic and biological factors affecting infectivity success.

Amoebophryidae infections were considered as an important cause of microdinoflagellate mortality in natural environments, with population outbreaks observed in less than 10 days (Chambouvet et al., 2008). Consequently, the high abundance of dinospores at station 27 could be the result of an infection of the microdinoflagellate populations that occurred few days before our sampling.

The presence of other potential hosts for dinospores, overlooked during this study, such as <20 µm thecate and naked dinoflagellates, could explain the high abundances of dinospores recorded at station 27. Nano- and picoplanktonic dinoflagellates can be an important component of coastal dinoflagellate assemblages (Siano et al., 2009; Siokou-Frangou et al., 2010), and some <20 µm dinoflagellate genera (e.g. *Karlodinium*, *Heterocapsa*, *Oxytoxum* etc.) are recognized as potential host of Amoebophryidae (Park et al., 2004). However, specific counts of <20 µm phytoplankton community have not been performed, thus data on the nano- and picodinoflagellate populations are not available. Unfortunately, the hypothesis of an infection of the small dinoflagellate populations cannot be neither verified on net samples collected at station A, B, C since a 60 µm-mesh size net-tow has been used. In addition other protists (e.g. ciliates) or pluricellular organisms (e.g. copepods) could have been infected by Amoebophryidae, being a further source of dinospores. If the infection of ciliates by Amoebophryidae is doubtfull (Salomon et al., 2009; this study), the possibility that Amoebophryidae infect metazoans is still to be verified.

Abiotic factors such as nutrient concentration may play an important role in the distribution of Amoebophryidae. Physiological experiments showed that *Amoebophrya* sp. cultured with dinoflagellate hosts grown in nutrient-replete medium produced 3–4 times more dinospores than those infecting hosts maintained under low-nutrient conditions (Yih and Coats, 2000). In addition, dinospores produced by parasites cultivated under high nutrient concentration had a higher infectivity success than those formed by parasites grown at low nutrient values (Yih and Coats, 2000). According to the results from these later studies, one can expect less dinospores produced in oligotrophic waters and rates of parasitism inferior to those of waters with more elevated nutrient concentrations. The difference in $NO_3 + NO_2$ concentration observed in the top 50 m (Fig. 3) of the water column between station 27 and all other stations might explain the higher Amoebophryidae dinospore abundance recorded at station 27. Indeed, a strong positive correlation was found between dinospores and $NO_3 + NO_2$ concentrations. Besides chemical components, other abiotic (light intensity, photoperiod, etc.),

but also biotic factors influencing host conditions (host immunological capabilities, complex life cycles) could explain the success of infections. Light intensity is a factor which has been suggested to play an important role in parasitic infection. Prevalences of infected host cells inoculated during the day were higher than when inoculated during the night, suggesting that infection rates might be related with environmental light conditions or diurnal biological rhythm of host species (Park et al., 2007). Finally it is worth considering that infectivity and the resulting production of dinospores depends on the encounter rate between hosts and free-living parasitoid stage (dinospores). This encounter may depend not only on the abundance of the host cells, but also on the physical factors triggering this encounter. Turbulence was shown to lead to a 25–30% reduction in the maximum infection rate of *Parvilucifera sinerae* on dinoflagellates populations (Llaveria et al., 2010), the role of other physical forcings (water density, circulation) is still to be elucidated. The production of attractive allelopathic molecules, that could trigger the host-parasite encounter, is also a possibility that remain to be explored.

4.2 Host diversity and prevalence in oligotrophic waters

Dinoflagellates diversity is rather high in the Mediterranean Sea (Gómez, 2003, 2006) but their biomasses and relative importance in the planktonic assemblages rarely reach considerable values (Siokou-Frangou et al., 2010). All dinoflagellate species identified in this study have been previously found in the Mediterranean Sea (Schiller, 1937; Rampi and Bernard, 1980; Gómez, 2003). Our sampling and detection strategy allowed the identification of clear Amoebophryidae infections only inside large thecate dinoflagellates. The different maturation steps detected are all congruent with the description of the genus *Amoebophrya*. In particular, typical beehive stages characteristic of that genus have been observed in several host species. Other hosts such as delicate ciliates and unarmored dinoflagellates may not have been properly preserved by the formaldehyde fixation procedure used. In addition, our study was restricted to cells retained by our 60 µm mesh size net-tow.

Fifteen species over the 38 identified dinoflagellate were infected along the three studied stations (A, B, C), and among them 13 had never been previously identified as potential hosts for Amoebophryidae. This rises to 48 the number of potential host species belonging to dinoflagellate, which was previously established at 35 taxa (Park et al., 2004). Our study provides additional evidences that infections are frequent, and occur toward a broad spectrum of host diversity. This is congruent with environmental genetic libraries showing the presence of several genotypes of MALV II at a single sampling site and from a wide range of ecosystems, including oligotrophic areas (Groisillier et al., 2006; Guillou et al., 2008).

Significant ($n > 50$) prevalences (i.e. % of infected cells of a given species) observed in the three oligotrophic stations sampled (A, B, C) were generally between 2% to 10% (average 4.6%), with a notable exception for *Blepharocysta paulsenii*, for which 25% of the cells were infected. These values are comparable to previously reported data for Brazilian oligotrophic waters, where percentages of early infection and late infection stages ranged from 1–7% and 20–50%, respectively (Salomon et al., 2009). In eutrophic environments, averaged prevalence of cells infected by *Amoebophrya* varied between 1% and 6%, with peaks of infected cells of 81% (Rhode River, Coats et al., 1996) and 40% (Chesapeake Bay, Coats and Bockstahler, 1994). It has been suggested that parasitic prevalence is strongly dependent on the host abundance (Park et al., 2004). In this study, abundances of microdinoflagellates (>20 µm in size) did not significantly varied between stations (average less than 1 cells ml^{-1}, F. Gómez, personal communication, 2010) and an important number of species were infected with relatively low prevalences (ranging from 1 to 3%). Such low prevalences might be linked to unspecific infections. For example, although these parasites have been detected to be essentially host specific in the Penzé estuary, other non-primary host species could also be infected at low prevalences by the same parasite (Chambouvet et al., 2008). However, complete maturation of these parasites infecting non-primary hosts were never observed, a fact that was also reported in cultures (Coats and Park, 2002). This capacity to initiate infections in different hosts may be a key aptitude in terms of adaptation and survival. However, we cannot exclude that some early stages (dinospores) observed inside dinoflagellates simply resulted from the feeding activity of dinoflagellates.

4.3 Integration of Amoebophryidae within the microbial food webs and biogeochemical cycles

Oligotrophic systems are characterized by the dominance of the microbial food web, where picophytoplankton are recognized as the major contributors to primary production and heterotrophic bacteria are the principal recyclers of the organic matter (Turley et al., 2000). Heterotrophic nanoflagellates (HNF) play also an important role in these ecosystems, being able to remove 45 to 87% of bacterial biomass (Christaki et al., 2001). Competitions for orthophosphate between heterotrophic bacteria and cyanobacteria, and predation by HNF on bacteria, have been suggested as the two main biological processes which regulate the structure of microbial food webs in oligotrophic systems (Thingstad and Rassoulzadegan, 1999). Our results indicate that parasitism by Amoebophryidae is also an important process influencing microbial food webs structure and dynamic in such ecosystems. Amoebophryidae dinospores are non-photosynthetic biflagellate cells which can be easily misinterpreted as HNF, but in contrast to "regular" HNF, dinospores were confirmed to not consume bacteria (this study). For instance,

at station 27, very small HNF of 1.8–2.2 µm in size were more abundant than dinospores with 2×10^3 cells ml^{-1} and 1.0×10^3 cells ml^{-1}, respectively (Christaki et al., 2011). Because of their slightly larger size and general shape, dinospores were distinguished from typical HNF and thus they were not included in the HNF counts (U. Christaki, personal communication, 2010). Since dinospores are larger, they finally represented an important available biomass for higher trophic levels.

Both field observations and computer based models suggested that Amoebophryidae do have the capacity to control their host population at short and long terms (Montagnes et al., 2008; Coats et al., 1996; Chambouvet et al., 2008) similarly to viruses (Sandaa, 2008). For viruses the so-called "killing the winner" model of community structure has been proposed (Thingstad and Lignell, 1997). The model implies that viruses control the most abundant and fastest-growing host population, enabling less competitive or slower growing host population to coexist with the dominant, fast growing hosts and consequently they act locally to the species richness and diversity (Sandaa, 2008). However, viruses are very resistant and can wait months for their host once released in the water. This is definitely not the case for dinospores which suffer predation. Grazing of Amoebophryidae dinospores by ciliates has been shown to limit host infections (Maranda, 2001; Johansson and Coats, 2002). Such grazing pressure is suggested also by the detection of dinospores within tintinnids by positive probe signal in FISH analyses (Salomon et al., 2009; this study). Dinoflagellates themselves may directly feed on dinospores, as it was discussed previously.

This study shows that Amoebophryidae dinospores densities follow total eukaryotic community trends rather than dinoflagellates abundances and moreover that in open oligotrophic settings dinospores are not host density dependent as infections occurred even at relatively low host abundances. This fact turns to a paradox, as it is reported from cultures that dinospores can only survive few days outside their host (Coats and Park, 2002). Nevertheless, the fate of these dinospores is unknown in natural environment. The use of alternative host (acting as reservoir), and the production of resistant cysts, would be extremely important strategies for such parasites, especially in oligotrophic environments, where cell densities are lower and consequently dinospore-host encounter is infrequent.

Like viral attack, parasitism by eukaryotes should be included in biogeochemical models of carbon flux as a source of particulate organic matter (POM). In cultures, only 5 to 20% of the dinospores successfully infect a novel host (Coats and Park, 2002). Considering their short time life, most of this biomass will be recycled through the microbial food web by grazers or release as particulate organic matter and then exposed to bacterial attack. Indeed, dinospores definitely represent a trophic link between hardly consumed dinoflagellates (like most of species observed to be infected in this study) and microzooplankton in oligotrophic waters. Similar trophic link has been also point out in freshwater where zoospores of the parasitic fungus *Zygorhizidium planktonicum* infect the inedible diatom *Asterionella formosa*, supporting the growth of the cladocera *Daphnia* (Bruning, 1991; Kagami et al., 2007). Yet, the relevance of this putative dinoflagellate-dinospore-microzooplankton pathway should be evaluated in future works on the basis of specific grazing experiments and observations in order to estimate better the amount of carbon transferred between these trophic levels.

5 Conclusions

The detection of Amoebophryidae dinospores at all stations sampled and the detection of infected dinoflagellate hosts demonstrate both the presence and activity of these eukaryotic parasites in the oligotrophic to ultraoligotrophic waters of the Mediterranean Sea. Our results stress the requirement to include parasitism processes in the modelling of microbial food webs and biochemical cycles structure and dynamic. The fate of dinospores and their survival time in waters are important parameters to assess in oligotrophic waters in order to understand the capacities of these parasites to propagate infections at low hosts concentrations.

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