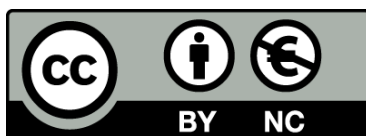




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Delivering insights into diversity and ecology of marine chytrids

Alan Denís Fernández Valero



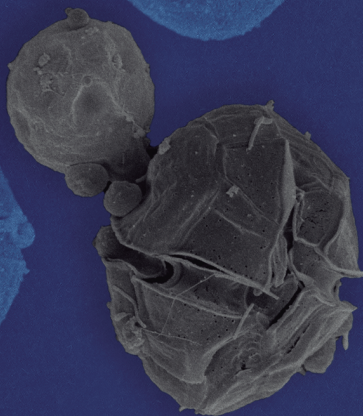
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Delivering insights into diversity and ecology of **marine chytrids**

Alan Denis Fernández Valero



2024



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The present thesis was conducted at the Institut de Ciències del Mar (ICM-CSIC; Barcelona, Spain). Alan Denis Fernández Valero was supported by a predoctoral fellowship from the Spanish government (PRE2018-084893). The research was funded by projects COPAS (CTM2017-86121-R) and SMART (PID2020-112978GB-I00) from the Spanish government. The Severo Ochoa Centre of Excellence accreditation (CEX2019-000928-S) from AEI 10.13039/501100011033 is acknowledged.

Recommended citation:

Fernández-Valero, A. D. (2024). Delivering insights into diversity and ecology of marine chytrids. Ph.D. Thesis, University of Barcelona.

Cover artwork by Alan Fernández-Valero.



Delivering insights into diversity and ecology of marine chytrids

Alan Denis Fernández Valero

Thesis presented to obtain the Doctoral Degree by the University of Barcelona
Ph.D. Program in Marine Sciences.

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*"La ciència és entendre el món i explicar-ho.
Si no ho expliques, no és ciència."*

Jordi Camp

*Para mi padre, mi madre
y mi familia.*

Agradecimientos

Quiero expresar mi profundo agradecimiento a quienes han sido fundamentales en este camino. A mi madre y a mi padre, cuyo amor y apoyo incondicional han sido, y siguen siendo, mi roca en cada una de mis decisiones y proyectos. Agradezco a toda mi familia por su constante aliento en estos años. A mi prima Giuliana, a mi tía Lucy, a mi tía Mary y a mi abuela Alida por embarcar juntos y mantenernos cerca. A mi abuela Lourdes y mis tíos Pedro, Ito, Richard por recibirme con brazos abiertos. A mis tíos Emilio y Carlos, a mi tía Teresa y a mi prima Carla por darme la bienvenida y estar atentos de mis pasos. Es de gran valor sentirse acompañado.

Quiero dar un especial agradecimiento a quienes estuvieron conmigo en primera línea durante estos años, a mis compañeras de trabajo, Jordina, Nagore, Laura y Natali, cuya colaboración no solo me ha ayudado en el desarrollo de esta tesis, sino que ha enriquecido mi experiencia en todo el proceso. Gracias por estar conmigo durante todos estos años.

También quiero agradecer a mis amigos Jon, Emi, Teresa, Sergio y Josep quienes han sido, y son, bocanadas de aire que me empujan a seguir. Y en especial a Isabel y a Jhonny quienes han sido mi consciencia y apoyo desde el colegio hasta hoy en día, muchas gracias por tantos años conmigo.

Quiero agradecer con especial cariño a todos los médicos y personal de OMIQ, así como a Rafa y Emilia. De corazón, muchas gracias por ayudarme a seguir viendo y viviendo.

Finalmente doy gracias a mis dedicados tutores, Esther Garcés y Albert Reñé, quienes apostaron en darme la oportunidad de hacer este trabajo y guiarme durante todo el desarrollo. Gracias a su compromiso y dedicación a mi persona, hoy soy capaz de entregar este manuscrito.

Sin ustedes, este logro no hubiera sido posible.

Resumen de la tesis

El conocimiento actual sobre los hongos marinos es limitado, marcado por la falta de conocimiento de su diversidad, patrones espacio-temporales y funciones ecológicas en los ecosistemas marinos. El desconocimiento sobre algunos grupos fúngicos específicos, como los quítridos presentes en proliferaciones fitoplanctónicas, y la falta de investigación en algunas regiones marinas, como el Mar Mediterráneo, enfatizan la necesidad de esta tesis.

En consecuencia, el objetivo principal de la tesis doctoral es investigar la composición de la comunidad fúngica en el ecosistema costero mediterráneo, y concretamente el grupo de hongos basales Chytridiomycota (quítridos). El estudio establece objetivos específicos: i) evaluar la distribución espacial de la diversidad de Chytridiomycota en diversas áreas costeras durante las proliferaciones de dinoflagelados; ii) evaluar la dinámica temporal de la comunidad fúngica en comunidades epífitas durante proliferaciones de dinoflagelados bentónicos e investigar su interacción con los huéspedes; y iii) identificar y describir nuevas especies marinas de Chytridiomycota.

La comunidad de quítridos en ambientes costeros es particularmente dinámica y diversa, con una marcada especificidad hacia sus huéspedes preferentes. Estas comunidades son más diversas y abundantes en los sedimentos y en superficies que en la columna de agua. La abundancia relativa de los quítridos aumenta en la columna de agua durante la proliferación de dinoflagelados, sugiriendo su comportamiento parasítico oportunista en situaciones de alta abundancia del huésped. En el laboratorio, se han cultivado e identificado cepas de cuatro especies de quítridos, incluyendo las especies más abundantes detectadas por secuenciación de metabarcodificación, mediante técnicas de co-cultivo con dinoflagelados. Tres cepas corresponden a especies aún no descritas y los cultivos han permitido la caracterización morfológica y molecular de dos de ellas, *Dinomyces gilberthii* y *Paradinomyces evelyniae*, las cuales han sido formalmente descritas en esta tesis. En términos de distribución geográfica de las especies, *Dinomyces gilberthii*, está ampliamente distribuido en hábitats costeros mediterráneos, particularmente prevalente durante las proliferaciones estivales de dinoflagelados. En cambio, especies como *Paradinomyces evelyniae* muestran una distribución más limitada en estos hábitats. La investigación sobre la dinámica temporal de la comunidad fúngica en nichos poco estudiados, como los biofilms en macroalgas, ha permitido observar una alternancia en la abundancia de los grupos fúngicos, influenciada significativamente influenciada por la dinámica de dinoflagelados epífitos. Especies dominantes de quítridos en estas comunidades, como *Dinomyces arenysensis*, exhiben adaptabilidad en sus estrategias de supervivencia, incluida versatilidad trófica.

Los resultados obtenidos demuestran que los quítridos desempeñan un papel significativo en la estructuración de las comunidades microbianas planctónicas y bentónicas de los ecosistemas costeros.

Abstract of the thesis

Current knowledge of marine fungi is limited, marked by gaps in the understanding of their diversity, spatio-temporal patterns, and ecological roles within marine ecosystems. The underexplored nature of specific fungal groups, like chytrids occurring during phytoplankton blooms, and the lack of research in most marine regions, such as the Mediterranean Sea, emphasise the need for this thesis.

Consequently, the main objective of this doctoral thesis is to investigate the composition of the fungal community in the Mediterranean coastal ecosystem, with a focus on the basal fungal group Chytridiomycota (chytrids). The study sets for specific objectives: i) to evaluate the spatial distribution of Chytridiomycota diversity in various coastal areas during dinoflagellate proliferations; ii) to evaluate the temporal dynamics of the fungal community in epiphytic communities during benthic dinoflagellate proliferations and to investigate their interaction with hosts; and iii) to identify and describe new parasitic Chytridiomycota species associated with their host proliferations.

The chytrid community in coastal environments is particularly dynamic and diverse, with a marked specificity towards their preferred hosts. These communities are more diverse and abundant in sediments and on macroalgae surfaces than in the water column. The relative abundance of chytrids increases in the water column during dinoflagellate proliferations, suggesting their opportunistic parasitic behaviour. In the laboratory, strains of four chytrid species, including the most abundant species detected by metabarcoding sequencing, have been cultured and identified by co-culture techniques with dinoflagellates. Three strains correspond to as yet undescribed species and the cultures have allowed the morphological and molecular characterisation of two of them, *Dinomyces gilberthii* and *Paradinomyces evelyniae*, which have been newly described in this thesis. In terms of geographic distribution of species, *Dinomyces gilberthii* is widely distributed in coastal Mediterranean habitats particularly during summer dinoflagellate blooms. Conversely, species like *Paradinomyces evelyniae* exhibit a more limited distribution in these habitats. Investigations into the temporal dynamics of the fungal community on poorly explored niches such as biofilms in macroalgae, revealed an alternation in the abundance of fungal groups, significantly influenced by the epiphytic dinoflagellates dynamics. Dominant chytrid species in these communities, such as *Dinomyces arenysensis*, exhibit adaptability in their survival strategies, including trophic versatility.

The findings demonstrate that chytrids play a significant role in the structuring of planktonic and benthic microbial communities in the coastal ecosystems.

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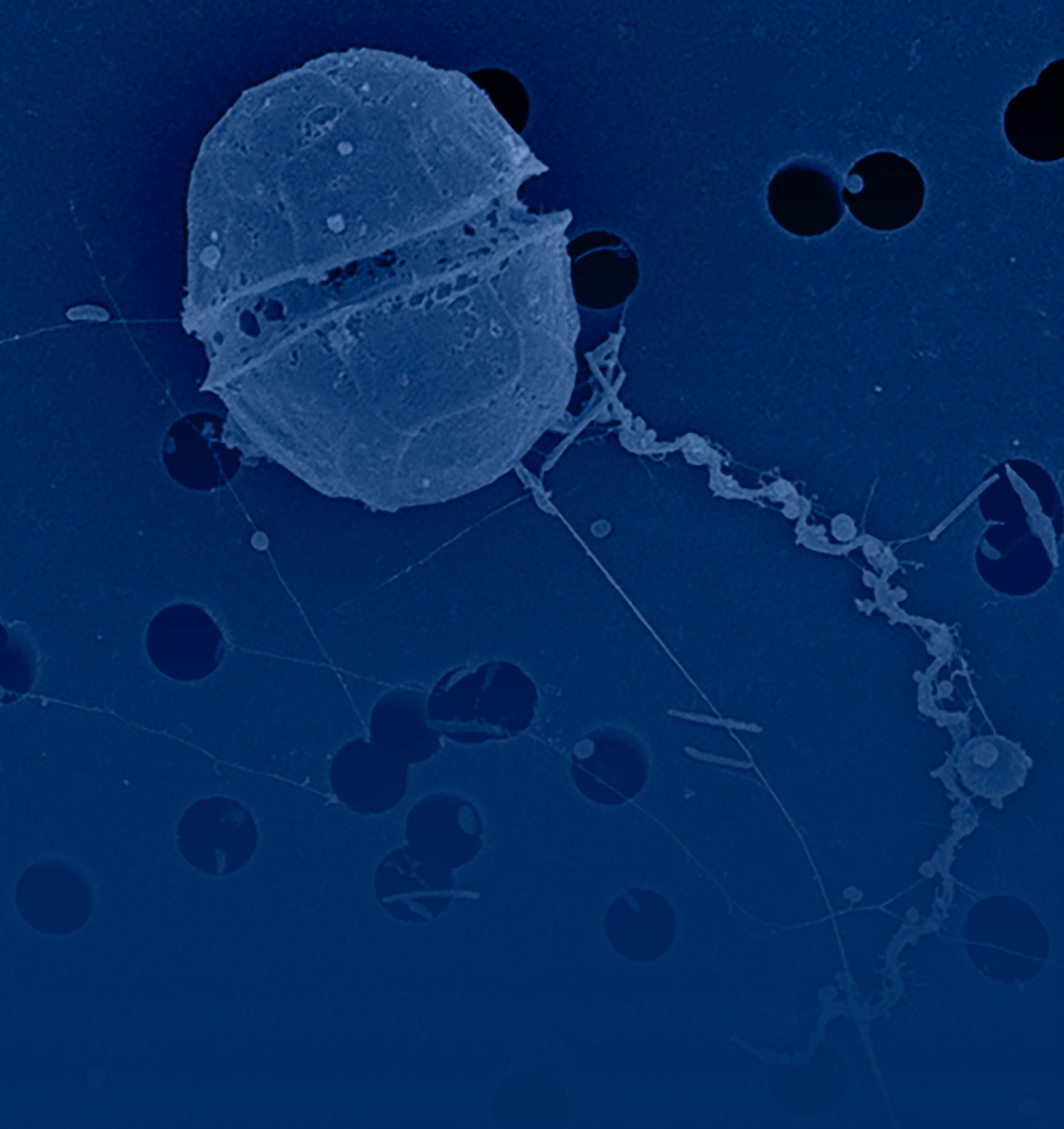
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1. General introduction

1 General introduction

1.1 Diversity and classification of fungi

Around 2-4 million species of fungi are estimated to exist, with only 3% - 8% currently described (Hawksworth and Lücking, 2017). These eukaryotes are characterised by a cell wall composed of glucan and chitin and a uni- or multinucleate cell state. Their life cycles exhibit considerable complexity, and they acquire nutrients through extracellular digestion (Dix, 1995; Jones et al., 2014). In ecosystems, fungi play fundamental ecological roles, including saprophytic, symbiotic mutualistic, parasitic and even hyper-parasitic interactions. Nutrient uptake occurs from a vegetative state, either on or within a host or substrate, through filamentous structures called hyphae. These filaments are the physiological structural basis for most fungi and serve a variety of functions involving habitat exploration, food resource acquisition and both, sexual, asexual reproduction, as well as fungal expansion.

Observation of their morphological and physiological structures, and more recently molecular techniques, have enabled the classification of the fungal kingdom into 18 primary clades (Tedersoo et al., 2018) (Figure 1.1). The Ascomycota and Basidiomycota, referred to as "higher fungi" form the Dikarya group. This group is characterised by cytoplasmic fusion of two haploid monokaryotic hyphae, resulting in dikaryotic hyphae, which is the group's defining feature (Hibbett et al., 2018). Ascomycota, the largest fungal phylum comprises over 64,000 formally described species (Kirk et al., 2008) with 1,000 to 1,500 new taxa identified annually (Wijayawardene et al., 2017). Basidiomycota, the second largest group, accounts for about 30% of described fungal species (Coelho et al., 2017). The remaining clades are named as "basal fungi". This category includes groups at the base of the fungal phylogenetic tree or early divergence, retaining ancestral traits such as the absence of septate hyphae or a free-living stage characterised by motile spores with a posterior flagellum (Blackwell and Spatafora, 2004; Ribichich et al., 2006). These fungi, also known as zoosporic fungi, often require a surface or aquatic environment for dispersal (Sparrow, 1960). The Dikarya group has received a predominant focus over the years and the ecological function of basal fungi is still largely unknown. Consequently, their study has acquired relevance in recent years. They only represent 3% of all described fungal species (James et al., 2020) and include 16 clades such as Blastocladiomycota, Neocallimastigomycota, Monoblepharomycota, Olpidiomyota, Aphelidiomycota, Rozellomycota, and Chytridiomycota (Wijayawardene et al., 2018). In addition, environmental sequencing studies indicate a large number of yet-undescribed taxa in these two latter groups (Manohar and Raghukumar, 2013; Tedersoo et al., 2017; Hurdeal et al., 2021). Recent systematic revisions resulting from the application of molecular techniques have generated debate over the classification of certain clades (e.g. Aphelidiomycota, Rozellomycota), questioning whether they should be considered part of the fungal kingdom or a distinct phylum.

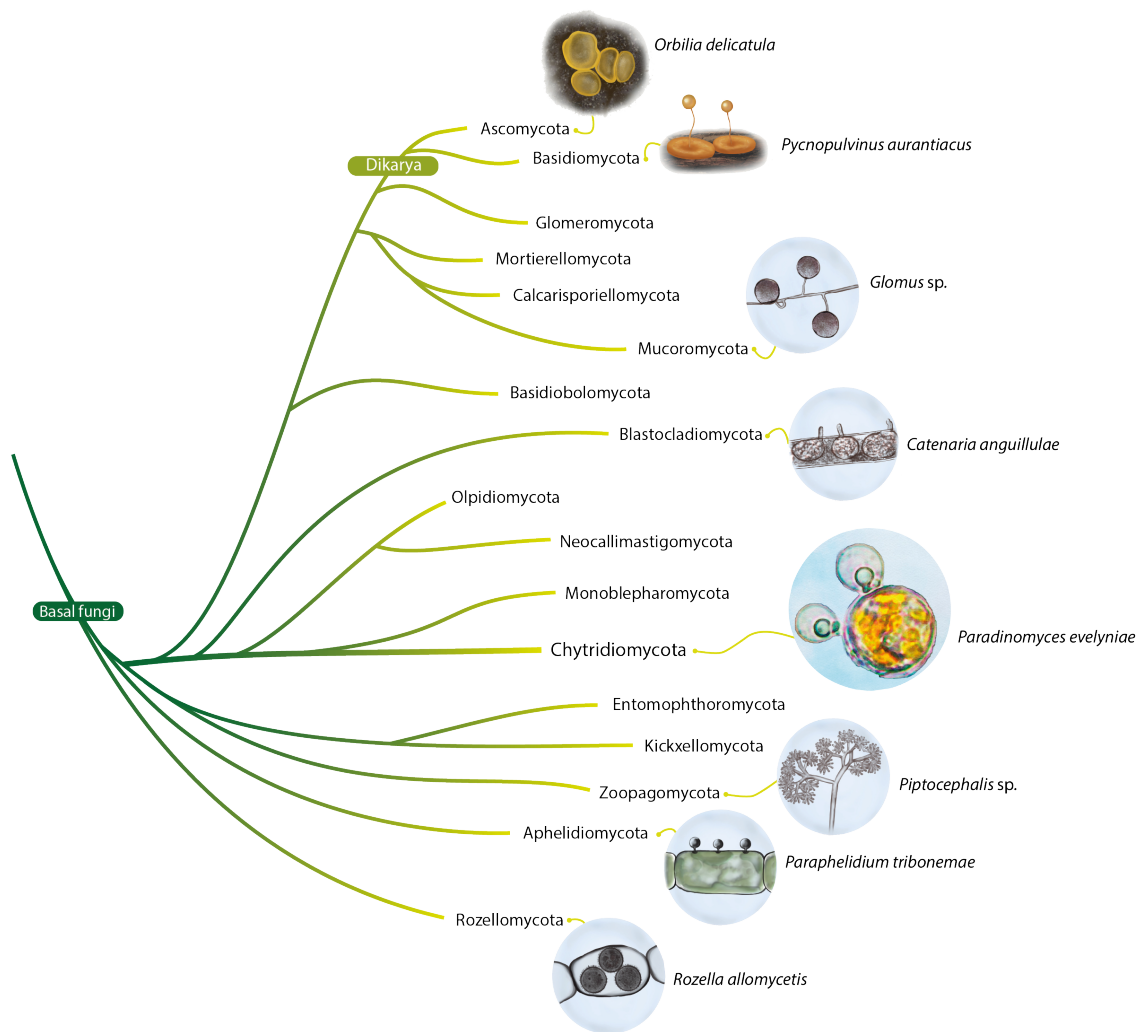


Figure 1.1: **Diagram of the Fungal Tree of Life** based on a phylogenetic tree of 18S rRNA and 28S rRNA gene regions from Hurdeal et al. (2021). Illustrations from Fungal tree of life poster by the László Nagy Laboratory.

1.2 Fungal diversity and ecology in marine habitats

Our understanding of fungal diversity and ecology has expanded from terrestrial ecosystems to marine environments (Comeau et al., 2016). Initially, "marine fungi" were defined as species exclusively thriving in saline environments (Johnson and Sparrow, 1961). However, this definition was reconsidered due to the observation of versatile species (Morrison-Gardiner, 2002). The current definition encompasses species frequently found in marine environments and include those capable of growing in marine environments, forming relationships with other marine organisms or exhibiting metabolic activity in these environments (Pang et al., 2016). Despite criticism of this definition for its lack of phylogenetic basis, there is consensus on the need to understand the genetic basis of these organisms (Hassett et al., 2019), which may lead to future redefinitions.

The first marine fungi were reported 130 years ago (Murray, 1893). Early research focused on identifying these species through isolation and culture from field samples, showing the adaptability

of various fungi in marine, freshwater and terrestrial environments (Johnson and Sparrow, 1961; Kohlmeyer and Kohlmeyer, 1979). These techniques led to the discovery of new taxonomic groups and the description of nearly 500 species, including 209 ascomycetes, 177 basidiomycetes and fewer than 100 species from other minor groups (Kohlmeyer and Kohlmeyer, 1979). Most of the species described in the marine environment were recognized as either saprophytic or parasitic species on macroalgae and other substrates like driftwood debris (Johnson and Sparrow, 1961). At the end of the 20th century, a decrease in newly identified species had been catalogued. It was assumed that the total number of species would remain relatively stable, even when including less accessible habitats such as seafloors (Kohlmeyer and Kohlmeyer, 1979; Jones, 2011). However, the introduction of molecular techniques dramatically shifted this perspective. These methods have revolutionised our understanding of marine fungal diversity, as well as that of many other previously underestimated organisms in aquatic ecosystems (de Vargas et al., 2015; Lima-Mendez et al., 2015). In addition, advancements in molecular tools for studying eukaryotic plankton diversity have led to the recognition of the ubiquity of fungi in various ecosystems, from the Arctic to the Antarctic, in both water columns and in sediments (Jones, 2011; Mohamed and Martiny, 2011; Ogaki et al., 2019; Kiliyas et al., 2020).

To date, 1,947 marine fungal species have been described, with about 600 of these discovered in the past three years (Jones et al., 2015, 2019). The predominant clades in marine environments are Ascomycota, Basidiomycota and Chytridiomycota (Tisthammer et al., 2016; Hassett et al., 2019; Debeljak and Baltar, 2023). Their dominance varies depending on community succession (Taylor and Cunliffe, 2016; Banos et al., 2020) and the specific habitat (Manohar and Raghukumar, 2013). Specifically, Dikarya species (Ascomycota and Basidiomycota), are particularly predominant in deep waters characterised by extreme conditions such as absence of light, low temperatures, high pressures and reduced diversity (Richards et al., 2012). These fungi inhabit diverse niches, ranging from sediments and macrobenthos in the Yap Trench of the Pacific Ocean (Nagahama et al., 2008), to offshore sediments in the Japan Trench, and hydrothermal fields in the mid-Atlantic (López-García et al., 2007). Saprophytic and anaerobic fungi have also been observed in hydrothermal vents (Moreira and López-García, 2003; López-García et al., 2007), although some of them have also been reported in marine surface waters, as well as in terrestrial aquatic environments, suggesting great adaptability (Bass et al., 2007; Lai et al., 2007). However, a vast unexplored fungal diversity with potential new phylogenetic affiliations remains. This fungal diversity has been termed "dark matter fungi" and underscores the need for further research to understand their ecology and impact on the marine environment (Nagano et al., 2010; Richards et al., 2012; Grossart et al., 2016; Picard, 2017).

In addition to deep-sea ecosystems, fungi are found in marine epipelagic zones. Unlike the deep sea, these environments exhibit significant seasonal variations, influenced by the fluctuations of biotic and abiotic factors. These fluctuations influence the succession of species and communities in these habitats, mainly composed of filamentous Ascomycetes and Basidiomycetes (Banos et al., 2020). These organisms have the ability to digest polysaccharides present in algae (Cunliffe et al., 2017) and act as organic matter decomposers. Therefore, this fraction of fungi is mostly considered to fulfil the role of saprophytic organisms. Another important fungal group is represented by Chytridiomycota (chytrids). Although they have received special attention in terrestrial environments (Hanrahan-Tan et al., 2023), being responsible for the decline of several amphibian species (Tessa et al., 2023), chytrids are also present in aquatic environments, including streams (Duarte et al., 2015), lakes (Monchy et al., 2011) and marine environments, as recently determined (Frenken et al., 2017; Amend et al., 2019).

The biogeography of chytrids in the marine environment is poorly known. Hassett et al., (2019) examined the diversity of both cultured and observed marine planktonic fungi globally and

did not observe any latitudinal gradients in marine fungal richness and diversity but found significant differences among oceanographic regions. The regions with the most reported chytrids diversity are those where atypical salinity regimes (varies by over 5 units from the global average), including the Red Sea, the Baltic Sea and Antarctic Sea ice in comparison to open ocean, that are dominated by the Ascomycota, Basidiomycota. Furthermore, the highest fungal richness in planktonic communities is observed in both the Baltic and the Mediterranean Seas (Hassett et al., 2019). These findings corroborate existing knowledge about the impact of salinity on the organisation of marine fungal communities, as documented in studies like Mohamed and Martiny (2011), and particularly in the Baltic Sea context, as noted by Rojas-Jimenez et al. (2019). The observed high fungal richness in the Baltic and Mediterranean Seas provides marginal support for the hypothesis that fungi are evolving from terrestrial to marine ecosystems. This concept, proposed by Spatafora et al. (1998) and Hibbett and Binder (2001), implies that coastal areas and enclosed seas, heavily influenced by terrestrial factors, may be key hotspots for marine fungal diversity at different stages of this evolutionary shift.

Current research is also aiding understanding in the temporal variations in fungal diversity across various marine environments, with a particular focus on coastal waters. Ongoing temporal studies currently focus on discerning the primary drivers of seasonality in marine ecosystems, specifically determining whether biotic or abiotic factors play a more significant role. Several temporal dynamics studies reveal seasonal and monthly fluctuations in marine fungal communities. For instance, research conducted in China's Bohai and Yellow Seas showed alternating dominance between Basidiomycota and Chytridiomycota in winter and summer, respectively, while groups as Ascomycota and Rozellomycota (referred to in the publication as Cryptomycota) remained stable throughout the year (Wang et al., 2017). Other studies have suggested a weak correlation between seasonality in fungal groups and abiotic factors, suggesting a stronger influence of biotic factors (Christmas et al., 2023). Specifically, Chytridiomycota consistently appear to be recurrent in coastal environments (Richards et al., 2015; Jeffries et al., 2016; Duan et al., 2018) typically peaking during the spring phytoplankton bloom in marine environments at higher latitudes, as observed by Cleary et al. (2017). Despite their generally low relative abundance in the eukaryotic community, often under 1% (Kilias et al., 2020; Reñé et al., 2023), notable interannual increases in abundances have been reported, concurrent with an increase in the abundance of phytoplankton species. While these peaks in relative abundance of Chytridiomycota sometimes respond to diatom blooms (Gutiérrez et al., 2016; Taylor and Cunliffe, 2016; Kilias et al., 2020), they are not consistently associated with them (Käse et al., 2021; Reñé et al., 2023). This suggests that the temporal patterns of chytrids may be linked to other less-studied phytoplanktonic groups (Gleason et al., 2014), such as a second major group in the marine system, the dinoflagellates.

1.3 Fungal relevance in food webs and biogeochemical cycles: trophic interactions of marine fungi

Early research on marine fungi provided insights on their diversity, as well as their spatial and temporal organisation in marine habitats. The increasing significance of fungi in aquatic ecosystems has encouraged research to understand their impact on food webs and biogeochemical cycles. Marine fungi employ various ecological strategies, ranging from saprotrophism, i.e., the ability to exploit senescent hosts or deceased organic organisms, to parasites that exploit a host to obtain nutrients and other benefits including transportation or protection (Barnard, 1990). While often detrimental to hosts, parasitism can also promote diversity, regulate population dynamics, and trigger immune responses during infection (Thomas et al., 2005). Therefore, parasites

profoundly influence the biological communities organisation. There are certain parasitic groups, termed parasitoids, that depend on a host to complete their life cycle, consuming and eventually killing the host to grow and reproduce (Eggleton and Gaston, 1990). Frequently, these parasitoids are zoosporic, characterised by the production of motile spores serving as their infectious agents.

The ability of fungi to synthesise a wide variety of enzymes allows them to utilise diverse compounds as a source of energy (Jones et al., 2014), which facilitates their survival as saprophytes in deep-sea and coastal benthic ecosystems, and acting as decomposers in most marine ecosystems (Kutty and Philip, 2008). This interaction is particularly prevalent in Ascomycota and Basidiomycota species (Jones and Choeyklin, 2007; Raghukumar, 2017). By contrast, the lifestyle of chytrids varies on a continuum from obligate parasites to obligate saprophytes. It remains uncertain whether the degree of parasitism or saprophytism is inherent to each taxon, or varies depending on ecosystem conditions (Frenken et al., 2017). Species of chytrids have been found exploiting both phytoplanktonic resources and organic substrates, such as pollen or artificial media, indiscriminately (Alster and Zohary, 2007; Leshem et al., 2016; Van den Wyngaert et al., 2022). Parasitism could be considered as a strategy for accessing higher-quality resources compared to dead organisms or organic substrates in decomposition. However, it may pose risks to their survival considering the multiple immune responses of the hosts (Schmid-Hempel, 2008). Therefore, understanding the continuum between parasitism and saprophytism in these organisms is crucial to comprehend their impact on ecosystems.

Chytrids, as zoosporic parasites, are known to establish parasitic interactions with several planktonic species, including marine microalgae such as diatoms (Scholz et al., 2014) and dinoflagellates (Lepelletier et al., 2014), and it is suspected that they are capable of infecting other groups of microalgae as is the case of cyanobacteria in freshwater environments (Sønstebo and Rohrlack, 2011). In the context of biogeochemical cycles, the mycoloop concept (Kagami et al., 2014) suggests that chytrids infect large phytoplankton species, typically inedible, making them accessible as a nutritional resource to other trophic levels, through zoospores released after infection (Figure 1.2). This has been demonstrated in freshwater habitats, where parasitic chytrids during biomass blooms of "non-edible" diatoms (not exploited by planktonic herbivores), contributed significantly to 50-57% of the zooplankton diet (Rasconi et al., 2014). However, research primarily focused on freshwater ecosystems neglecting the dynamics of marine environments, or the biotic and abiotic features that are present in the water column, estuarine zones or benthic substrates. Furthermore, indirect effects of chytrid infections on the development of other communities, such as bacteria and archaea have been demonstrated (Gruninger et al., 2014; Gleason et al., 2017), as well as in regulating the size and the fitness of the host population and influencing its ecophysiology (Gleason et al., 2012, 2015; Karpov et al., 2014).

Other factors modulating the role of parasitic interactions rely on host specificity. Parasites are categorised into specialists and generalists. Specialists show a strong affinity for specific hosts, often influenced by host dynamics or unique host characteristics such as life cycle, abundance and its ecology (Adamson and Caira, 1994). An example is the Angulomycetaceae family of chytrids, highly virulent parasites exclusive to the freshwater cyanobacterium *Planktothrix* (Sønstebo and Rohrlack, 2011). Conversely, generalists can infect various organisms, including a primary or preferred host, typically the most abundant in the community, and other alternative hosts. For example, chytrids observed in Lake Stechlin (Germany) preferentially infect the dominant diatom *Fragilaria* but can also infect other genera like *Ulnaria* (Van den Wyngaert et al., 2022). Infectivity depends on factors like host and parasite population densities, species composition in the same ecological niche and the host-parasite encounter rate (Lootvoet et al., 2013).

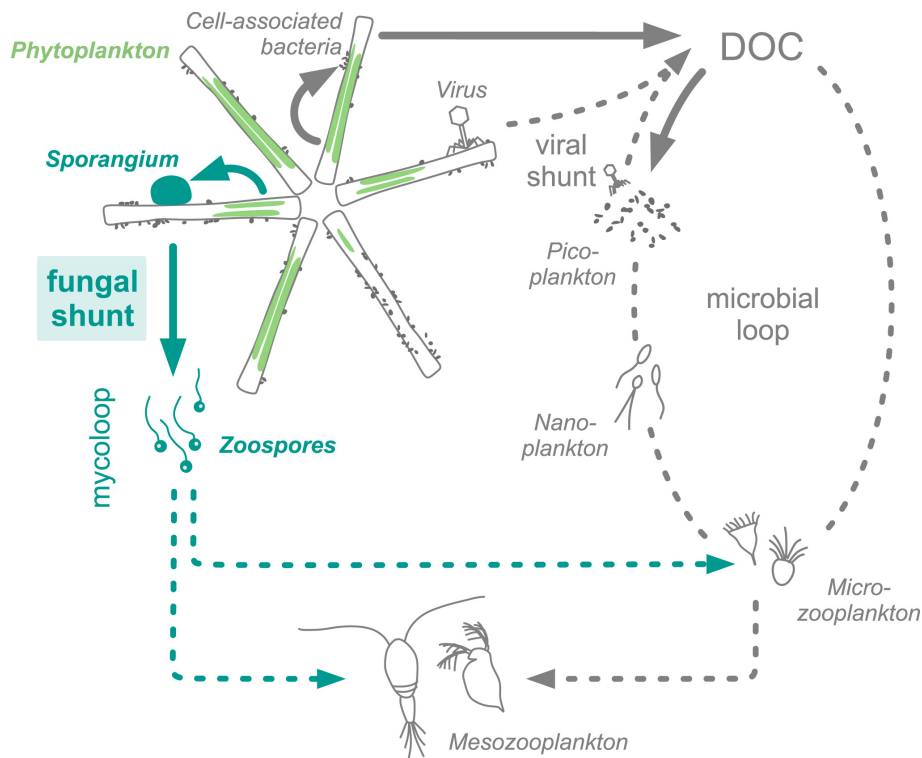


Figure 1.2: **Diagram of the transfer pathways of newly fixed carbon (C).** The fungal shunt (solid, turquoise arrows) diverts phytoplankton-derived C to chytrid sporangia and their free-swimming zoospores, promoting C transfer to higher trophic levels and bypassing the microbial loop (schematically shown as dashed ellipse) and viral shunt. The fungal shunt together with the subsequent consumption of zoospores by micro- and mesozooplankton are described as the mycoloop. DOC: Dissolved organic carbon. Diagram from Klawonn et al. (2021).

In summary, the global prevalence and significance of Chytridiomycota are still largely unknown, given their low recoverability and associated diversity in culture-based studies (Jones et al. 2015), specifically in marine habitats. Therefore, further studies are needed to understand the function of chytrids in the marine environment, enabling more accurate modelling of their impact on marine food web and biogeochemistry.

1.4 Impact of zoosporic parasites during phytoplankton blooms in coastal ecosystems

Coastal areas are characterised by a remarkable variability, the result of continuous interactions between the continent and the ocean. These areas present peculiarities with respect to open or deep-sea habitats, such as an abundant amount of nutrients of continental origin, a greater influence of wave or tidal cycles, or the influence of local climatic conditions (Ray, 1988). This combination of factors creates a wide variety of niches conducive to the development of diverse communities, including epibionts, benthic and planktonic communities. In addition, the interactions that are established in these habitats are fundamental to the development of the life cycle of the species that inhabit them, as well as their contribution in biogeochemical

cycles, primary production (Raghukumar, 2017) and in increasing the occurrence of proliferations (Burkholder et al., 2006).

The coastal ecosystems play a crucial role in the study of food webs due to their capacity to give rise to events of high phytoplankton biomass, known as algal blooms. In fact, the coastal zone is home to the greatest diversity of dinoflagellates, especially toxic species capable of triggering harmful proliferations (Graham and Wilcox, 2000). These proliferations, known as harmful algal blooms (HABs), have a direct impact on the food web and can affect human health and cause significant economic losses, either by affecting aquaculture or by closing beaches.

Parasitism plays a crucial, yet not fully understood, role in the study of phytoplankton proliferations in coastal ecosystems. It significantly contributes to "top-down" control and regulation of dinoflagellate proliferations (Jephcott et al., 2016). Other zoosporic parasite groups, such as the Syndiniales and Perkinsozoa, have demonstrated the capacity to regulate and reduce the dinoflagellate population during proliferation events (Chambouvet et al., 2008; Velo-Suárez et al., 2013). This regulatory effect is supported by mathematical models, experiments (Llaveria et al., 2010) and field observations (Salomon et al., 2009; Velo-Suárez et al., 2013). In particular, Perkinsozoa of the genus *Parvilucifera* have demonstrated similar contributions to the role of modulating proliferations compared to other biological factors, such as grazing or encystment (Alacid et al., 2017). In the case of Syndiniales, a study conducted in the Sargasso Sea revealed that these parasites accounted for 48% to 78% of the food web connections, based on covariance estimates and statistical inference. Furthermore, it suggests that these organisms attenuate carbon flux through remineralization, highlighting the importance of Syndiniales and, in general, parasites in biogeochemical cycles (Anderson et al., 2023). Concerning zoosporic parasitic fungi (Chytridiomycota), only three species known to infect marine dinoflagellates have been identified: *Dinomyces arenysensis* (Lepelletier et al., 2014) in the Mediterranean Sea, and *Paradinomyces triforaminorum* (Reñé et al., 2022) and *Ericiomyces syringoforeus* (Karpov et al., 2021) in the Baltic Sea. Moreover, the extent of the influence of chytrids on marine dinoflagellate proliferations remains largely unexplored. However, it is hypothesised that their impact could be similar to that observed for other zoosporic parasite groups, or to the previously explained influence of zoosporic fungi in freshwater ecosystems.

In summary, our understanding of marine fungi remains limited, encompassing various aspects: from the inadequate knowledge of their diversity to the insufficient research on their spatio-temporal patterns, specificity and prevalence. There exists significant uncertainty regarding their ecological roles and interaction with other biological groups in marine ecosystems. The study of specific groups of fungi, such as zoosporic parasites during phytoplanktonic bloom, remains underexplored. Moreover, the lack of research in regions of significant fungal diversity, particularly the Mediterranean Sea, highlights the need to explore these areas. Therefore, this thesis aims to establish foundational knowledge on the taxonomic characterization and the spatio-temporal composition of marine fungal communities in the northwestern Mediterranean Sea. It places a special emphasis on the parasitic interactions between Chytridiomycota species and dinoflagellates, encompassing both benthic and planktonic forms. This focus is expected to significantly advance the fields of marine mycology and ecology.

1.5 Objectives of the thesis

The main objective of the thesis is to investigate fungal community diversity in Mediterranean coastal ecosystems, focusing on the diversity of Chytridiomycota species during dinoflagellate species proliferations.

To achieve this general objective, the following specific objectives are proposed:

1. To evaluate the spatial distribution of Chytridiomycota diversity in contrasting coastal areas during dinoflagellate proliferations.
2. To evaluate the temporal dynamics of the fungal community in epiphytic communities during a benthic dinoflagellate proliferation and to investigate their interaction with hosts.
3. To identify and describe new parasitic Chytridiomycota species associated with host proliferations.

1.6 Structure of the thesis

The thesis is organised into nine chapters, with chapters two, three and four corresponding to previously published scientific articles, aligned with each of the specific objectives established. A summary of the chapters is briefly presented here:

The **first objective** was focused on assessing the diversity and distribution of Chytridiomycota species in different coastal areas during dinoflagellate proliferations. Different dinoflagellate species proliferations were sampled at several areas along the Catalan coast, providing crucial information on the diversity of Chytridiomycota in such events. We characterised the associated chytrid communities and isolated and identified new undescribed Chytridiomycota species. The results are shown in **Chapter 2** and have been published in the following scientific article:

Fernández-Valero, A. D., Reñé, A., Timoneda, N., Sampedro, N., & Garcés, E. (2022). Dinoflagellate hosts determine the community structure of marine Chytridiomycota: Demonstration of their prominent interactions. *Environmental Microbiology*, 24(12), 5951-5965. doi.org/10.1111/1462-2920.16182.

The **second objective** focused on determining the temporal dynamics of the fungal community during a benthic dinoflagellate bloom. Metabarcoding was used for the analysis of the epiphytic community. The *in situ* prevalence of Chytridiomycota infections during these proliferations was determined. In addition, a new, as yet undescribed species of Chytridiomycota was isolated and identified, and laboratory experiments were conducted to gain insight into the infection dynamics of these microorganisms. The results are shown in **Chapter 3** and have led to the publication of the work:

Fernández-Valero, A. D., Reñé, A., Timoneda, N., Pou-Solà, N., Gordi, J., Sampedro, N., & Garcés, E. (2023). The succession of epiphytic microalgae conditions fungal community composition: how chytrids respond to blooms of dinoflagellates. *ISME communications*, 3 (1), 103. doi.org/10.1038/s43705-023-00304-x

The **third objective** consisted of an exhaustive characterization of two Chytridiomycota species identified in the previous objectives. All phases of the life cycle were documented, phenotypic characteristics were described by light and electron microscopy, and their phylogenetic relationships were determined. The spatial and temporal distribution of the new species was also reported. The results are shown in **Chapter 4** and have led to the publication of the paper:

Fernández-Valero, A. D., Karpov, S. A., Sampedro, N., Gordi, J., Timoneda, N., Garcés, E., & Reñé, A. (2024). Newly identified diversity of Dinomycetaceae (Rhizophydiales, Chytridiomycota), a family of fungal parasites of marine dinoflagellates. *European Journal of Protistology*, 126053. doi.org/10.1016/j.ejop.2024.126053

Subsequently, a summary of the different methodologies used in the different chapters and a synthesis of the results obtained are presented. The thesis continues with a general discussion and the general conclusions of the research are presented. The thesis concludes with the bibliography cited in the Introduction and General Discussion sections.

1.7 Methodological summary

In this thesis, a multifaceted approach was followed to investigate chytrids diversity in the NW Mediterranean Sea. The research incorporated field sampling, laboratory cultivation of the species, microscopic and molecular analyses, as well as statistical inferences and laboratory experiments.

Field sampling to cover temporal and spatial distribution of chytrids diversity

Sampling was conducted during periods of high dinoflagellate biomass along the Catalan coastline, specifically during the spring and summer seasons from 2019 to 2021, and focusing on coastal environments, i.e. harbours and beaches. Three types of samples -seawater, sediment, and macroalgal epiphytes- were collected. An integrated seawater sample was collected and pre-filtered to exclude metazoans and large particles. Sediment samples were collected from shallow depths. Macroalgae samples were procured from rocky terrains at depths between 1 and 1.5 metres and stored for later laboratory processing.

Field samples processing and chytrid cultivation

Optical microscopy was used to determine the phytoplankton composition and abundances of the natural samples. All samples were also processed to detect and isolate fungal parasite species. Seawater from diverse samplings was allocated into well-plates, while sediment samples were placed in double-bottom plates. Cells of different dinoflagellate cultured strains were added to the samples and incubated under controlled temperature and light conditions to promote infections. Macroalgae samples underwent vigorous shaking to dislodge the surface epiphytic biofilm. The resuspended material was then filtered and incubated with potential dinoflagellate hosts. Infected cells detected by microscopy were manually isolated to establish monoclonal parasitic fungal cultures, sustained by periodic replenishment of healthy hosts.

Identification and morphological characterisation of chytrid species: microscopic analyses, molecular and phylogenetic analyses

Multiple microscopy techniques were employed for the characterization of chytrid strains. Transmission and scanning electron microscopy (TEM & SEM) techniques were employed to characterise the ultrastructure of zoospores and the external structure of sporangia, respectively. Epifluorescence microscopy, combined with Nile Red, Alcian Blue, and DAPI staining, was utilised to characterise their lipid and DNA features. Lastly, differential interference contrast (DIC) microscopy was used for visual recording of chytrid life cycle stages. The single-cell PCR technique was used to amplify the 18S, ITS and 28S rDNA regions of the isolated chytrids strains. The sequences obtained by Sanger sequencing were used for phylogenetic analyses to determine the taxonomic position of studied taxa.

Characterisation of the diversity of natural communities through metabarcoding sequencing

Microeukaryotic and fungal communities from seawater, sediment, and epiphytic on macroalgae were determined by metabarcoding. Seawater samples were subjected to serial filtrations,

segregating them into small (0.8-10 μm) and large (10-200 μm) fractions for assessing motile free-living stages (zoospores) and non-motile infections, respectively. For epiphytic resuspension, the 8-100 μm fraction was filtered, whereas sediment was directly analysed. DNA extractions were carried out and PCR using universal eukaryotic primers biased against metazoans were used to amplify the V4 18S rDNA region of the microeukaryotic community. Specific fungal primers amplifying the ITS1, 5.8 and ITS2 regions were used to determine the composition of the fungal community. Metabarcoding sequencing of the amplicons was performed using Illumina PE300.

Bioinformatics and statistical analyses

Eukaryotic and fungal communities were determined from metabarcoding data. Two datasets covering coastal locations from the Catalan coastline were generated, a first one representing a spatial sampling, belonging to the National research project COPAS CTM2017-86121-R, and a second one representing temporal sampling in some locations, belonging to the National research Project SMART PID2020-112978GB-I00. All datasets are publicly available at NCBI SRA repository under Bioproject numbers PRJNA891539, PRJNA89150 (SMART) and PRJNA630546 (COPAS). Environmental sequencing data were processed using DADA2 and the ASVs (Amplicon Sequence Variants) obtained were taxonomically assigned using the PR2 database. Metrics of diversity, i.e. Chao1 and Shannon indices, were assessed to gain a solid understanding of chytrids diversity. The relationship between chytrid and phytoplankton communities was studied through Pearson correlation analysis. Furthermore, Nonmetric Multidimensional Scaling (NMDS) and ADONIS analysis were performed to understand the spatial and temporal patterns within planktonic, benthic, and epiphytic communities.

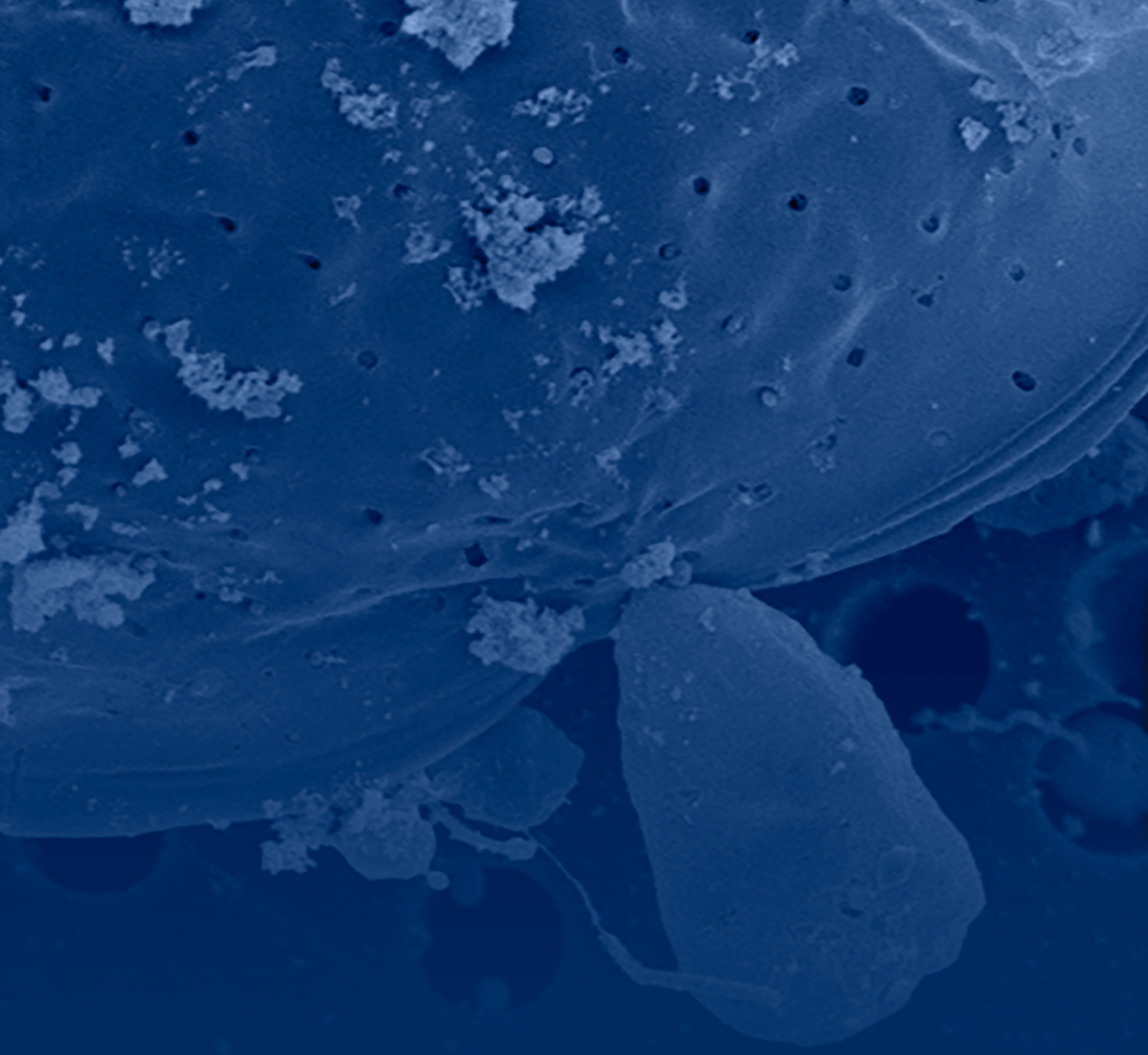
Global context through exploration of metabarcoding datasets

The sequences obtained from the newly discovered chytrid species were queried in the metaPR2 database, and the datasets generated during this research NCBI Sequence Read Archive Bioproject numbers PRJNA630546, PRJNA891539 and PRJNA89150, in order to determine their global positioning and spatial distribution in the Catalan coast, respectively.

Field and laboratory infection dynamics of chytrid strains and nutritional insights

The infection prevalence, referred to the percentage of infected cells with respect to the total host cells, was measured in natural samples to determine the infection dynamics of chytrids. Fixed subsamples collected in filters were stained with DAPI, allowing the identification of nuclei from sporangia, and counts were conducted by epifluorescence microscopy. Additionally, different strains of a chytrid species isolated from various locations were used to examine infection dynamics in the laboratory. Chytrid zoospores were added to healthy host cells and cultures were sampled along fixed time periods to determine chytrid prevalences and host abundances. This approach allowed for a detailed investigation into the infectivity and virulence of chytrid infections. Research was also conducted to explore chytrid growth using alternative nutrient sources (e.g pollen) aiming to elucidate the nutritional factors that influence chytrid development.

Together, all these methods offer a robust and multi-layered framework for studying chytrids diversity, biogeography and interactions with their hosts.

A microscopic image showing various marine microorganisms, including Chytridiomycota and dinoflagellates, in a blue-tinted overlay. The organisms are scattered across the frame, with some showing distinct cellular structures and others appearing as smaller, more diffuse forms.

2. Spatial variation of chytrids during blooming dinoflagellates

Fernández-Valero, A. D., Reñé, A., Timoneda, N., Sampedro, N., & Garcés, E. (2022). Dinoflagellate hosts determine the community structure of marine Chytridiomycota: Demonstration of their prominent interactions. *Environmental Microbiology*, 24(12), 5951-5965. doi.org/10.1111/1462-2920.16182.

2 Spatial variation of chytrids during blooming dinoflagellates

2.1 Abstract

The interactions of parasitic fungi with their phytoplankton hosts in the marine environment is mostly unknown. In this study, we evaluated the diversity of Chytridiomycota in phytoplankton communities dominated by dinoflagellates at several coastal locations in the NW Mediterranean Sea and demonstrated the most prominent interactions of these parasites with their hosts. The protist community in seawater differed from that in sediment, with the latter characterized by a greater heterogeneity of putative hosts, such as dinoflagellates and diatoms, as well as a chytrid community more diverse in its composition and with a higher relative abundance. Chytrids accounted for 77 amplicon sequence variants, of which 70 were found exclusively among different blooming host species. The relative abundance of chytrids was highest in samples dominated by the dinoflagellate genera *Ostreopsis* and *Alexandrium*, clearly indicating the presence of specific chytrid communities. The establishment of parasitoid-host co-cultures of chytrids and dinoflagellates allowed the morphological identification and molecular characterization of three species of Chytridiomycota, including *Dinomyces arenysensis*, as one of the most abundant environmental sequences, and the discovery of two other species not yet described.

2.2 Introduction

Members of the basal fungal division Chytridiomycota, informally called chytrids, are found in soil, freshwater, brackish, and marine habitats. They exist in a free-living zoosporic stage until they come into contact with a host/substrate resource, with which they physically interact using a rhizoid system (Laundon et al., 2020). Chytrids can be saprophytic, parasitic, or both and have a wide host range (Thompson et al., 2005; Rasconi et al., 2011, 2014; Sime-Ngando, 2012; Dunne et al., 2013; Hyde et al., 2020). Chytrid zoospores, produced as the result of a successful infection, are also a food source for grazing predators (Kagami et al., 2004, 2014). Consequently, chytrids not only act as a food web driver, they also define a particular trophic link (Rasconi et al., 2014, 2020; Agha et al., 2016; Gerphagnon et al., 2019; Sánchez Barranco et al., 2020). They contribute to shaping host populations as well as interspecific competition between their hosts and other species while also fully participating in trophic interactions (Sime-Ngando, 2012; Kagami et al., 2014; Rasconi et al., 2014).

The chytrid-host relationship has been studied mainly in freshwater models, which evaluated the dynamics between chytrids and phytoplankton (Kagami et al., 2014). Chytrid infections have been reported in diatoms (Canter, 1969; Seto and Degawa, 2018), chlorophytes (Karpov et al., 2010; Vélez et al., 2011), cyanobacteria (Agha et al., 2016), and dinoflagellates (Leshem et al., 2016). In marine ecosystems, the few studies conducted on chytrids showed that chytrid biomass correlates positively with phytoplankton (Gutiérrez et al., 2016; Lepère et al., 2016).

Environmental studies based on metabarcoding of the SSU rRNA and rDNA of marine microbial eukaryotes have revealed an unexpected and uncharacterized diversity of chytrid-like fungal sequences (Richards et al., 2012, 2015; Comeau et al., 2016), detected in ecosystems ranging from the Arctic and Antarctic (López-García et al., 2001; Scholz et al., 2016; Picard, 2017; Kiliyas et al., 2020), to tropical zones (Hassett et al., 2017, 2020) and in habitats ranging from sediments to deep as well as shallow waters (Hassett and Gradinger, 2016; Scholz et al., 2016; Banos et al., 2020; Käse et al., 2021; Priest et al., 2021; Yang et al., 2021; Zäncker et al., 2021). A greater fungal diversity has been detected in sediments than in the water column, and in the epipelagic than in the mesopelagic zone (Xu et al., 2014; Guo et al., 2015; Li et al., 2019).

Studies of the parasitic relationships between marine chytrids and diatoms (Scholz et al., 2014; Worden et al., 2015) have shown that chytrid parasitism affects the dynamics of marine diatom blooms (Gutiérrez et al., 2016). In situ experiments on the susceptibility of diatoms to chytrid infections demonstrated the influence of light stress and host abundance, as well as the preferential infection of different host species (Scholz et al., 2017). However, far less is known about the interactions of chytrids with dinoflagellates in the marine environment and very few species of dinoflagellate-associated marine chytrids have been described. The parasitic chytrid *Dinomyces arenysensis* was isolated from a bloom of *Alexandrium minutum* and in laboratory studies was shown to infect different species of dinoflagellates, most of them belonging to the genus *Alexandrium* (Lepelletier et al., 2014). Other species of marine Chytridiomycota isolated, cultured, and formally described so far include members of *Thalassochytrium* (Nyvall et al., 1999), *Rhizophyidium* (Amon, 1984) and *Chytridium* (Küpper and Müller, 1999). Recently, species belonging to the *Ericiomyces* and *Paradinomyces* were isolated from a brackish environment in the Baltic Sea (Karpov et al., 2021; Reñé unpublished).

Despite the growing number of chytrid species identifications and insights into host-parasite interactions, the impact and prevalence of chytrid infections of marine dinoflagellates and the role of chytrids in the marine food web are largely unknown, mainly due to the scarcity of field studies at the community level, including those examining biological interactions within consortiums. Such studies have also been hindered by the lack of annotated reference sequences of marine chytrids in DNA databases, which has made it difficult to determine the identity, ecological role (parasitic or saprophytic), and host interactions of marine chytrids. Moreover, correlations inferred from environmental DNA data cannot be systematically interpreted as indicators of biotic interactions (Blanchet et al., 2020). Therefore, studies that combine cultivation, host range determinations, single-cell PCR techniques, and genome sequencing, among others (Grossart et al., 2016; Garvetto et al., 2019; Kagami et al., 2021) are needed to fully understand the structure and function of the chytrid community, the parasitic and competitive strategies of chytrids, as well as the ecological or temporal niches of chytrids (Van den Wyngaert et al., 2022).

Therefore, in this study we examined the diversity of Chytridiomycota during dinoflagellate blooms at several coastal locations of the NW Mediterranean Sea and then used a combination of metabarcoding, phylogenetic identification, and laboratory cultivation approaches to characterize the pairwise interactions of Chytridiomycota and their hosts.

2.3 Experimental procedures

Procedures for metabarcoding sequencing and analyses

Nineteen samplings were carried out along the Catalan coast (NW Mediterranean Sea) in 2018 and 2019, including during high-biomass blooms of five dinoflagellate species (*Alexandrium minutum*, *Ostreopsis* sp., *Alexandrium taylori*, *Gymnodinium litoralis*, and *Kryptoperidinium foliaceum*)

and under no-bloom conditions. Sediment and pre-filtered ($>200\ \mu\text{m}$) seawater samples were collected at each coastal location (Table 9.1: Suppl. Table 1). Detailed information about the sampling procedures was provided in a previous study (Reñé et al., 2021). Briefly, two replicates of each seawater sample were immediately filtered by serial filtration through $10\ \mu\text{m}$ and $0.8\ \mu\text{m}$ polycarbonate filters, resulting in two fractions differing in size: small ($0.8\text{--}10\ \mu\text{m}$) and large ($10\text{--}200\ \mu\text{m}$). The filters and sediment samples were stored frozen at -80°C until processed.

Metabarcoding analyses were performed by extracting genomic DNA from the environmental sample filters and from 10 g of each sediment sample using the DNeasy PowerMax soil Dneasy kit (Qiagen) according to the manufacturer’s instructions. After DNA extraction, a first PCR was performed using universal eukaryotic primers designed against metazoan EK-565F (Simon et al., 2015) and 18S-EUK-1134-UnonMet (Bower et al., 2004), resulting in an amplicon of about 560 base pairs. This was followed by metabarcoding sequencing of the V4 region of SSU rDNA on an Illumina PE300, using the primers EK-565F and E1009R (Comeau et al., 2011), to determine the protist community, which yielded an amplicon of 346 base pairs. Raw molecular data were processed using DADA2 (Callahan et al., 2016), and the obtained ASVs were taxonomically assigned using the PR2 version 4.12 database (Guillou et al., 2013). Raw metagenomic reads were submitted to the NCBI Sequence Read Archive under Bioproject number PRJNA630546. Detailed information was provided in a previous study (Reñé et al., 2021).

Differences in richness and diversity according to the size fraction were assessed by testing the significance of the Chao1 and Shannon indexes using Wilcoxon tests. Potential host-parasite interactions were assessed by analysing the correlations between Chytridiomycota and dinoflagellates using a relative abundance matrix constructed independently of both phylogenetic groups. Only data from the large seawater fraction were used for this analysis, in order to consider potentially active chytrid sequences. Correlations were calculated using the `cor` function of the Rcran stats package, applying the Pearson correlation coefficient. Only correlations with a p-value <0.05 were further examined.

Isolation and culture of chytrids during dinoflagellate blooms

Additional coastal locations were sampled from 2019 to 2021, focusing on blooms of the dinoflagellates *Ostreopsis* sp. and *A. minutum*. Samplings were performed when dinoflagellate abundances in the water column were $>10^4$ cells L^{-1} . Details of the sampled locations can be found in Table 2.1. *Ostreopsis* sp. was sampled at four different locations: Cubelles, Llavanneres, Tossa, and La Fosca. Since *Ostreopsis* sp. is an epiphytic dinoflagellate, macroalgal samples were collected at shallow depths in bottles containing 1 L of $0.2\ \mu\text{m}$ filtered seawater. Additionally, 2.5 L of seawater was collected at a depth of 1–1.5 m. *A. minutum* proliferations were sampled in the harbors of Arenys, Vilanova, and L’Estartit. At each location, a tube connected to an electric pump was used to collect 5 L of integrated seawater from the water column (2.5 to 4 m depth) and 5 L from the deeper layer. Surface sediments were obtained using a sediment corer connected to a pole, with ~ 30 g of sediment placed in a 50 mL Falcon tube using a scoop.

In the laboratory, seawater samples from both blooms were pre-filtered through an $80\ \mu\text{m}$ mesh to remove metazoans and large particles. One liter of each seawater sample was concentrated to 10 mL and aliquots of the concentrate were incubated in 6-well plates. For the macroalgal samples from the *Ostreopsis* blooms, the macroalgae were washed in the flask three times with 1 L of seawater to resuspend the epiphytic community. The resuspended samples were filtered through an $80\ \mu\text{m}$ mesh, and 5 mL aliquots of each supernatant were then transferred to 6-well plates for incubation. Lastly, portions of the surface sediment sampled were placed in double-bottomed

tubes separated by an 8 μm mesh. *A. minutum*, as the host, was placed on top to promote vertical swimming of the zoospores through the mesh and thus separate the parasite from the sediment.

All incubations were maintained in chambers at $20\pm 1^\circ\text{C}$ with a 12:12 h (light:dark) photoperiod. Illumination was provided by LED fluorescent tubes with a photon irradiance of $\sim 40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The incubations were periodically checked for the presence of chytrid infections for up to 2 weeks. Infected dinoflagellate cells were manually isolated to establish monoclonal chytrid strains. Chytrids were cultured in 24-well plates under the same conditions as the incubations. Cultures were established by transferring a 1 mL aliquot of mature sporangia every 3-4 days to a well containing 1 mL of an exponentially growing uninfected host stock culture. The stock cultures of *A. minutum* (ICMB292) and *Ostreopsis* sp. (ICMB293) used to feed the respective chytrid strains (Table 2.1) were obtained from the culture collection of the Institut de Ciències del Mar, Barcelona, Spain. A drop taken from each chytrid culture was placed on a glass slide and observed using a Leica DMIRB inverted microscope. Photographs were taken at $40\times$ magnification using a Jenoptik ProgRes C10 Plus camera; ProgRes CapturePro v2.8.8 software was used to capture the images.

DNA extraction from cultures and single-cell PCRs

Molecular information on the parasitoid strains was obtained by isolating individual mature sporangia. Glass micropipettes were used to physically separate the sporangium from the host dinoflagellate cell. The single-cell protocol was implemented following (Reñé and Hoppenrath, 2019) and (Reñé et al., 2021). A first PCR was performed using the primers EK82F and 28S-1611R to amplify the entire ribosomal operon (López-García et al., 2001; Moreira et al., 2007). The PCR product was then used as a template to amplify the partial SSU rDNA gene, using the specific primers CRYPTO2-2F and EUK-1520R (López-García et al., 2003; Lazarus and James, 2015). Four μL of each product was electrophoresed on a 1.2% agarose gel. The gel was stained with SYBR Safe (Invitrogen) to visualize the bands and confirm the correct amplicon length. For samples with poorly visualizable bands, a third semi-nested PCR was performed using the specific primers CRYPTO2-2F and AU4v2 (Lazarus and James, 2015) and the product of the previous PCR as template. Additionally, the product of the first PCR was used to amplify the LSU rDNA region, using the primers D1R and D3B (Scholin and Anderson, 1994; Nunn et al., 1996). The specific SSU and LSU rDNA regions were amplified in 25 μL reactions containing 1 μL of template DNA, 2.5 μL of buffer 10, 1.5 mM MgCl_2 , 0.2 mM of each dNTP, 0.4 μM of direct and reverse primers, and 2 U of Taq DNA polymerase. The PCR program was as follows: initial denaturation at 94°C for 2 min, 35 cycles including denaturation at 94°C for 15 s, polymerization at 55°C for 30 s, and elongation at 72°C for 60 s, followed by a final elongation at 72°C for 60 s (Reñé et al., 2021). Finally, Sanger sequencing with forward and reverse primers was performed by an external service (Genoscreen, France). The obtained molecular sequences were deposited in GenBank under the accession numbers ON081632-ON081642 (SSU rDNA) and ON113503-ON113504 (LSU rDNA *Dinomyces arenysensis*).

Table 2.1: Cultured chytrid strains, sampling date and location, dinoflagellate host, and the molecular sequences obtained. The last column reports the match between strain sequences and ASVs obtained by metabarcoding.

Cultured chytrid	Strain name	Location	Coordinates	Sampling date	Host	18S rDNA gene	ASV match
<i>Dinomyces arenysensis</i>	ICMB1101	Cubelles	41°11'51.8"N; 1°40'23.0"E	27-08-2019	<i>Ostreopsis</i> sp.	ON081632; ON081633	ASV46-ASV66
<i>D. arenysensis</i>	ICMB1102	Llavaneres	41°33'07.5"N; 2°29'31.6"E	22-07-2020	<i>Ostreopsis</i> sp.	ON081634; ON081635	ASV46-ASV66
<i>D. arenysensis</i>	ICMB1103	Tossa	41°42'55.9"N; 2°55'56.4"E	11-08-2020	<i>Ostreopsis</i> sp.	ON081636	ASV46-ASV66
<i>D. arenysensis</i>	ICMB1104	La Fosca	41°51'27"N; 3°8'38"E	20-08-2020	<i>Ostreopsis</i> sp.	ON081637	ASV46-ASV66
<i>D. arenysensis</i>	ICMB1105	Estartit harbor	42°3'5"N; 3°12'9"E	10-03-2021	<i>Alexandrium minutum</i>	ON081638	ASV46-ASV66
Rhizophydiales sp.	ICMB1106	Vilanova harbor	41°12'54.9"N; 1°43'57.9"E	01-04-2021	<i>A. minutum</i>	ON081639; ON081640	ASV84
Rhizophydiales sp.	ICMB1107	Arenys harbor	41°34'45.5"N; 2°33'19.8"E	10-03-2021	<i>A. minutum</i>	ON081641; ON081642	ASV613

Phylogenetic analysis

SSU rDNA sequences from the cultures were queried for sequence similarity using NCBI-BLAST against all ASVs from the metabarcoding dataset (Altschul et al., 1990). A dataset was constructed using the SSU rDNA sequences recovered from GenBank and covering the diversity of Chytridiomycota orders. The dataset was aligned using MAFFT v.7 (Katoh and Standley, 2013) and the -auto option and then trimmed with trimAL v.1.2 (Capella-Gutiérrez et al., 2009), using the -automated option to remove poorly aligned regions, resulting in 85 sequences and 1,685 positions. The best substitution model was tested using jModelTest 2 (Darriba et al., 2012) and a maximum-likelihood phylogenetic tree was inferred using RAxML v.8 (Stamatakis, 2014), based on all parameters estimated by the program and the best tree inferred from 1,000 different starting trees. Once the best ML tree was obtained, the ASV sequences were added to the previous alignment using the -add option from MAFFT and trimmed again to remove poorly aligned positions in the V4 region. A maximum-likelihood phylogenetic tree was inferred again using the previously obtained tree, with reference sequences as a constrained tree. Bootstrap statistical support was evaluated using 1,000 replicates.

2.4 Results

Diversity and relative abundance of the protist community

Metabarcoding analyses evidenced differences in protist community composition between seawater and sediment. While the sediment was characterized by a greater heterogeneity that included dinoflagellates (class Dinophyceae), diatoms, and other groups, such as Perkinsida and Spirotrichea (Figure 2.1), the seawater fraction was dominated by dinoflagellates, with a relative abundance per sample of 21.3-97% versus 1-69.3% in the sediment. The dominant dinoflagellate species identified in seawater coincided with those responsible for blooms and confirmed by microscopy (*Alexandrium minutum*, *Ostreopsis* sp., *Alexandrium taylori*, *Gymnodinium litoralis*, and *Kryptoperidinium foliaceum*). The dinoflagellate species composition in samples obtained during no-bloom conditions was heterogeneous, dominated by *Prorocentrum triestinum*, *Scrippsiella* sp., *Ansanella* sp., and *Levanderina fissa*. A previous study compared the community composition of dinoflagellates based on microscopy and metabarcoding findings (Reñé et al., 2021). It showed that, after dinoflagellates, diatoms (class Bacillariophyta) were the second most abundant group, comprising 0.04-54.2% of the seawater samples and 1.7-56.8% of the sediment samples. Like dinoflagellates, diatoms were present in all studied samples. Other phytoplankton groups, such as Chlorophyta (class Chlorodendrophyceae), were poorly represented, as their relative abundance was generally <7.5% in both seawater and sediment and their distribution was much more localized (Figure 2.1).

Diversity and relative abundance of the chytrid community

Metabarcoding analyses showed that the fungal community was dominated by members of the Chytridiomycota (chytrids), representing 1.04% of the total eukaryotic sequences (Figure 2.1). Chytrid sequences were detected in 72 of the 101 samples from all studied locations and accounted for a median 0.07% of the total relative abundance of the eukaryote community in seawater and a median 0.75% of that in sediment. In the large seawater fraction, the highest relative abundance of chytrids was in the sample from Sitges (14.48%) whereas in the small seawater

fraction, low percentages were registered in all locations. Among the sediment samples, chytrid relative abundance was highest (>10%) in the samples from Cambrils (Figure 2.2).

Alpha-diversity analyses (Chao1 richness and Shannon indices) of the chytrid community showed a higher diversity of Chytridiomycota sequences in sediment samples than in samples collected from the water column ($p < 0.05$ for both indices) (Figure 2.3).

Chytrids accounted for a total of 77 ASVs. Among the 63 ASVs in sediment, 42 were exclusively detected therein. The large seawater fraction contained 25 ASVs and the small seawater fraction 19 ASVs, including 7 unique ASVs in each fraction. The majority of the reads belonging to chytrids corresponded to only three ASVs (Figure 2.2): ASV46, with relative abundances of up to 6.7% (seawater) and 3.0% (sediment); ASV66, with relative abundances of up to 7.0% (seawater) and 2.0% (sediment); and ASV84, with relative abundances of up to 0.3% (seawater) and 8.2% (sediment). The presence of chytrid ASVs varied depending on the locality and therefore the blooming dinoflagellate species in the sample (Figure 2.4). During *A. minutum* blooms, 27 chytrid ASVs were detected, 25 of which were unique to this bloom type. During *Ostreopsis* sp. blooms, 19 chytrid ASVs were obtained, of which 17 were unique. The non-bloom samples contained 17 chytrid ASVs, of which 11 were found only in these samples. During blooms of the dinoflagellates *Alexandrium taylori*, *Gymnodinium litoralis*, and *Kryptoperidinium foliaceum*, fewer than seven chytrid ASVs were obtained from each one. These results showed that most chytrid ASVs were exclusive to a particular bloom type.

A. minutum and *Ostreopsis* sp. blooms had the highest relative abundances of chytrids (Figure 2.4). ASV46 and ASV66 dominated the chytrid communities in all samples collected from *Ostreopsis* sp. blooms and were detected exclusively in those samples. Among the samples corresponding to *A. minutum* blooms, ASV84 had the highest relative abundance and was present in three of the five studied locations. However, this chytrid sequence was not exclusive to *A. minutum* as it was also present in blooms of *G. litoralis* and in the no-bloom samples (Figure 2.4).

Demonstration of chytrid-dinoflagellate interactions

The potential associations between dinoflagellate hosts and the chytrid sequences obtained in this study were evaluated in a correlation analysis. Figure 2.5 shows the 23 chytrid ASVs that correlated significantly with at least one dinoflagellate in the large seawater fraction. Among the dominant chytrid sequences, ASV46 co-occurred with *Amphidinium* sp. (0.91), *Scrippsiella* sp. (0.89), *Coolia* sp. (0.89), *Cryptothecodinium* sp. (0.98), and *Ostreopsis* sp. (0.49); ASV66 with *Ostreopsis* sp. (0.9), *Zooxanthellae nutricula* (0.81) and *Fragilidium* sp. (0.8); and ASV84 with *Alexandrium tamarense* (0.77) and *Polykrikos tanit* (0.79) (Figure 2.5).

Based on analyses of diversity, relative abundance, and host-parasite co-occurrences with respect to the Chytridiomycota community, *Ostreopsis* sp. and *A. minutum* blooms were selected as the best candidates to investigate the chytrid-dinoflagellate interactions at the studied locations. Additional samplings performed during *Ostreopsis* sp. and *A. minutum* blooms allowed the establishment of seven chytrid strains in culture, with four strains isolated from *Ostreopsis* and three from *A. minutum* (Table 2.1).

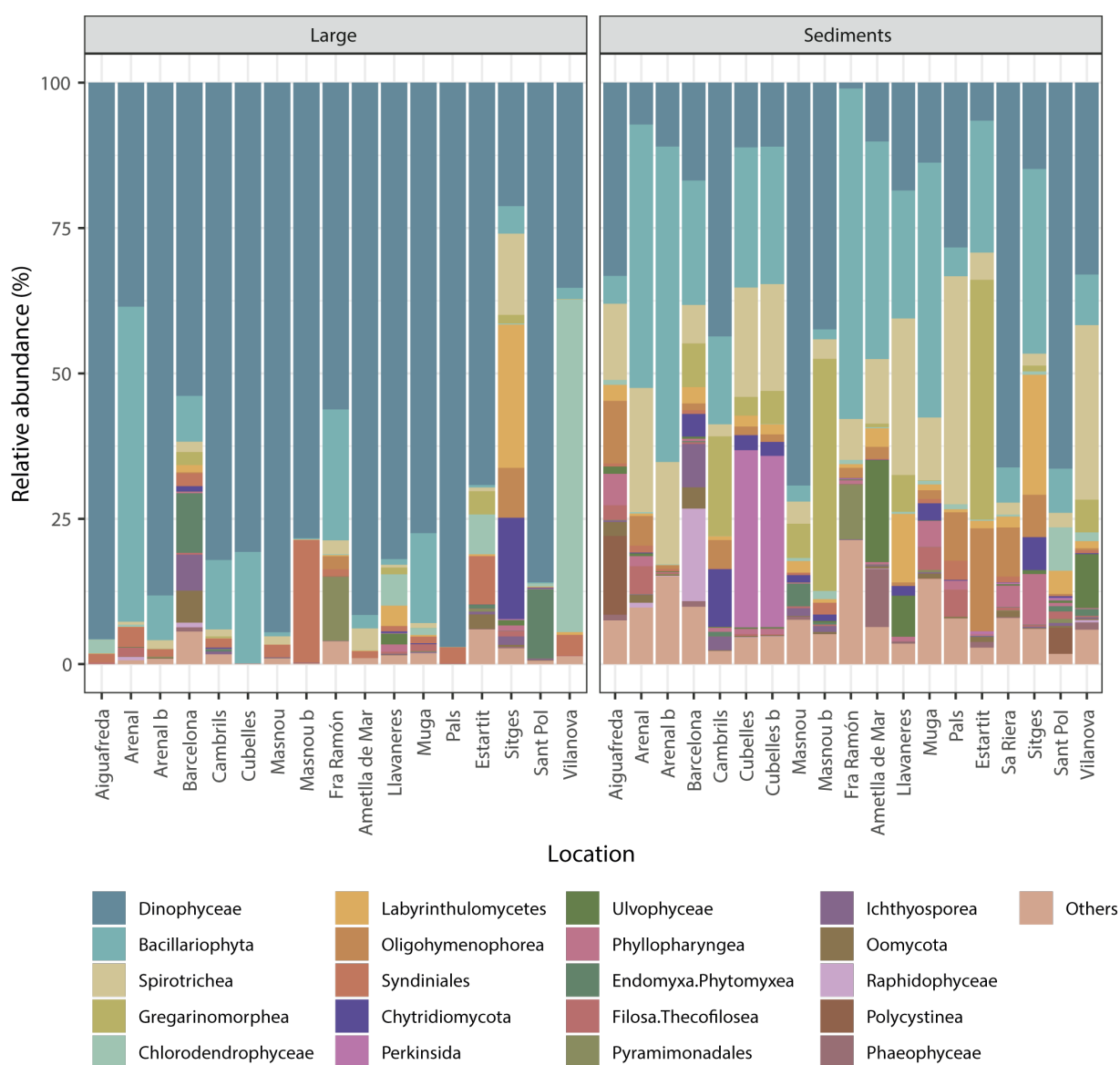


Figure 2.1: **Bar-plot showing the relative abundance of the protist community in each sample as determined by metabarcoding.** On the x-axis, samples from each location are grouped according to the fraction type: seawater >10 μm (left) and sediment (right). Repeated samplings at the same location were standardized and averaged. The bars are divided according to the taxonomic class.

Identification of cultures strains and the phylogenetic classification of chytrid sequences detected by metabarcoding

Light microscopy revealed three distinct morphologies among the isolated strains. The first, exemplified by strain ICMB1106, consisted of mature sporangia with papillae, which allow zoospore discharge (Figure 2.6A). The second, exemplified by strain ICMB1107, consisted of a single lipid globule that was present during the long period of early sporangium development (Figure 2.6B). The third, exemplified by strains ICMB1101, ICMB1102, ICMB1103, ICMB1104, and ICMB1105, also consisted of a mature spherical sporangium, but without papillae (Figure 2.6C).

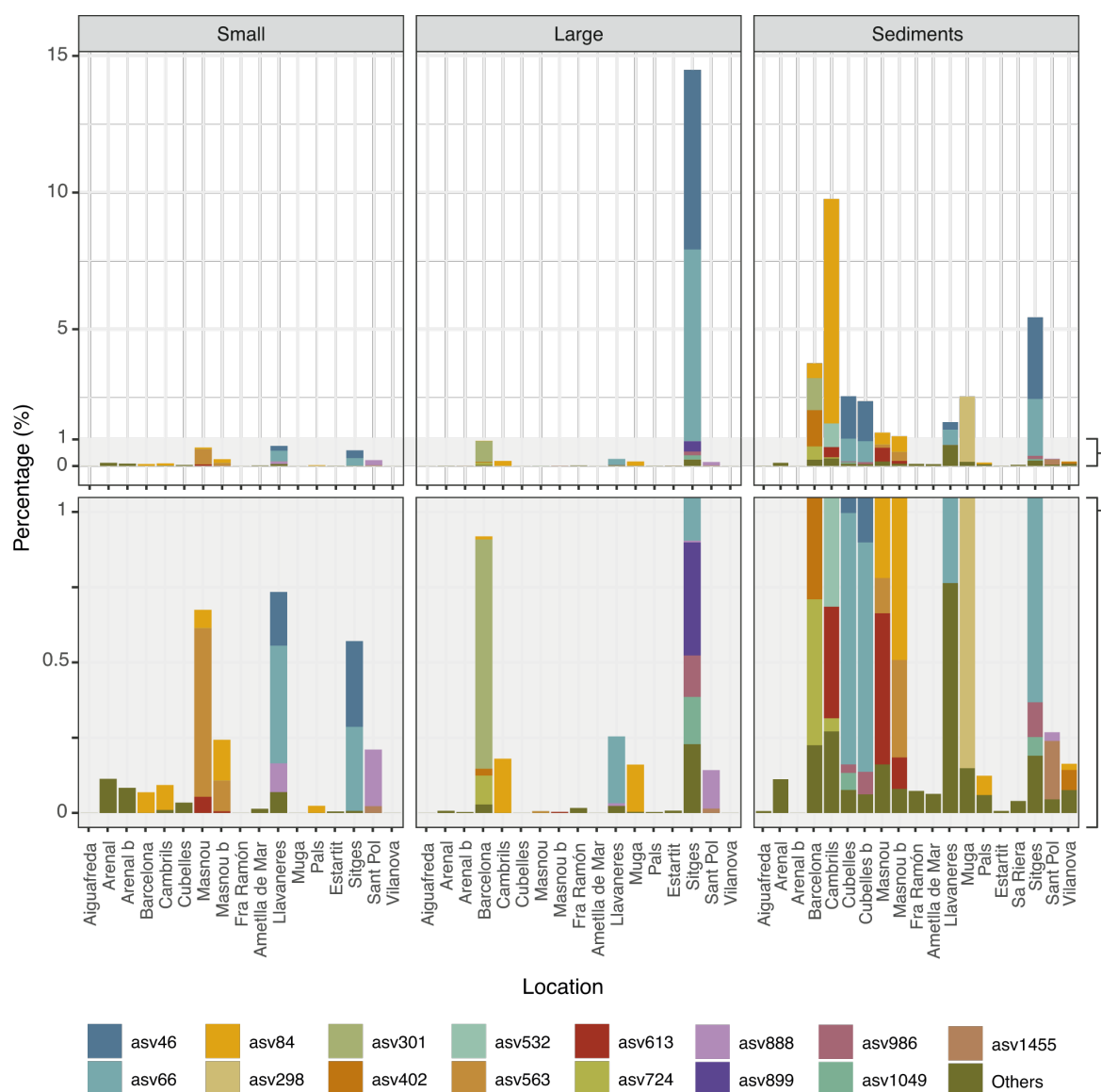


Figure 2.2: **Relative abundance of the Chytridiomycota community obtained from 19 locations and the corresponding fraction.** Seawater small (0.8-10 μm), seawater large ($>10 \mu\text{m}$), and sediments. The relative abundances of the 15 most abundant ASVs are shown. The remaining 62 chytrid ASVs are grouped into the “Others” category. Repeated samplings at the same location were standardized and averaged. The lower graph shows a zoom of the relative abundance between 0 and 1% (grey area).

The SSU rDNA sequences obtained from strains ICMB1101-1105 were identical and had 99.72% and 100% identity with ASV46 and ASV66, respectively. The analysis of the V4 SSU rDNA barcodes of ASV46 and ASV66 showed that the sequences were homologous, differing by only one nucleotide. This finding and the detection of a polymorphic position in sequences obtained from the cultures indicated that the two ASVs represent intraspecific variability of the same species (Table 2.1). Strains ICMB1101-ICMB1105 morphologically resembled *Dinomyces arenysensis*, a species previously detected in the study area. However, only the LSU rDNA and ITS sequences of *Dinomyces arenysensis*, not its SSU rDNA sequences, are publicly available. The LSU rDNA

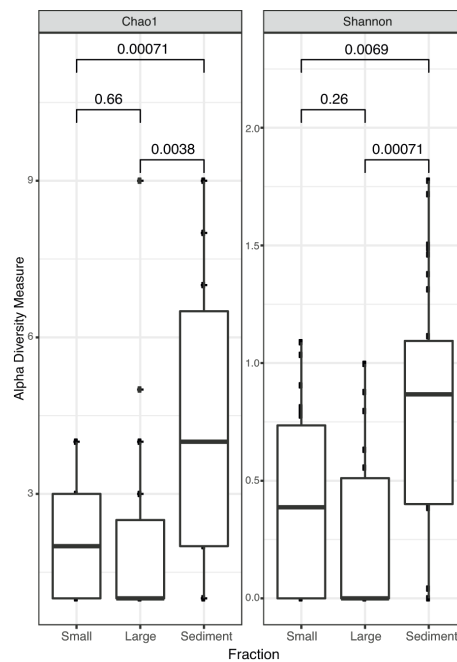


Figure 2.3: **Bar-plot of the Chao1 and Shannon alpha-diversity indices.** Bar-plot of the Chao1 and Shannon alpha-diversity indices, comparing the ASVs of sediment and seawater (small and large fraction) samples.

sequences of strains ICMB1101 and ICMB1102 were therefore obtained for comparison with the sequences available in GenBank. The sequences of both strains showed 100% identity with the LSU rDNA sequence of *Dinomyces arenysensis* (KJ027548). Consequently, these cultured strains, and thus ASV46 and ASV66, were assigned to *Dinomyces arenysensis*.

The morphological features of strains ICMB1106 and ICMB1107 differed from those of strains ICMB1101-ICMB1105 (Figure 2.6). The correspondence analysis showed that the SSU rDNA fragment of strain ICMB1106 matched ASV84 (100% identity), and that of strain ICMB1107 matched ASV613 (100% identity). The latter ASV had a relative abundance of <0.5% and was found in only two of the five *A. minutum* blooms sampled. Both strain ICMB1106 and strain ICMB1107 corresponded to newly detected marine chytrid species that interact parasitically with *A. minutum*. Detailed morphological and molecular information as well as formal descriptions will be provided in a future study.

The phylogenetic positions of the 77 chytrid ASVs in the Chytridiomycota phylum were analyzed (Figure 7). Overall, most of the sequences were included in Rhizophydiales (23 ASVs, 30,866 reads) and Lobulomycetales (25 ASVs, 2,348 reads). The three species isolated in this study clustered within Rhizophydiales. Eight of the ASVs corresponding to Rhizophydiales belonged to *Dinomyces arenysensis*, specifically ribotypes ASV46 (10,867 reads) and ASV66 (8,936 reads), which, as noted above, differed in a single position. Five ASVs could be assigned to Rhizophydiales sp.1 Vilanova (ICMB1106), mainly represented by ASV84 (7,388 reads), while ASV613 (532 reads) corresponded to Rhizophydiales sp.2 Arenys (ICMB1107). The sequences of *D. arenysensis* and Rhizophydiales sp. 1 Vilanova were closely related, but the position of Rhizophydiales sp. 2 Arenys was more distant. Nine additional ASVs could not be assigned to any of those species. Among Lobulomycetales, two ASVs clustered at the base of the clade, with the remaining 23 ASVs clustering together and forming a sister group with sequence EF443138. Representatives of other

orders were also determined, including Zygothryctales (1 ASV, 5 reads) and Rhizophlyctidales (6 ASVs, 38 reads). The phylogenetic position of some ASVs could not be assigned with certainty. A clade of four ASVs (952 reads) formed a sister clade with Mesochytriales and Gromochytriales; three ASVs (72 reads) clustered in the clade *incertae sedis* 1 sensu (Seto et al., 2020), and eight ASVs (1503 reads) formed a sister clade with Neocallimastigomycota. Some ASVs did not show a clear relationship with known orders. Three ASVs, represented by 24 reads, clustered at the base of the Chytridiomycota.

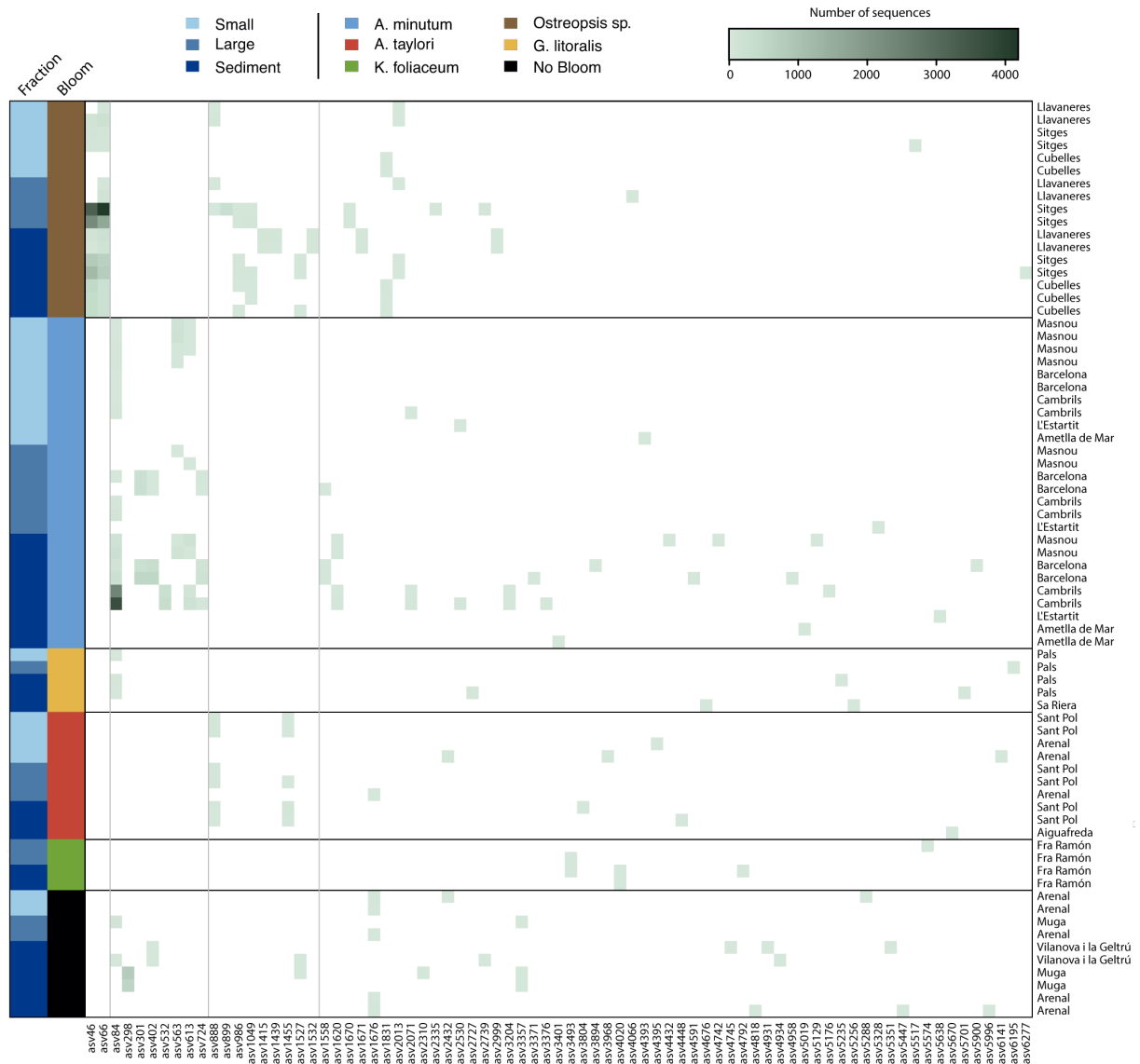


Figure 2.4: **Heatmap of the chytrid ASVs (x-axis) identified in all samples (y axis).** The left bars report the fraction and bloom type. The green color gradient represents the number of sequences in the protist community, ranging from 0 (white) to 4183 (dark green).

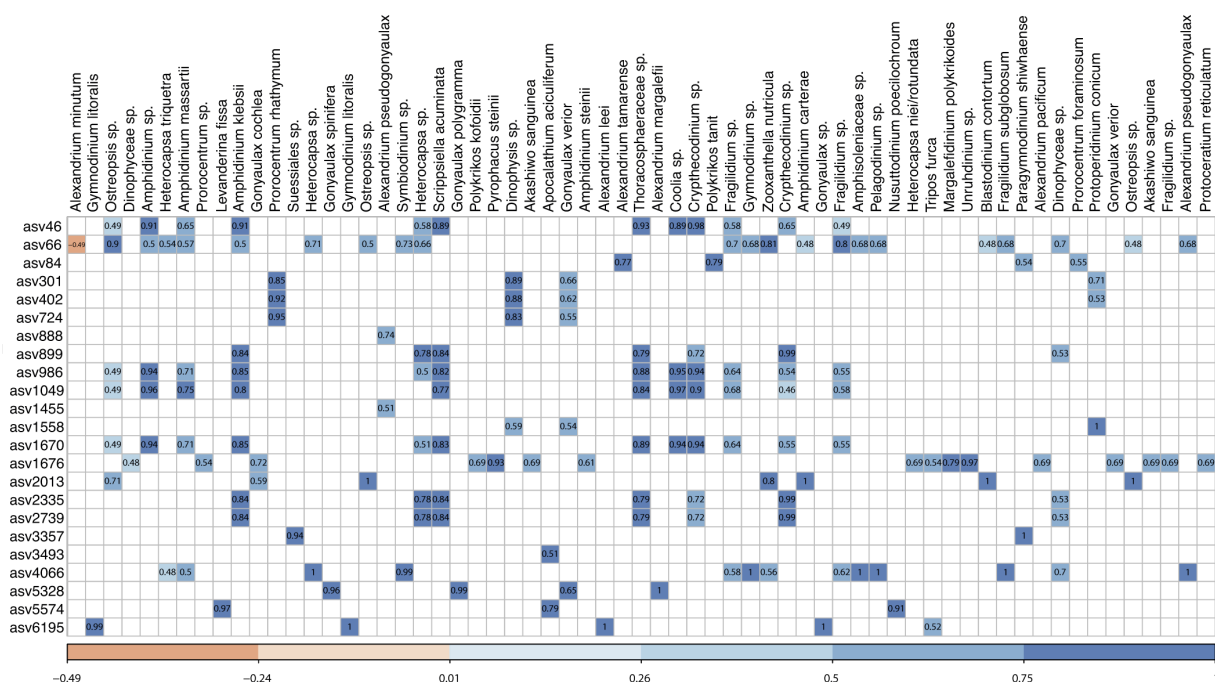


Figure 2.5: **Evidence of potential host-chytrid co-associations based on correlations between dinoflagellate and chytrid ASVs.** The Spearman correlation coefficients for the dinoflagellate and Chytridiomycota sequences, considering samples corresponding to the large seawater fraction and sediment, are shown. A significance threshold of 0.05 was applied. The correlation percentage is shown as a gradient ranging from dark blue (1) to orange (-0.5).

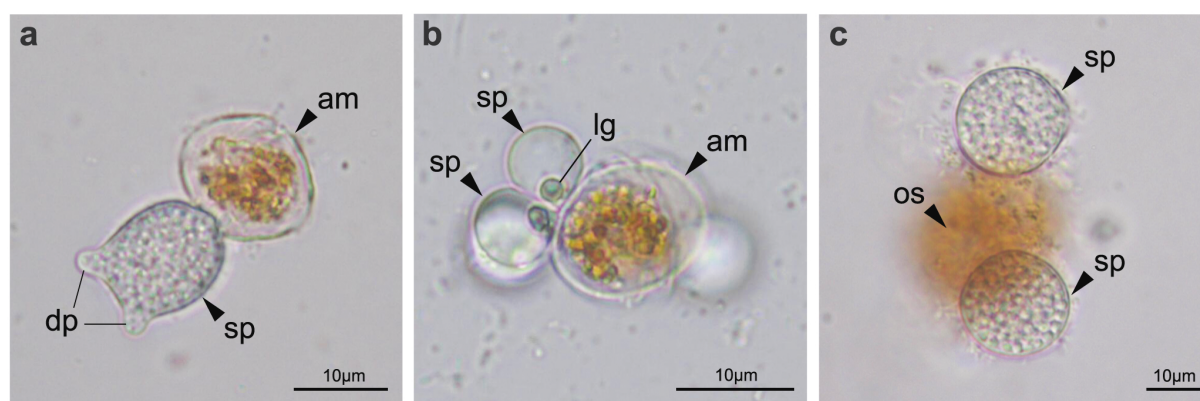


Figure 2.6: **Microscopy images of chytrids on dinoflagellates.** (a) Strain ICMB1106 infecting *Alexandrium minutum*. (b) Strain ICMB1107 infecting *A. minutum*. (c) Strain ICMB1104 (*Dinomyces arenysensis*) infecting *Ostreopsis* sp. Abbreviations: dp, discharge papillae; lg, lipid globule; sp, sporangium; am, *Alexandrium minutum*; os, *Ostreopsis* sp.

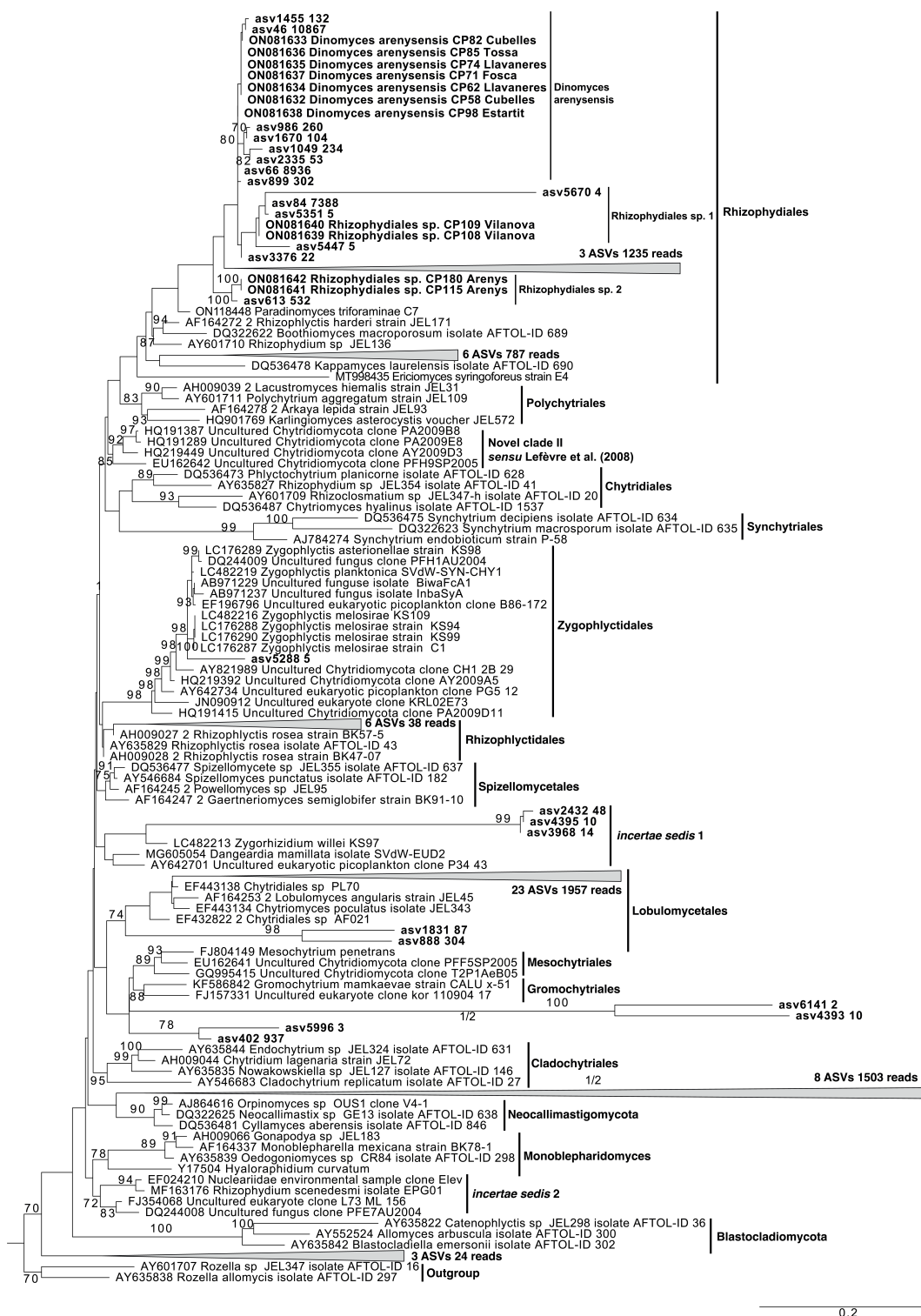


Figure 2.7: Maximum-likelihood phylogenetic tree of SSU rDNA sequences, including representatives of most orders belonging to Chytridiomycota. New sequences obtained from cultured strains and the ASVs from metabarcoding are shown in bold. The numbers in parentheses correspond to the reads obtained for the ASVs. Values in the nodes represent bootstrap (%) statistical support. Only values >70% are shown.

2.5 Discussion

In this study, the structure of Chytridiomycota communities under conditions of dinoflagellate blooms in coastal environments was characterized. Among the sequences obtained from the eukaryotic community, 1.04% belonged to Chytridiomycota. In studies of other marine environments, the relative abundance of this group similarly accounted for 1-2% of the eukaryotic community (Edgcomb et al., 2011; Stern et al., 2015; Hassett et al., 2017, 2020; Picard, 2017; Celussi et al., 2018; Enberg et al., 2018). However, in our samples the relative abundance of Chytridiomycota greatly varied depending on the location, the dinoflagellate bloom, and the sample source (small and large seawater and sediment fractions), ranging from a lack of detections to chytrids abundances that accounted for 14.48% of the total eukaryote sequences. A large variation in relative abundance was also observed in previous studies of coastal waters. For example, in a study of microbial community structure in the Arctic Ocean, fungal environmental sequences accounted for a mean 1.35% of eukaryotic sequences, but they ranged from 0.01% to 59%, with Chytridiomycota and Chytridiomycota-like sequences constituting >50% of the fungal fraction (Comeau et al., 2016). A large variation in chytrid relative abundance was also found in the Plymouth coastal region (UK), where a specific chytrid sequence ranged from non-detectable to comprising 100% of the fungal community (Taylor and Cunliffe, 2016). The large oscillations in relative abundance may be primarily due to the availability and abundance of the chytrid host, as both influence the community composition not only of Chytridiomycota (Ibelings et al., 2004; Gutiérrez et al., 2016) but also that of other marine zoosporic parasite groups (Alacid et al., 2017). Other factors, such as predator grazing on free-living zoospores (Johansson and Coats, 2002) and the genetic diversity of the host and parasite populations (Blanquart et al., 2016), may also lead to oscillations in the relative abundances of chytrids in marine environments.

The results also showed the unequal distribution of chytrids between the water column and sediment in the same location, as in all cases the diversity Chytridiomycota sequences was higher in sediment than in planktonic samples. These results are in agreement with other studies in which Chytridiomycota, as well as other zoosporic parasite groups such as Perkinsea, were consistently detected in the sediment and their community structure was stable over time (Chambouvet et al., 2011; Orsi et al., 2013; Xu et al., 2014; Picard, 2017; Reñé et al., 2021). These observations support the hypothesis that sediments are a reservoir of diversity of zoosporic parasites, which remain in a latent phase until favorable conditions enable their activation (Picard, 2017; Reñé et al., 2021).

The community structure of Chytridiomycota in all samples was represented by 77 different ASVs, but only ASV46, ASV66, and ASV84, were highly abundant; the rest were either rarely detected or of low relative abundance. The dominant ASVs belonged to Rhizophydiales, consistent with its inclusion of several known parasites of dinoflagellates (Lepelletier et al., 2014; Karpov et al., 2021; Reñé et al., 2021). ASVs belonging to other orders, such as Lobulomycetales, were also obtained. Consistent with previous studies, our results suggest that the dominance of certain chytrid species is determined by host abundance (Picard, 2017; Scholz et al., 2017) and by the presence of different niches, which in turn depends on the available host/substrate resource, as demonstrated for freshwater chytrids (Van den Wyngaert et al., 2022). In our study, despite its temporal and geographical bias, the chytrid community associated with proliferations of *A. minutum* highly differed from that associated with proliferations of *Ostreopsis* sp., as >80% of the chytrid taxa were exclusive to each bloom type. This difference suggests that the structure of the chytrid community is driven by specific chytrid-host interactions. This was also observed for the marine fungal community under Arctic ice, in which members of the Lobulomycetes were dominant and parasitized diatoms, with a preference for *Pleurosigma elongatum* (Hassett et

al., 2017). In a study in the Solthörn tidal flat, parasitic interactions between different chytrid strains infecting specific taxa of benthic diatoms were likewise observed (Scholz et al., 2016).

The chytrid-host co-occurrence determined by metabarcoding were further investigated in culture, and specifically with the host species *A. minutum* and *Ostreopsis* sp. From the *A. minutum* bloom samples, a new, as yet undescribed chytrid species (ICMB1106) was isolated and cultured. This species corresponded to the most representative chytrid sequence detected (ASV84) in that bloom. However, the sequence correlated with the dinoflagellates *Polykrikos tanit*, *Paragymnodinium* sp., *Prorocentrum* sp., and *A. tamarense* but not with its putative host *A. minutum*. This may be due to the fact ASV84 was not always present in blooms of *A. minutum*, including those at L'Estartit and L'Ametlla de Mar (Figure 2.4), as other specific biotics factors may not have favoured its presence.

The cultured strain ICMB1107, linked to ASV613, was also isolated from *A. minutum* bloom samples. The low relative abundance of this chytrid, the absence of ASV613 in other bloom samples, and the lack of correlation with, at least, dinoflagellate sequences suggest that ASV613 represents a rare species whose interactions are thus far unknown. Establishing a link between interactions and co-occurrence is often challenging, especially for rare or transient species, independent of the method used (Calatayud et al., 2020). However, the establishment of lab cultures allows the interactions of these species and thus their dynamics in the marine environment to be studied in detail.

Among the ASVs assigned to *Dinomyces arenysensis*, both the metabarcoding sequences and the four strains obtained at different blooms of *Ostreopsis* sp. provide evidence of a prominent interaction between the chytrid parasite and this dinoflagellate. This finding also supports a previous lab host-range study, in which a moderate level of *Ostreopsis* sp. infection was demonstrated, although in that study *D. arenysensis* was isolated from an *A. minutum* bloom and was shown to infect that species at a high level (Lepelletier et al., 2014). The metabarcoding sequences of *D. arenysensis* also correlated with *Heterocapsa* sp., and *Scrippsiella* sp., thus supporting the same lab host-range experiment, which additionally found that the sensitivity of infection of these dinoflagellates was, respectively, moderate and high (Lepelletier et al., 2014). Correlations of *D. arenysensis* with *Amphidinium* sp., *Coolia* sp., and other dinoflagellates based on metabarcoding sequences have also been reported. However, neither culturing efforts nor host-range studies capture the interactions in nature (Blanchet et al., 2020). Thus, whether the correlations between *D. arenysensis* and these species were due simply to the usual appearance of the species in this epiphytic assemblage or to a true parasitic interaction with the dinoflagellate is unclear.

Our study showed that the community structure of Chytridiomycota in coastal environments is highly dependent on the blooming dinoflagellate species. It also demonstrated that insights into chytrid-dinoflagellate interactions can be gained through a combination of environmental sequencing, such as metabarcoding, and detailed laboratory studies of confirmed host (dinoflagellate)-parasite (chytrid) interactions. This combined approach can contribute to providing an accurate picture of the chytrid community and therefore of its host preferences as well as the consequences for the marine eukaryotic community.

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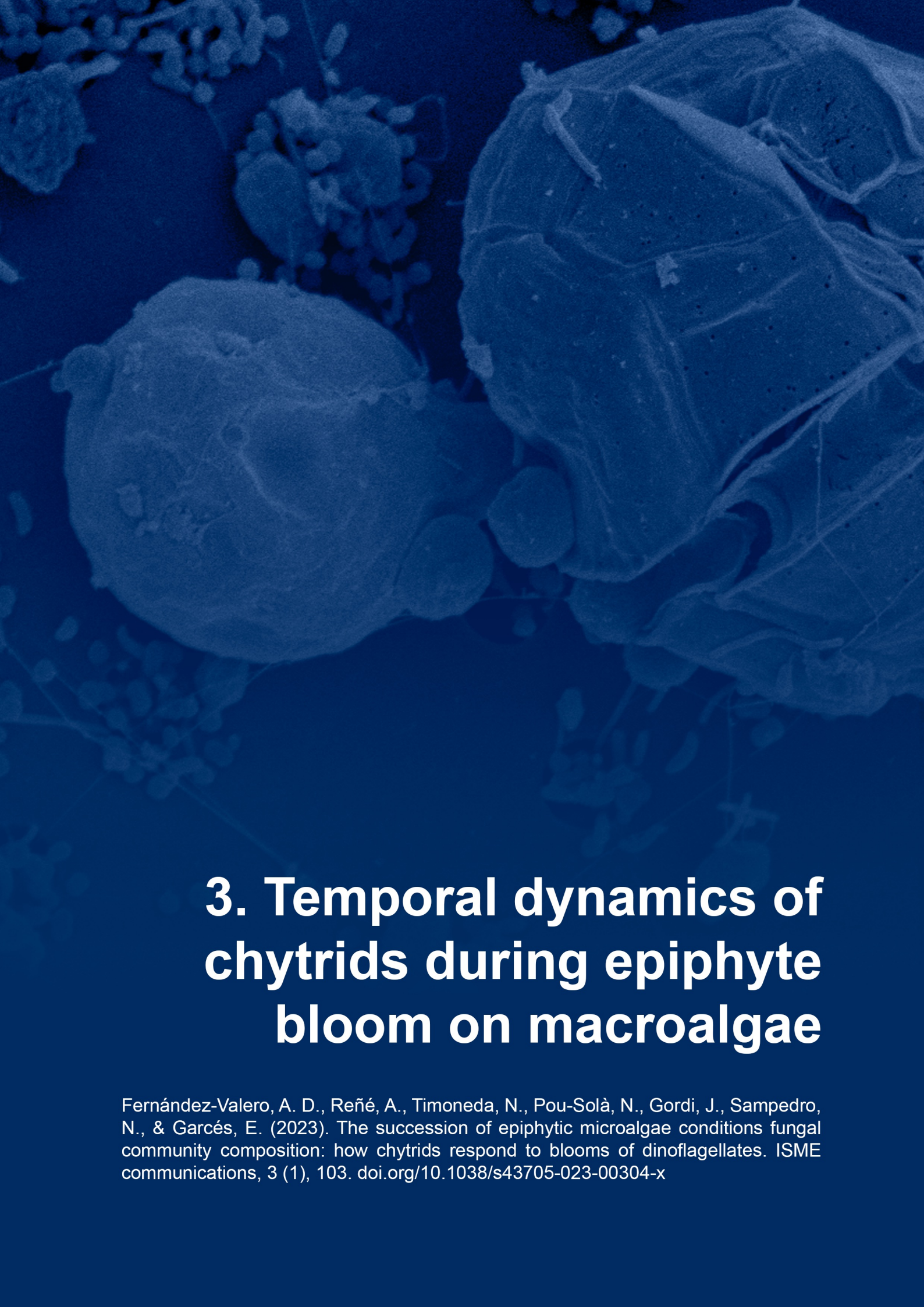
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A blue-tinted microscopic image showing various biological structures. In the foreground, there are large, rounded, and somewhat irregular structures, possibly chytrid zoospores or macroalgal cells. In the background, there are smaller, more numerous, and more structured clusters, likely representing a bloom of dinoflagellates. The overall texture is complex and organic.

3. Temporal dynamics of chytrids during epiphyte bloom on macroalgae

Fernández-Valero, A. D., Reñé, A., Timoneda, N., Pou-Solà, N., Gordi, J., Sampedro, N., & Garcés, E. (2023). The succession of epiphytic microalgae conditions fungal community composition: how chytrids respond to blooms of dinoflagellates. *ISME communications*, 3 (1), 103. doi.org/10.1038/s43705-023-00304-x

3 Temporal dynamics of chytrids during epiphyte bloom on macroalgae

3.1 Abstract

This study aims to investigate the temporal dynamics of the epiphytic protist community on macroalgae, during the summer months, with a specific focus on fungi, and the interactions between zoosporic chytrid parasites and the proliferation of the dinoflagellates. We employed a combination of environmental sequencing techniques, incubation of natural samples, isolation of target organisms and laboratory experiments. Metabarcoding sequencing revealed changes in the dominant members of the epiphytic fungal community. Initially, fungi comprised <1 % of the protist community, mostly accounted for by Basidiomycota and Ascomycota, but with the emergence of Chytridiomycota during the mature phase of the biofilm, the fungal contribution increased to almost 30%. Chytridiomycota became dominant in parallel with an increase in the relative abundance of dinoflagellates in the community. Microscopy observations showed a general presence of chytrids following the peak proliferation of the dinoflagellate *Ostreopsis* sp., with the parasite, *D. arenysensis* as the dominant chytrid. The maximum infection prevalence was 2% indicating host-parasite coexistence. To further understand the *in-situ* prevalence of chytrids, we characterised the dynamics of the host abundance and prevalence of chytrids through co-culture. These laboratory experiments revealed intraspecific variability of *D. arenysensis* in its interaction with *Ostreopsis*, exhibiting a range from stable coexistence to the near-extinction of the host population. Moreover, while chytrids preferentially parasitized dinoflagellate cells, one of the strains examined displayed the ability to utilize pollen as a resource to maintain its viability, thus illustrating a facultative parasitic lifestyle. Our findings not only enrich our understanding of the diversity, ecology, and progression of epiphytic microalgal and fungal communities on Mediterranean coastal macroalgae, but they also shed light on the presence of zoosporic parasites in less-explored benthic habitats.

3.2 Introduction

Fungi are an important component of the microeukaryotic community in aquatic environments. They engage in a wide variety of parasitic and saprophytic interactions (Jones et al., 2015) involving diverse members of the microbial community. Studies conducted in freshwater habitats have shown that fungal interactions range from competitive strategies for resource acquisition to the development of coexistence mechanisms based on niche differentiation (Van den Wyngaert et al., 2022). While abiotic factors exert an impact on fungal community (Wang et al., 2017; Li et al., 2019), biotic factors, especially the influence of other organisms within the microbial community, also play a significant role (Cheung et al., 2018). Within the microbial community, the temporal patterns of fungal species composition range from seasonal and repetitive cycles to brief peaks in abundance. For instance, sudden increases in the abundance of zoosporic fungi belonging to the Chytridiomycota (referred to as chytrids) occur nearly simultaneously

with diatom blooms (Taylor and Cunliffe, 2016; Van den Wyngaert et al., 2022). In addition, infections by members of the Chytridiomycota during the proliferations of other microalgal groups, including cyanobacteria (Gerphagnon et al., 2013; Xue et al., 2018) and dinoflagellates (Fernández-Valero et al., 2022; Reñé et al., 2022), have also been documented.

Fungi have been identified in diverse niches across the marine environment (Grossart et al., 2019), including the benthic habitat (Tisthammer et al., 2016). The identification of a large number of fungal sequences in the benthic habitat indicates its role as a reservoir of zoosporic parasites, including chytrids, thus contributing to the overall diversity of the marine environment. In a previous study, the diversity of benthic microbial communities and the prevalence of specific fungal groups have revealed gradients spanning coastal, offshore, and deep-sea regions (Rubin-Blum et al., 2022). The authors observed that Chytridiomycota were dominant in the coastal zone, whereas Ascomycota and Basidiomycota were more prevalent on the seafloor. Another study specifically conducted within coastal area highlighted higher diversity of marine chytrid in sediment compared to the water column (Fernández-Valero et al., 2022).

The characteristics of the benthic habitat, including inorganic substrate such as rocky surfaces, and organic substrates such as macroalgae and seagrass surfaces, exert significant influence on shaping the microbial community that develops into a biofilm. Notably, the organic substrates within the benthic habitat, primarily consisting of macroalgae and seagrass surfaces, create a nutrient-rich milieu abundant inorganic carbon. This, in turn, facilitates microbial colonization and promoting biofilm formation on available surfaces (Steinberg and De Nys, 2002; Goecke et al., 2010). The community composition of these epiphytic microorganisms is shaped by multiple biotic interactions that reflects the dynamic and complex interplay between the epiphytic community and the substrate (Roth-Schulze et al., 2016; Wichard and Beemelmanns, 2018). In the case of epiphytic fungi, these interactions involve colonization of the substrate through zoospores or hyphae, leading to the establishment of parasites, saprophytes, mutualists or endosymbionts (White, 2005). An example of these interactions was observed in the study of epibiotic fungi on seaweed in the Red Sea, Egypt, wherein different available host seaweed species exerted strong selective pressure and temporal changes on the associated fungal populations (Abdel-Gawad et al., 2014). Specific interaction among epiphytic species modulate the community composition on macroalgae. As an illustration, the fungus *Aspergillus versicolor*, found in the brown seaweed *Sargassum thunbergii*, generates compounds with anti-bacterial properties, thereby selectively limiting the growth of prokaryotes (Miao et al., 2012). Furthermore, the epibiotic fungus *Trichoderma virens*, isolated from the red alga *Gracilaria vermiculophylla*, has been reported to secrete compounds that inhibit the growth of phytoplankton species such as *Chattonella marina*, *Heterosigma akashiwo*, *Karlodinium veneficum* and *Prorocentrum donghaiense*, while also displaying toxicity towards certain zooplankton species (Shi et al., 2018).

In our study system, the colonization of macroalgae by epiphytic protist is a well-established process (Vila et al., 2001; Moncer et al., 2017). The initial stages of epiphytic assemblage development on Mediterranean macroalgae are typically characterized by the presence of microalgae, particularly diatoms of the *Navicula*, *Nitzschia*, and *Coscinodiscus* genera. These diatoms possess the ability to attach to both inorganic or organic substrates and withstand hydrodynamic stress (Romagnoli et al., 2007). Moreover, certain benthic diatom species such as *Navicula* sp. and *Proschkinia* sp. have a negative impact on the growth of other microalgae, including the dinoflagellate *Ostreopsis*. As a result, they influence the composition of the epiphytic protist assemblage (Pichierri et al., 2017). In the later stages of epiphyte biofilm formation on macroalgae, dinoflagellates of the genera *Gambierdiscus*, *Coolia*, *Prorocentrum*, *Amphidinium* and the aforementioned *Ostreopsis* become dominant.

Despite having knowledge of species succession within benthic epiphytic assemblages, the succession patterns of other heterotrophic protist groups, such as fungi, remain incompletely described and understood. Similarly, the temporal dynamics and interaction between chytrids and epiphytic dinoflagellates have yet to be investigated. We hypothesized that temporal changes in the composition of the epiphytic assemblage influence the presence and abundance of co-occurring fungi. Therefore, this study combines field sampling and laboratory experiments to investigate into the temporal succession of the epiphytic assemblage. Moreover, it explores the ecological interaction between Chytridiomycota and dinoflagellates over time, focusing on the dynamics of the most abundant chytrid, *Dinomyces arenysensis*, during its infection of its host, the bloom-forming dinoflagellate *Ostreopsis* sp.

3.3 Materials and methods

Field sampling

Twelve weekly samplings were conducted from June to September 2021 at one station in the coastal locality of Sant Andreu de Llavaneres, NW Mediterranean Sea (41°33'07.5"N; 2°29'31.6"E) to determine the temporal dynamics of the epiphytic assemblage on macroalgae. The collection and treatment of the samples were based on the standard protocol for studying epiphytes associated with macrophytic substrates (Yasumoto et al., 1980). The macroalgal growing season extends from early June to late August [(Medrano et al., 2020)]. At each sampling, samples of macroalgae were collected from the rocky surface of the study area at depths of 1-1.5 m. The sampled macroalgae represent the predominant species in the study area: *Halopteris scoparia*, *Jania rubens*, *Padina pavonica*, *Dictyota dichotoma* and *Cladophora* sp., and reductions in macroalgal cover or changes in epiphyte composition were not observed throughout the sampling period. The samples were placed in flasks containing 1 L of autoclaved seawater, which was used to wash and resuspend the epiphytic protist community. The macroalgae were then separated from the epiphytic resuspension and the fresh weight of the macroalgae (gFW) was determined. The resuspended samples were vigorously shaken until all biofilm was disintegrated. The epiphytic resuspensions were filtered through a 100 µm mesh and a 150 mL aliquot was fixed with Lugol's iodine to determine the cell abundance of diatoms and dinoflagellates, by settling 10 mL of each fixed sample in Utermöhl chambers for 24 h (Utermöhl, 1958). Sedgewick-Rafter counting chambers were used when the cell abundance of certain species was too high. Cells were counted using a Leica-Leitz DMIRB inverted microscope equipped with a Jenoptik ProgRes C10 Plus camera. The dimensions of ten cells of each taxon were measured using the ProgRes CapturePro v2.8.8 software. Biovolumes were calculated using the appropriate geometric formulae (Hillebrand et al., 1999), and measurements were taken for 15 cells per taxon. The results were expressed as cubic millimetres per gram of fresh weight of macroalgae (mm³ gFW⁻¹).

Metabarcoding sequencing and analyses

Each water sample derived from resuspension of the macroalgae was immediately filtered in two replicates using non-cytotoxic Nalgene™ polysulfone bottles and 8 µm polycarbonate filters with a diameter of 47 mm. The organisms on the filters represented the epiphytic assemblage spanning the 8 to 100 µm size fraction. The filters were stored frozen at 70 °C until processing. Genomic DNA from the filtered samples was extracted using the DNeasy PowerSoil kit (Qiagen) according to the manufacturer's instructions, Metabarcoding sequencing of the V4 region of

the 18S rDNA was performed using the universal primers EK-565F (Comeau et al., 2011) and EUK-1134R-UnonMet biased against metazoans (Bower et al., 2004) to determine the protist community, defined as all eukaryotic microorganisms, including fungi, ranging from 8 μm to 100 μm . The 8 μm filter would exclude fungal zoospores and consequently result in an underestimation of fungal proportions. The fungal-specific primers ITS3-KYO2 (Toju et al., 2012) and ITS4 (White et al., 1990) were used to amplify the 5.8S and ITS2 fragments of the rDNA operon (here in after referred to ITS region). This region represents the best option for amplification and taxonomic identification of fungi (Tedersoo et al., 2015). Both the V4 18S rDNA and the ITS regions were sequenced on an Illumina PE300 system. The raw reads were trimmed from the adaptors using CUTADAPT (Martin, 2011). The DADA2 pipeline (Callahan et al., 2016) was used to filter and dereplicate the reads, trim low-quality sequences, merge paired-ends, and remove chimeras. The parameters maxEE = 4, 6 and truncLen = 270, 250 were applied to both regions (V4 18S rDNA and ITS), and minOverlap = 20 for V4 18S rDNA and minOverlap = 50 for ITS. The obtained ASVs were taxonomically assigned using a simple non-Bayesian taxonomy classifier, VSEARCH v2.8.1 (Rognes et al., 2016), with the PR2 database version 4.12 (Guillou et al., 2013) customized with new in-house sequences for the V4 region and the full UNITE + INSD dataset from UNITE community v.8.3 for the ITS segment (Abarenkov et al., 2021) and with bootstrap cut-off = 0.6. Unassigned ITS sequences that could not be classified as belonging to fungal organisms were removed from the dataset and were not considered for the fungal community analysis. The amplicons were 490 bp for V4 18S rDNA and 450 bp for ITS. The data were normalized according to the relative abundance of ASVs per sample. Raw metagenomic reads were submitted to the NCBI Sequence Read Archive under Bioproject numbers PRJNA891539 and PRJNA891506, corresponding to the V4 18S rDNA and ITS region, respectively. The ASV tables, including the FASTA sequences and taxonomic classifications, are available in Supplementary File 1. Metabarcoding data were analysed using the package Phyloseq and all graphs were generated using the package ggplot2 from Rcran (McMurdie and Holmes, 2013; R Core Team, 2013; Wickham, 2016). Nonmetric multidimensional scaling (NMDS) using Bray-Curtis distances was then performed to analyse the dissimilarity between samples and permutational multivariate analysis of variance using distance matrices (ADONIS) was performed to assess differences between sample groups.

Dinoflagellate and chytrid sporangium cell counting by epifluorescence microscopy

Chytrid presence, defined herein as the detection of *Ostreopsis* cells infected with *Dinomyces*, was assessed using the filters representing the temporal samplings at the coastal locality. Infections were attributed to *Dinomyces* based on previously reported metabarcoding results, isolation of the strains, the identification of single cells, and the morphological identification of sporangia from laboratory cultures of *D. arenysensis*. A 10 mL fraction of the sample derived from the macroalgal resuspension was fixed with 10% formaldehyde (v:v) for 24 h, filtered through 10 μm polycarbonate filters, and stored at -70°C. The filters were covered with a 0.1% agarose permeable film to prevent cell loss during further manipulation. The filters were then cut into triangular sections and placed on slides with a drop of DAPI oil to stain the DNA strands. The oil solution was prepared from 1.25 μL of 0.5 mg DAPI (4',6-diamidine-2'-phenylindole dichlorohydrochloride) mL^{-1} and 125 μL of a pH 9.5 solution composed of 100 mL of glycerol, 2 mL of Vectashield, and 1 mL PBS. Stained filter sections were observed at 1000 $\times$ magnification using an Olympus Bx61 epifluorescence microscope connected to an Olympus DP72 camera (Olympus America Inc.). The following fluorescence filters were used for DAPI detection: excitation BP330-385, dichroic mirror DM400, and barrier filter BA420. The infection prevalence (%) was calculated as the percentage of infected *Ostreopsis* cells with respect to the total number of cells. The

determination of infection prevalence in natural samples was based solely on the presence of mature sporangia, visualized using epifluorescence microscopy.

Isolation, culture of chytrids, and identification of cultured strains by single-cell PCR

All cultured strains used in this study were obtained from the stock culture collection of the Institut de Ciències del Mar (ICM-CSIC). In the laboratory, chytrid cultures were established by transferring 5 mL aliquots of the resuspended epiphytic assemblages to 6-well plates for incubation. For samples with *Ostreopsis* abundances $<10^3$ cells g FW⁻¹, healthy cells from an *Ostreopsis* sp. culture (strain ICMB293) were added to the incubations to increase host density and promote parasite infection. All incubations were maintained in chambers at $20\pm1^\circ\text{C}$ and subjected to a 12:12h photoperiod (light:dark). Illumination was provided by LED fluorescent tubes with a photon irradiance of ~ 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The incubations were periodically checked for the presence of chytrid infections for up to 2 weeks. Infected dinoflagellate cells were manually isolated to establish clonal chytrid strains. The chytrids were grown in 24-well plates by transferring a 1 mL aliquot of mature sporangia every 3–4 days to a well containing 1 mL of an exponentially growing uninfected host stock culture. An *Ostreopsis* sp. strain (ICMB293) maintained under non-axenic conditions in F/2 medium and a salinity of 36 was used to maintain the chytrid strains. Samples from the chytrid cultures were collected and stained with Alcian blue (1% solution) to identify the polysaccharide structures constituting the mucilage of the host. The isolated strains were observed using a Leica DMIRB inverted microscope. Photographs were taken at 40–60 $\times$ magnification using a Jenoptik ProgRes C10 Plus camera.

Individual mature sporangia were isolated from the parasitoid strains using glass micropipettes and then subjected to further molecular characterization. The single-cell protocol was implemented following (Reñé and Hoppenrath, 2019) and (Reñé et al., 2021). A first PCR was performed using the primers EK82F and 28S-1611R (López-García et al., 2001; Moreira et al., 2007), to amplify the entire ribosomal operon. The PCR product served as a template to amplify the partial V4 18S rDNA gene, using the specific primers CRYPTO2-2F and EUK-1520R (López-García et al., 2003; Lazarus and James, 2015). Four μL of each product was electrophoresed on a 1.2% agarose gel. The gel was stained with SYBR Safe (Invitrogen) to visualize the bands and confirm the correct amplicon length. For samples with poorly visualizable bands, a third semi-nested PCR was performed using the specific primers CRYPTO2-2F and AU4v2 (Lazarus and James, 2015) and the product of the previous PCR as template. Finally, Sanger sequencing with forward and reverse primers was performed by an external service (Genoscreen, Lille, France).

Infection dynamics

The temporal dynamics of *Dinomyces arenysensis* infection were examined using strains isolated during *Ostreopsis* proliferations at three different locations of the Catalan coast. Strain ICMB1101 was isolated from Cubelles Beach ($41^\circ 11' 51.8''$ N, $1^\circ 40' 23.0''$ E) in September 2019; strain ICMB1102 was isolated from Llanvaneres Beach ($41^\circ 33' 07.5''$ N, $2^\circ 29' 31.6''$ E) in July 2020; and strain ICMB1104 was isolated from La Fosca Beach ($41^\circ 51' 27''$ N, $3^\circ 8' 38''$ E) in August 2020. Cell abundances of the host *Ostreopsis* sp. strain ICMB293 and the chytrid zoospores in each stock culture were determined. Culture flasks were inoculated in triplicate with healthy host cells and zoospores from each chytrid strain (ICMB1101, ICMB1102, ICMB1104), at a

host:parasite ratio of 1:10 and a final volume of 20 mL. The initial abundances of *Ostreopsis* cells and zoospores were 300 mL⁻¹ and 3000 mL⁻¹, respectively. This low initial parasite abundance allowed to study the development of the infection from its early stage, and thus host-parasite dynamics to be monitored over time. The incubations were maintained at 21°C and a 10h:14 light:dark cycle. The infections were followed for 20 days, with the flasks sampled on days 2, 5, 10, 16, and 20. The collected samples (2 mL) were fixed with formaldehyde (final concentration 3.6%) and then counted in a Sedgewick-Rafter chamber by light microscopy to determine the number of healthy and infected *Ostreopsis* cells as well as chytrid prevalence.

Growth of chytrids using an alternative nutrient source

The ability of the chytrids to grow on organic sources other than dinoflagellate cells was examined using *D. arenysensis* strains ICMB1101, ICMB1103, and ICMB1104. Strain ICMB1103 is a monoclonal culture of *D. arenysensis* isolated from Tossa Beach (41°42'55.9" N, 2°55'56.4" E) in August 2020. Pine pollen (*Pinus pinea*) was added to the chytrid culture in a 10 mL 6-well plate. The presence of infections was checked by inverted light microscopy of the plates after 4 and 6 days of incubation. Thereafter, an inoculum of healthy *Ostreopsis* culture (strain ICMB293) was added to the wells to determine the viability of the sporangia and their ability to infect dinoflagellate cells.

3.4 Results

Diversity and relative abundance of the eukaryotic community

The epiphytic protist community (8 to 100µm fraction) on macroalgae was represented by 2153 ASVs, based on metabarcoding of the V4 18S rDNA region. The metabarcoding data could be categorized into three temporal phases according to community composition and the relative abundance of specific groups, including as Dinoflagellata, Ochrophyta (diatoms), Ciliophora, and Fungi (Figure 3.1). The three phases of the community structure were observed from a Non-Metric Multidimensional Scaling (NMDS) analysis (Figure 9.1: Suppl. Figure 1) and its statistical significance was confirmed through a Permutational multivariate analysis of variance using distance matrices (ADONIS. R²=0.48, p-value<0.001).

The differentiation between these phases was mainly attributed to the presence of distinct dominant taxonomic groups in each respective phase. The first phase, corresponding from June 14th to July 8th, was characterized by the dominance of Ochrophyta (diatoms), which accounted for >50% of the relative abundance. This was followed by other groups of microalgae, including Dinoflagellata (dinoflagellates) and Ciliophora (Figure 3.1a). In the second phase, observed from July 15th to July 22nd, the relative abundance of dinoflagellates ranged from >60% to nearly 100%. During the third phase, from July 29th to September 1st, the relative abundance of dinoflagellates remained high (~50%) but that of other groups, including Fungi, Apicomplexa, Chlorophyta, Sagenista, Mesomycetozoa, and unassigned taxa, increased (Figure 3.1a). However, microscopy of the samples showed slightly different dynamics of the epiphytic protist assemblage. The proportions of diatoms and dinoflagellates determined by microscopy were based on the biovolume (Figure 3.1c). Thus, during the first phase, the biovolume of epiphytic diatoms and dinoflagellates was <0.1 mm³ gFW⁻¹. In the second phase, the fraction of epiphytic dinoflagellates increased to 21,8 mm³ gFW⁻¹ while that of epiphytic diatoms remained unchanged. In the third phase, the dinoflagellate fraction reached a maximum of 14,7 mm³ gFW⁻¹ (August 4th)

followed by a progressive decrease to $0.2 \text{ mm}^3 \text{ gFW}^{-1}$. During the month of August, the dominant fraction of organisms was dinoflagellates, consistent with the metabarcoding results. However, the biovolume of diatoms gradually increased such that by September the epiphyte assemblage was dominated equally by dinoflagellates and diatoms (Figure 3.1c), which was not observed in the metabarcoding data.

Diversity and relative abundance of the dinoflagellate assemblage

Given that dinoflagellates were the most abundant taxonomic group on macroalgae along the epiphytic succession, the structure of the epiphytic dinoflagellate assemblage was evaluated in detail. The metabarcoding results showed structural differences between the three phases described above (Figure 3.1b), with a high diversity of species, including those belonging to the genera *Amphidinium*, *Coolia*, *Symbiodinium* and unassigned taxa, during the first phase. *Ostreopsis* sp. appeared in early July, with a relative abundance of <6%. During the second phase, the relative abundance of this species increased from 40% to almost 100% of the dinoflagellate and protist sequences. During the third phase, the relative abundance of *Ostreopsis* sp. decreased from 25% (July 29th) to <6% in subsequent samples. The composition of the dinoflagellate sequences during the third phase was more diverse than during the first and second phases and was dominated by Suessiaceae, unidentified taxa, and, to a lesser extent, *Prorocentrum* after August 4th (Figure 3.1b). By contrast, according to the microscopic analyses *Ostreopsis* sp. dominated among the dinoflagellate during both the second and the third phases, accounting for 89% to almost 100% of the epiphytic dinoflagellate biomass (Figure 3.1c). Other dinoflagellate species, belonging to the genera *Coolia*, *Amphidinium* and *Prorocentrum*, were detected at low abundance beginning on July 15th, consistent with the metabarcoding results.

Diversity and relative abundance of fungal and chytrid assemblages

The fungal community based on metabarcoding of the V4 18S rDNA region was represented by 203 ASVs. During the first and second phases, fungi accounted for <1% of the protist community, with the exception of the samples taken on June 30th and July 8th, in which the inter-replicate values ranged from 2% to 30%. However, on July 29th, corresponding to the third phase, fungi accounted for almost 30% of the protist community, followed by a gradual decrease to <5% by September 1st. (Figure 3.1a). The analysis of the fungal assemblage revealed that the elevated relative abundances during the first phase were due to the contributions of Basidiomycota (June 30th) and Ascomycota (July 8th). During the third phase, Chytridiomycota emerged as the dominant group in the fungal community, but its contribution gradually declined and its dominance eventually alternated with that of Ascomycota (Figure 3.2a). Between mid-August and September, overall fungal diversity increased.

The diversity of the fungal community was further examined using the ITS region of fungal representatives (Figure 3.2b). Seventy-seven percent of the ITS sequences were discarded as they were not identified as belonging to fungi. A total of 466 ASVs were obtained, revealing differences in community composition during the three phases. During the first and second phases, the community was dominated by fungal sequences with unknown taxonomic affiliations, albeit with Ascomycota and Basidiomycota present to a lesser extent. Between July 29th and August 4th, and thus during the third phase, the proportion of Chytridiomycota significantly increased and accounted for almost 100% of the fungal community. However, the relative abundance of Chytridiomycota gradually declined, accompanied by an increase in other groups, such as Ascomycota, Basidiomycota, and sequences of unclassified taxonomy.

3 Temporal dynamics of chytrids during epiphyte bloom on macroalgae

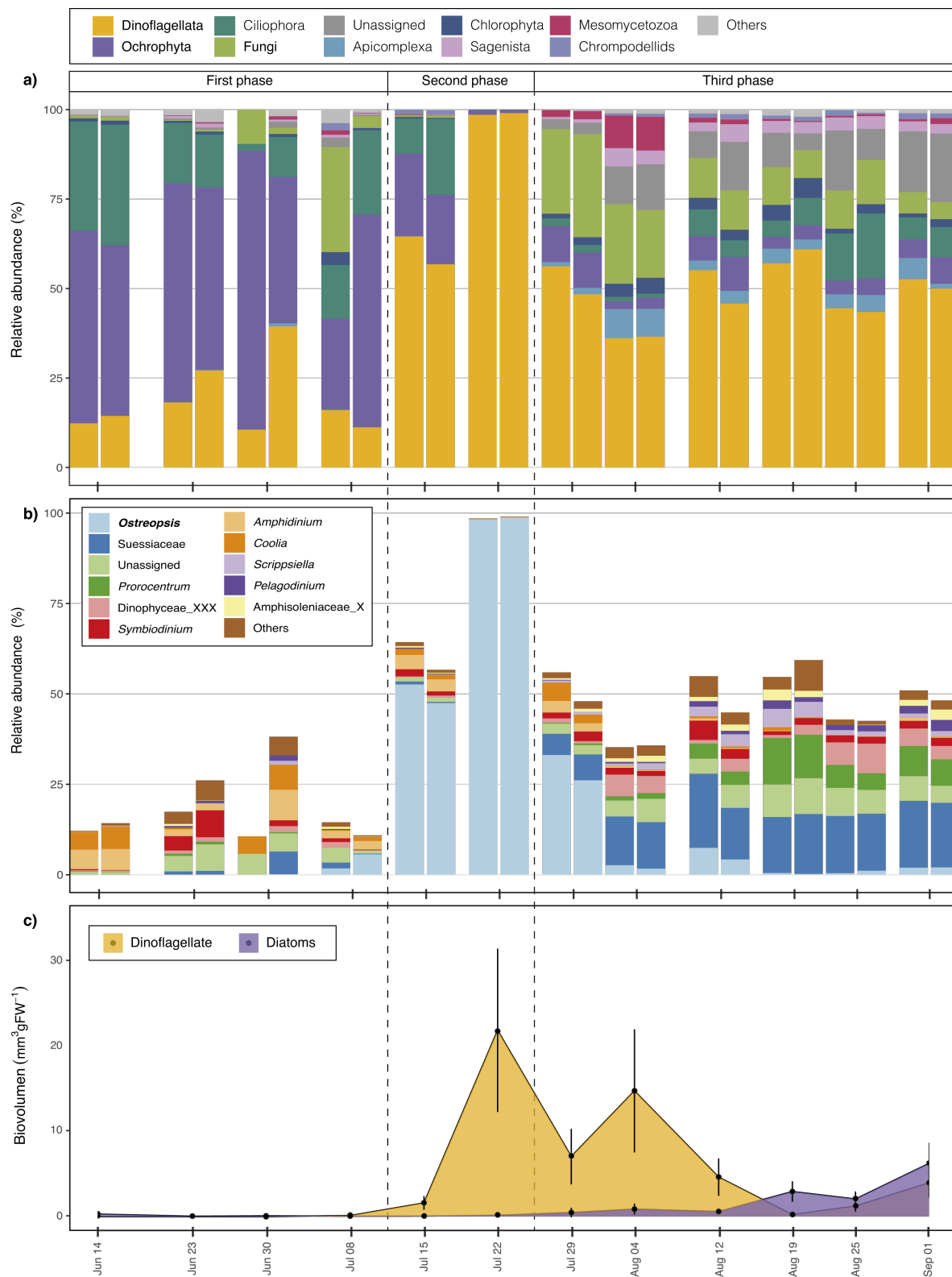


Figure 3.1: Temporal dynamics of the epiphytic assemblage on macroalgae.
Legend in the next page.

Figure 3.1: Temporal dynamics of the epiphytic assemblage on macroalgae. (a) Bar plot showing the variation (%), in replicates, of the different epiphytic eukaryote groups at each sampling date. (b) Bar plot showing the variation in the epiphytic dinoflagellate assemblage on macroalgae. Relative abundances (%) are based on the total protist community. (c) Non-stacked area plot showing the variations in the biovolumes of epiphytic diatom and dinoflagellate groups. Vertical dashed lines represent the three temporal phases described in the text.

Further analysis of the Chytridiomycota based on ITS region analysis revealed 19 ASVs (Figure 3.2c). During the first and second phases, the composition of Chytridiomycota was diverse but the total relative abundance was <5%. During the third phase, ASV2 emerged as the dominant Chytridiomycota sequence, accounting for almost 100% of the total Chytridiomycota and fungal sequences during the samplings of July 29th and August 4th, followed by ASV29 at much lower relative abundance.

Although ASV2 remained the dominant Chytridiomycota sequence, its abundance relative to total fungal sequences gradually decreased. On August 25th and September 1st, the relative abundance of ASV2 was lower, and the relative abundance of ASV83 increased. ASV2 and ASV29 were only differentiated by one single nucleotide, showing an identity of 99.7%. Both sequences were taxonomically assigned to the species *Dinomyces arenysensis*, likely representing intraspecific variants. ASV83 was assigned as an unknown species of Lobulomycetales.

Identification and prevalence of chytrids in field samples

Chytrid infections were detected by light and epifluorescence microscopy from the second phase (beginning July 15th) onwards (Figure 3.3a, b and Table 3.1). During this period, two species of chytrids were identified by the molecular characterization of cultured strains established, or single cell isolates from natural samples (*Dinomyces arenysensis* and Lobulomycetales sp.). The last one was isolated, cultivated and identified as a new, yet undescribed species of Lobulomycetales (strain ICMB1108) (Figure 3.3e, f and Table 3.1).

Only the prevalence of one species, *D. arenysensis*, was determined. The morphology of mature sporangia observed by epifluorescence was compared and confirmed with sporangia from cultures (Figure 3.3a, b). The prevalence of *Dinomyces* infection during this second phase was <0.5%. In the third phase, after August 4th, chytrid infections were recurrently detected in field samples by light microscopy, facilitating the establishment of chytrid cultures and thus their molecular characterization. Spherical epibiotic sporangia without discharge papillae were observed, and the subsequent molecular characterization of these strains revealed them to be *D. arenysensis* (ICMB1109–ICMB1112) (Figure 3.3c, d and Table 3.1). Infection prevalence at this stage reached up to 2.7% (Table 3.1). Infections in the lab of the two chytrid species found in this study (*D. arenysensis* and Lobulomycetales sp.) were equally observed in the mucilage of *Ostreopsis* sp. (Figure 3.3i). All infections, isolations and cultures were originated from the dinoflagellate *Ostreopsis* sp. No infections by chytrids were observed in any other microalgae during the study period.

3 Temporal dynamics of chytrids during epiphyte bloom on macroalgae

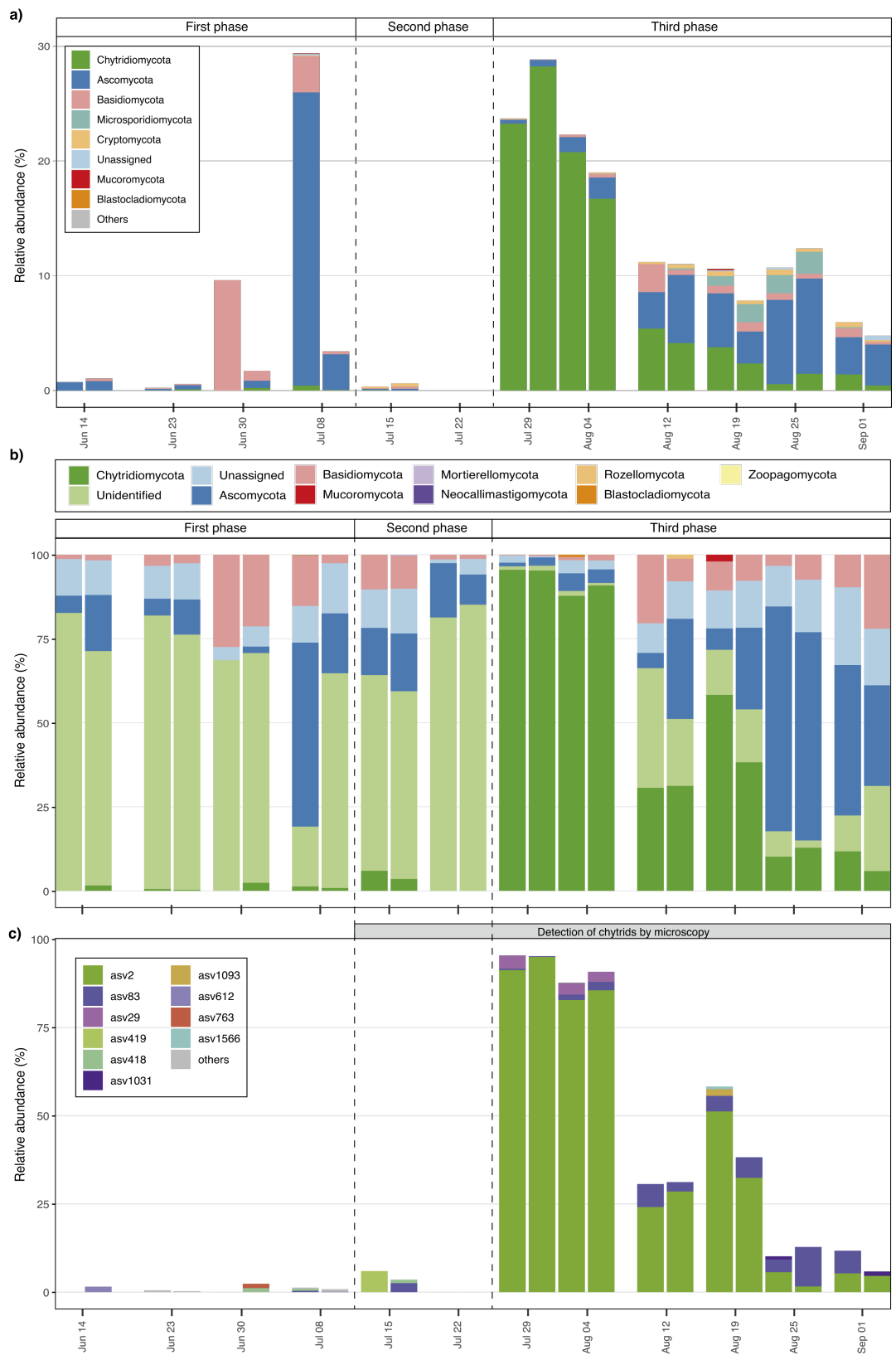


Figure 3.2: Temporal dynamics of the epiphytic fungal assemblage as determined using the V4 18S rDNA and ITS regions. Legend in the next page.

Figure 3.2: Temporal dynamics of the epiphytic fungal assemblage as determined using the V4 18S rDNA and ITS regions. (a) Bar plot showing the variation, in replicates, of the epiphytic fungal assemblage as determined using the V4 18S rDNA region. Relative abundances (%) are based on the total protist community. (b) Bar plot showing the relative abundance of the fungal groups as determined using the ITS region. (c) Bar plot showing the relative abundance of Chytridiomycota with respect to the fungal community based on the ITS region. The ten most abundant Chytridiomycota ASVs are shown; the remaining nine ASVs are included in the 'other' category. The shaded area between (b) and ((c) indicates the period when the presence of chytrids was detected by light microscopy, epifluorescence microscopy or incubation of natural samples.

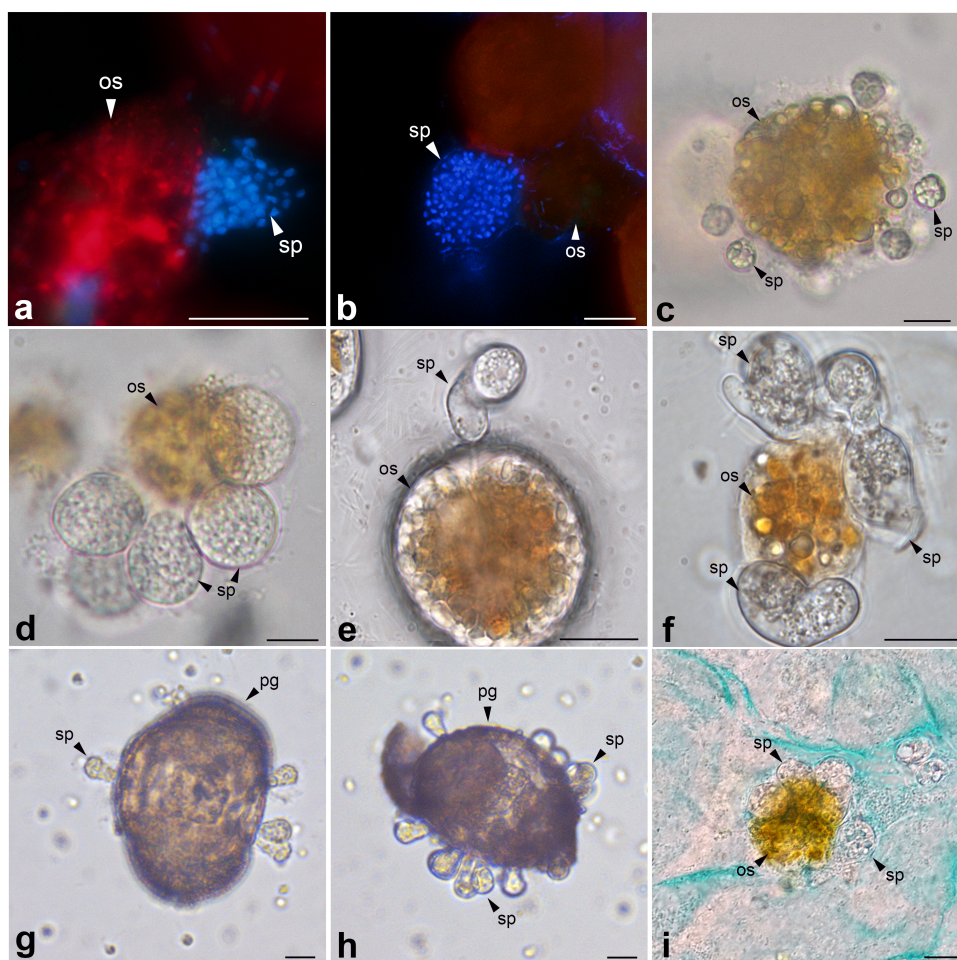


Figure 3.3: Microscopy images of chytrids. Epifluorescence microscopy: (a) DAPI staining of mature sporangia from natural samples. (b) DAPI staining of mature sporangia of *Dinomyces arenysensis* from laboratory cultures. Light microscopy. (c) Early infection stage of *D. arenysensis* strain ICMB1109 infecting *Ostreopsis* sp. (ICMB293). (d) Immature sporangium stage of strain ICMB1109 infecting *Ostreopsis* sp. (e) Early infection stage of Lobulomycetales strain ICMB1108 infecting *Ostreopsis* sp. (f) Immature sporangium stage of strain ICMB1108 infecting *Ostreopsis* sp. (g), (h) Pine pollen grains with multiple sporangia of *D. arenysensis* ICMB1103, observed 4 days after inoculation of the chytrid (i) Alcian blue staining of mucilage present in cultures of Lobulomycetales sp. sp: sporangium, os: *Ostreopsis* sp., pg: Pine pollen grain. Scale bars = 10 μ m.

Facultative parasitic strategy of *D. arenysensis*

The ability of *D. arenysensis* to utilize non-algal organic matter for its life cycle and propagation was investigated by incubating previously established chytrid strains (ICMB1101, ICMB1103 and ICMB1104) with pine pollen grains. After 4 days of incubation, ICMB1104 was the only strain that had formed sporangia on the pollen grains, with chytrids growing in the main body of the grain and forming single or multiple sporangia (Figure 3.3g, h). After 6 days, more mature sporangia were found, although no living *Ostreopsis* sp. cells from the initial inoculum were observed. The viability of the newly produced sporangia on the pollen was tested by inoculating new, healthy *Ostreopsis* sp. cells into the same well, which resulted in re-infection of the dinoflagellate by the chytrid 2 days later. These infections originated from zoospores released from the sporangia present on the pollen grains. Once the dinoflagellate cells were added and infected, no new infections on the pollen grains were observed.

Temporal dynamics of *D. arenysensis* strains and *Ostreopsis* sp.

The levels of infection development and virulence differed among *Dinomyces* strains (ICMB1101, ICMB1102, ICMB1104) in infections of *Ostreopsis* sp. (strain ICMB293) (Figure 3.4). In infections with strain ICMB1101, <2% of *Ostreopsis* sp. cells were infected during the first 10 days of the experiment and the abundance of healthy *Ostreopsis* sp. cells gradually increased from 300 cells mL⁻¹ to 900 cells mL⁻¹. Thereafter, the prevalence of infection increased exponentially such that by the end of the experiment 30% of the population was infected and the population of healthy *Ostreopsis* sp. cells decreased to >300 cells mL⁻¹. When *Ostreopsis* sp. was inoculated with chytrid strain ICMB1104, the infection rate did not exceed 1% and the growth of *Ostreopsis* sp. cells was comparable to that observed in an uninfected control treatment. In infections with strain ICMB1102, the highest prevalence of infection occurred after 5 days, at which time 5% of the population was infected. On subsequent days, the prevalence decreased gradually, to <1% on day 20. During the first 10 days of exposure, the *Ostreopsis* population remained stable, with cell numbers similar to the starting value. From day 10 until the end of the experiment, the *Ostreopsis* population increased from 300 cells mL⁻¹ to 900 cells mL⁻¹ while the prevalence of infection remained low.

3.5 Discussion

This study investigated the diversity and temporal dynamics of marine epiphytic protist assemblages on macroalgae, with a focus on fungi, Chytridiomycota and dinoflagellates. Our findings provide new insights into the complex interactions between marine fungi and their host communities. Specifically, the results showed that both the composition and the relative abundances of fungal communities are influenced by the succession of epiphytic microalgae. Over time, the community structure of the epiphytic fungi changed, evidenced by changes in the dominance of Ascomycota, Chytridiomycota, and unidentified fungal organisms. The abundance of the Chytridiomycota was highly dependent on epiphytic dinoflagellate species assemblages, with *Dinomyces arenysensis* and a new species of Lobulomycetales as the primary representatives of this group. The peak in the abundance of the dinoflagellate *Ostreopsis* sp. coincided with the highest relative abundance of chytrids, particular *D. arenysensis*. Laboratory experiments showed that the latter species is characterized by intraspecific variability in its prevalence, with a capacity of growth on pollen demonstrated in one of the tested strains.

Table 3.1: **Sampling dates, cell abundance of *Ostreopsis* sp. on macroalgae as determined by light microscopy counts, and chytrid prevalence on *Ostreopsis* sp. in field samplings.** -: not determined. Chytrids were isolated for culture and sequenced by single-cell PCR. Strains grown under laboratory conditions are named after the culture collection (ICMB). The 18S rDNA molecular sequences and the resulting taxonomic identities of the strains are indicated. White rows mark the first and third sampling-based phases of the epiphytic assemblage; shaded rows mark the second phase.

	Date	<i>Ostreopsis</i> sp.; abun. on macroalgae (cell*gFW ⁻¹)	Chytrid prevalence on <i>Ostreopsis</i> sp. (%)	Isolated chytrids and established cultures	GenBank Accession Number	Taxonomic identification of strain
First phase	2021-06-14	19	-			
	2021-06-23	13	-			
	2021-06-30	43	-			
Second phase	2021-07-14	13.509	0.59	ICMB1108	OQ911493	Unknown Lobulomy- cetales sp.
				Single-cell from natural sample	OQ911487	<i>Dinomyces arenysensis</i>
	2021-07-22	548.326	0.23			
Third phase	2021-07-29	379.949	0.86	Single-cell from natural sample	OQ911488	<i>D. arenysensis</i>
	2021-08-04	666.983	1.99	ICMB1109	OQ911489	<i>D. arenysensis</i>
	2021-08-12	213.928	1.63	ICMB1110	OQ911490	<i>D. arenysensis</i>
	2021-08-19	8.423	2.74			
	2021-08-25	43.783	1.20	ICMB1111	OQ9114916	<i>D. arenysensis</i>
	2021-09-01	64.711	0	ICMB1112	OQ911492	<i>D. arenysensis</i>

The identity and diversity of the eukaryotic protist community was explored using the V4 18S rDNA region, and that of the fungal community using the V4 18S rDNA and ITS regions. The two markers provide complementary information about the diversity of fungi present in the samples along with that of the other microbial components of the biofilm from which they were obtained. However, while V4 18S rDNA enables accurate assignments at high taxonomic levels, such as phylum and order, in fungal organisms the region is too conserved to allow species-level discrimination (Větrovský et al., 2016; Nilsson et al., 2019). The latter can be achieved with the ITS region, which allows precise taxonomic assignments at the genus and species levels. Nonetheless, the accuracy of the taxonomic assignments obtained with both markers heavily relies on the availability and quality of reference databases. The large number of unknown sequences in this study well demonstrates the need to expand our knowledge of fungal diversity.

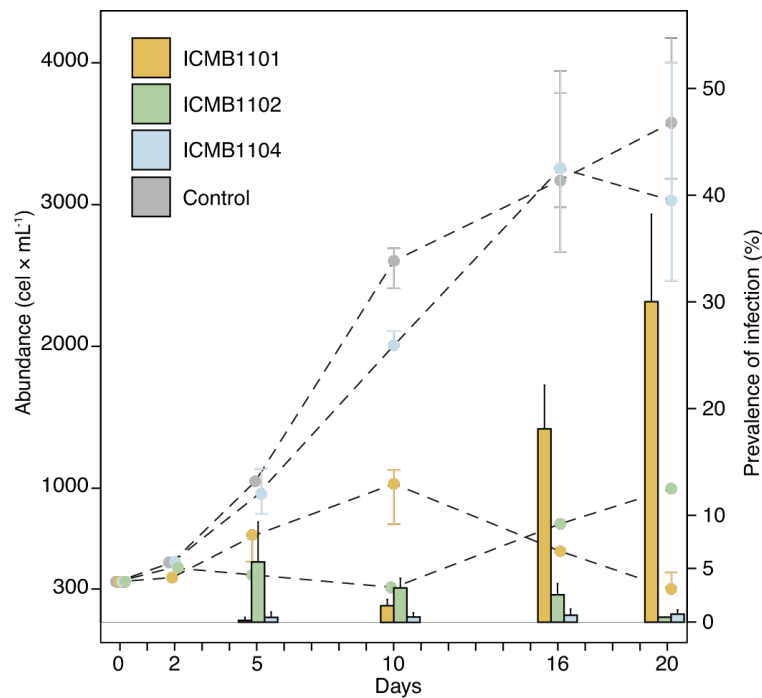


Figure 3.4: **Temporal dynamics of infection.** Cell abundance (cells mL⁻¹) of host *Ostreopsis* sp. strain ICMB293 and the prevalence (%) of strains of the chytrid parasite *D. arenysensis* (ICMB1101, ICMB1102 and ICMB1104) over time (days). Dashed lines correspond to cell abundance, and bars to prevalence. Whiskers represent the standard deviation.

Identification of unknown fungal species will provide valuable insights into their ecological roles.

The main finding of the study is that the composition of the fungal community is influenced by the temporal dynamics of epiphytic protists on macroalgae. During the first phase, diatoms and dinoflagellates accounted for very few of the epiphytic protists on macroalgae. This phase can thus be considered as the initial period of colonization and development of the microalgae that will subsequently dominate the epiphytic community (Vila et al., 2001). The fungal community on the macroalgae during the first phase was mainly composed of members of the Ascomycota and Basidiomycota, based on V4 18S rDNA. However, 70% of the fungal sequences obtained using the ITS region could not be attributed to any known fungal taxon, indicating a significant gap in our understanding of the diversity of marine fungal communities in these epiphytic assemblages, as has been reported in other studies (Jeffries et al., 2016; Ettinger and Eisen, 2019; Chen et al., 2023). As the protist biofilm on the macroalgae was not fully formed, this fungal assemblage may have been an epiphytic fungus of macroalgae rather than of the protist assemblage. In fact, Ascomycota and Basidiomycota are the main groups of marine fungi found in metabarcoding studies, and the most abundant and diverse fungal groups in benthic ecosystems (Grossart et al., 2019; Chen et al., 2022). Studies conducted in the Mediterranean Sea have shown that these two groups, including Ascomycetes saprophytes of *Posidonia oceanica* and the macroalga *Dictyota dichotoma* (Pasqualetti et al., 2020), represent 97.2% of the fungal community present on vegetal substrates (Poli et al., 2022)

During the second phase, the abundance of epiphytic dinoflagellates increased rapidly, from <25% to almost 100%. This proliferation corresponded specifically to the dinoflagellate *Ostreopsis*

sp., a well-known blooming species characterised in previous studies of the NW Mediterranean during the summer months (Vila et al., 2001). *Ostreopsis* sp. blooms are likely driven by the same factors that promote macroalgal growth, i.e., increases in temperature, irradiance, and substrate availability (Ballesteros, 1992). Furthermore, the mucilage secretions characteristic of these blooms enable the establishment of the microorganisms that will ultimately form the epiphytic summer biofilm on macroalgae (Accoroni et al., 2016). The proportion of Ascomycota, Basidiomycota, and the unidentified set of taxa in the fungal community during the second phase remained the same as during the first phase. However, a week after the increase in *Ostreopsis* abundance, the fungal composition changed such that by the beginning of the third phase the fungal fraction accounted for nearly 25% of the total protist community, with an increase in the number of Chytridiomycota members, specifically *D. arenysensis*. The time lag between the maximum cell abundance of the host and the increase in parasite abundance aligns with typical predator-prey dynamics, where predator (in this case, parasite) and prey (host) alternate in abundance over time. Starting from a growing host population, the increase in this food resource will promote the growth of the parasite population. Parasitic pressure, in turn, can adversely affect host population abundance, leading to a subsequent decline in parasite population. The specific observed time lag can be considered as the period necessary for the parasite to complete its infective life cycle, in a situation of high host abundance (Begon et al., 2005). In the case of chytrids, their complete development, from the onset of infection to the formation of new infective zoospores released into the environment, occurs within 2–3 days (Lepelletier et al., 2014; Reñé et al., 2022). A similar time lag was observed in studies that likewise evaluated the temporal dynamics of chytrids and their microalgal hosts (Picard, 2017; Xue et al., 2018).

The abrupt increase in the relative abundance of Chytridiomycota during the beginning of the third phase coincided with the period of maximum infection prevalence in the samples and the highest abundance of *Ostreopsis* sp. This observation was consistent with the blooming strategy of chytrid species during phytoplankton blooms, which has been described as a ruderal strategy based on chytrid-phytoplankton parasitism during bloom events (Dix, 2012). The subsequent decrease in dinoflagellate abundance occurred in parallel with an increase in other groups, such as Fungi, Apicomplexa, Mesomycetozoa, Sagenista and unassigned taxa. The dominant fungal group in this third phase was Chytridiomycota, although towards the end of this phase its abundance decreased and alternated with a relatively high abundance of Ascomycota. These changes, during the third phase, suggest that the decline of the dominant dinoflagellate *Ostreopsis* sp. allowed the development of other protist groups, thereby changing the composition of the fungal community. This trend reflects changes in community assembly following the proliferation and decline of previously dominant *Ostreopsis* sp.

Dinomyces arenysensis was the dominant species in the fungal community examined in our study. This species is found in benthic marine-coastal habitats and has been described as a generalist, biotrophic parasite with a preference for the dinoflagellate *Ostreopsis* (Lepelletier et al., 2014; Fernández-Valero et al., 2022). However, the prevalence of *D. arenysensis* infections during the period of maximum *Ostreopsis* sp. abundance was relatively low, ranging from 1% to 2.7%, which is comparable to the values reported for other host-parasite systems within plankton. For example, the prevalence of two chytrid species of the genus *Rhizosiphon* on the cyanobacterium *Anabaena* increased from non-detection to 6% (Gerphagnon et al., 2013). In a study examining chytrids associated with freshwater diatoms, the prevalence of infection varied widely among chytrid species, with those exhibiting specialized parasitic behaviour attaining an infection prevalence as high as 70%. By contrast, the infection prevalence of species with facultative parasitic behaviour, and thus able to assimilate food resources other than diatoms, such as pollen, was low, around 2% (Van den Wyngaert et al., 2022). The behaviour of *D. arenysensis* as observed in our study suggested that this species has a facultative parasitic life style. Previous

research on the growth dynamics of *D. arenysensis* has reported its inability to grow on agar media or soil extracts, indicating an obligate parasitic lifestyle (Lepelletier et al., 2014). However, our laboratory experiments demonstrated that this chytrid can successfully use pine pollen, present in abundance in the atmospheric pollen above the Catalan Coast, to complete a viable life cycle. This ability to utilize diverse resources suggest that *D. arenysensis* is a facultative parasite. Moreover, it provides further evidence of intraspecies variability in virulence, as the ability to infect pine pollen was observed in only one of the studied strains. Likewise, sporangia were not observed on pollen when *Ostreopsis* sp. was present, indicating a preference for a parasitic life-style and that pollen serves only as a temporary resource. The ability of chytrids to infect both phytoplankton and pollen is common in freshwater environments (Kagami et al., 2017; Van den Wyngaert et al., 2022). Our results suggest similar interactions in marine habitats, specifically in seasonal epiphytic assemblages, where other resources sustain the parasite until its preferred host again becomes available. However, survival of the parasite in winter, when the substrate of its host (macroalgae) is no longer available, suggests a reservoir within the plankton or benthonic compartment, allowing the recurrence of *Dinomyces* in *Ostreopsis* blooms (Fernández-Valero et al., 2022).

Low infection prevalence of *Dinomyces* in *Ostreopsis* sp. could indicate benign parasitism (as described in Ewald, 1987). This suggests that chytrids and dinoflagellates can coexist without significant harm to the host population, allowing for a recovery from parasite pressure. Previous studies suggested that marine chytrid outbreaks prematurely terminate or suppress phytoplankton blooms (Canter and Lund, 1951; Ibelings et al., 2011).

However, this was not supported by the prevalence of infection observed in the field in our study, as the infections observed in natural samples did not explain the decrease in *Ostreopsis* abundance. Rather, our findings are consistent with the coexistence of chytrids and dinoflagellates. In fact, laboratory studies have shown that parasites and dinoflagellate host populations can coexist if the equilibrium between them is sustained by a minimum host density threshold (Gerla et al., 2013). Coexistence at low infection prevalence was also observed in studies of other protist parasites, such as *Amoebophrya* sp., and can be interpreted as reducing the fitness of the dinoflagellate host without causing significant damage to the host population (Siano et al., 2011).

In contrast to the field studies suggesting coexistence, in the lab experiments the impact of parasitism ranged from harmless to lethal, depending on the host affinity of the different strains of *D. arenysensis*. Thus, the coexistence between chytrids and dinoflagellates observed in the field might reflect differences in the virulence of the different strains of chytrids. The presence in the field of multiple parasite strains with varying degrees of virulence would support parasite-host equilibrium and thus coexistence. Studies of the relationship between chytrids and cyanobacteria have shown that differences in the performance of multiple strains of parasitic chytrids can lead to changes in the infection strategy of these organisms (McKindles et al., 2023). Intraspecific diversity can also promote species coexistence and influence community dynamics, by promoting niche complementarity between genotypes (De Bruin et al., 2008; Agha et al., 2018).

Mucilage secretion, a prominent feature of *Ostreopsis* and another microalgal species, provides structure to the epiphytic assemblage (Reynolds, 2007; Honsell et al., 2013), and serves as a trap for prey acquisition (Tillmann et al., 2023). In a recent study, it has been proposed that the mucilage acts as a protective barrier against chytrid infection in diatoms (Van Den Wyngaert et al., 2023). However, the two chytrid species identified in our study (*D. arenysensis* and *Lobulomycetales* sp.) were capable of infecting the dinoflagellate *Ostreopsis* despite mucilage secretion. The ability to overcome the protective mucilage barrier suggests that these chytrid species have evolved specific mechanisms to infect their host. Understanding these mechanisms

could provide valuable insights into the interactions between fungi and dinoflagellates, and the competitive advantage that means using these resources.

In summary, this study characterized epiphytic protist succession on macroalgae with focus on chytrids and dinoflagellates. Our investigation of the host-parasite dynamics of *Dinomyces arenysensis* showed that the infection peak is influenced by the predominant resource, *Ostreopsis* sp., but also that the parasite is facultative with respect to its host resources. The coexistence of chytrids and dinoflagellates without significant harm to the host organism was supported by field studies. Concurrently, laboratory experiments indicated that this coexistence is partly attributed to the variability in the virulence levels exhibited by different strains of chytrids. Our findings contribute to a better understanding of the dynamics of epiphytic biofilm and host-parasite interactions in less-explored marine benthic habitats.

3.6 References

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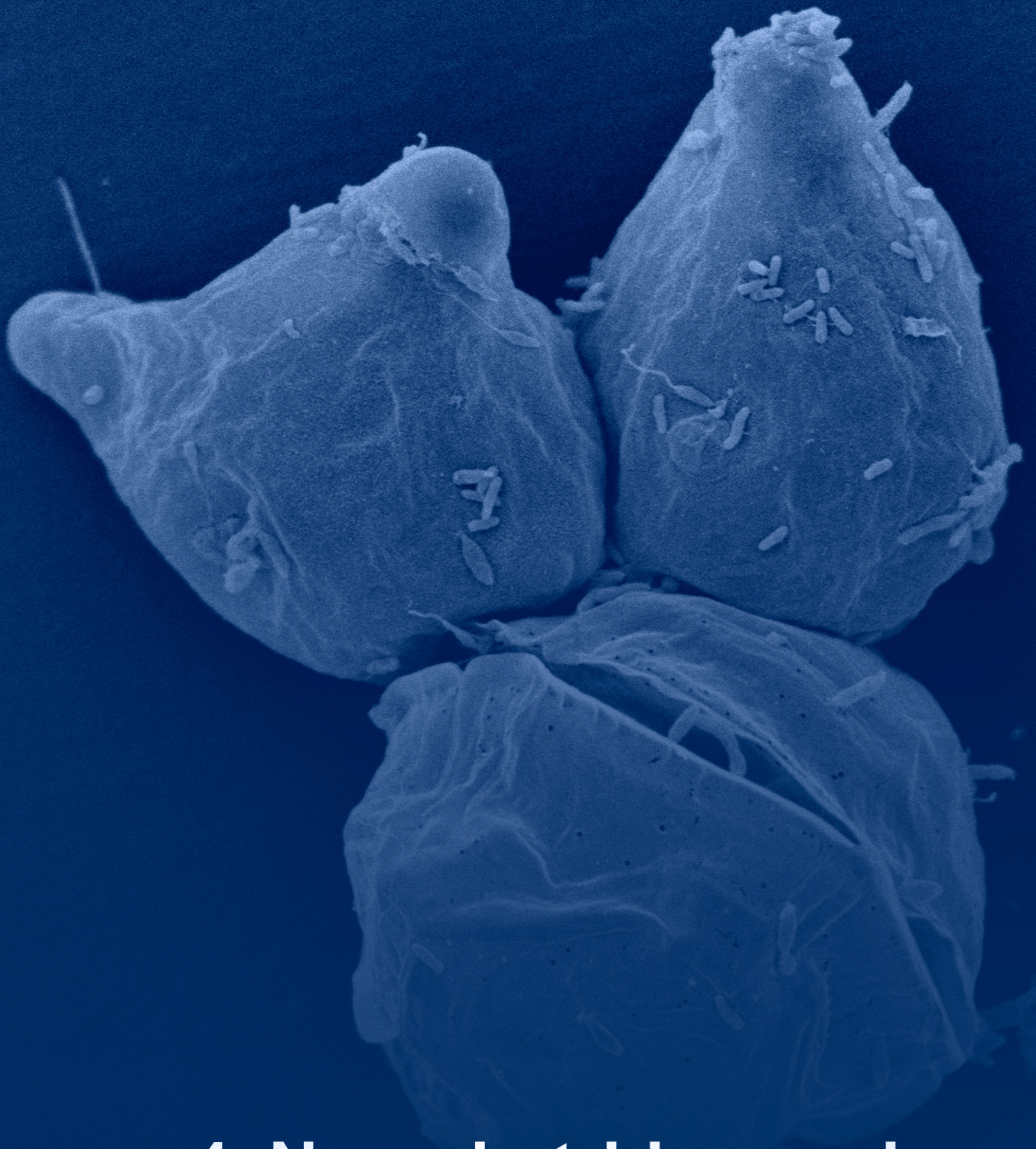
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4. New chytrids species in the coastal area

Fernández-Valero, A. D., Karpov, S. A., Sampedro, N., Gordi, J., Timoneda, N., Garcés, E., & Reñé, A. (2024). Newly identified diversity of Dinomycetaceae (Rhizophydiales, Chytridiomycota), a family of fungal parasites of marine dinoflagellates. *European Journal of Protistology*, 126053.
doi.org/10.1016/j.ejop.2024.126053

4 New chytrids species in the coastal area

4.1 Abstract

We identified two new parasite species of Chytridiomycota isolated during blooms of the dinoflagellate *Alexandrium minutum* in the coastal Mediterranean Sea. Light and electron microscopy together with molecular characterization of the nuclear 18S, ITS, and 28S rDNA regions led to their identification as two new species, *Dinomyces gilberthii* and *Paradinomyces evelyniae*, both belonging to the family Dinomycetaceae, order Rhizophydiales. *Dinomyces gilberthii* differs from the previously described *D. arenysensis* by the presence of discharge papillae and the development of a drop-shaped sporangium. *Paradinomyces evelyniae* differs from the previously described *P. triforamini* by the prominent lipid globule present in early sporangia and the pointed end producing a rhizoid. The two chytrids differed in their geographical distribution. *Dinomyces gilberthii* was detected in several Mediterranean habitats, including harbours and beaches, and was particularly prevalent during summer dinoflagellate blooms. This widespread occurrence in coastal ecosystems suggested a high level of adaptability in this environment. *Paradinomyces evelyniae* had a more restricted distribution in the coastal-marine environment, occurring in harbour sediments and only occasionally in the water column during winter and early spring. *Paradinomyces evelyniae* has previously been detected in the Baltic Sea, suggesting that its distribution encompasses contrasting coastal environments, although its presence is rare.

4.2 Introduction

Recent environmental sequencing studies of marine protist populations have led to a growing interest in fungi present in marine habitats and their ecological characteristics (Debeljak and Baltar, 2023; Hassett et al., 2019; Pernice et al., 2015; Richards et al., 2015; Tisthammer et al., 2016). The basal fungal clade of Chytridiomycota includes saprotrophs and parasites that infect diatoms (Duan et al., 2018), dinoflagellates (Fernández-Valero et al., 2022; Lepelletier et al., 2014; Reñé et al., 2022), and other marine microorganisms (Gerphagnon et al., 2013; Xue et al., 2018). Infections lead to the death of host cells and can cause the host population to decline (Van den Wyngaert et al., 2022), but parasite and host populations can also coexist (Fernández-Valero et al., 2023). Fungal-host interactions thus have the potential to shape the structure and diversity of marine microbial communities.

Parasitic Chytridiomycota, or chytrids, are a group of zoosporic fungi with a characteristic life cycle. Free-living zoospores attach to the host surface and encyst. After germination, they penetrate the host, often forming rhizoids to absorb nutrients. Cyst growth results in the formation of a sporangium, which contains several nuclei. During sporangium maturation, its multinuclear content differentiates into individual cells, which develop a posterior flagellum and become zoospores. The latter are released through the sporangial wall and infect new host cells (Medina and Buchler, 2020; Trinci et al., 1994). The dependence on a host to complete the life cycle of chytrids defines them as parasitoid fungi, i.e., parasites that consume and eventually kill the host in order to grow and reproduce (Eggleton and Gaston, 1990).

Chytrids have a greater presence in the marine environment than initially believed, which has motivated studies aimed at understanding their ecological role. However, while chytrids have been detected in a large number of environmental sequencing studies, the species identify of those sequences is largely unknown (Grossart et al., 2016), as is their ecological function. Identification of these organisms relies on cell isolation and culture efforts, together with microscopic observations. The integration of molecular techniques along with the morphological and ultrastructural characterization allows a correct taxonomic assignment of fungal zoosporic parasites and insights into their specific ecological functions. Indeed, characterizing the diversity of zoosporic fungi in marine environments has been the focus of several studies, covering localities ranging from the Baltic Sea, where *Ericiomyces syringoforeus* Karpov & Reñé (Karpov et al., 2021) and *Paradinomyces triforaminorum* Reñé & Karpov (Reñé et al., 2022) were identified, through the Hebrides Sea, where chytrid strains infecting diatoms have been described (Garvetto et al., 2019), to the Mediterranean Sea, where Fernández-Valero et al. (2022, 2023) examined a broad spectrum of habitats, including seawater, sediment, and epiphytic niches.

Nonetheless, Chytridiomycota are often overlooked in ecological studies, and their role in the marine ecosystem food web, and consequently in nutrient cycling remains poorly understood. In this study, we describe a new diversity in the family Dinomycetaceae Karpov & Guillou (Chytridiomycota), based on the identification of two new species of Chytridiomycota isolated from marine water samples of the coastal Mediterranean Sea. Both parasitize the dinoflagellate *Alexandrium minutum* Halim. These findings have implications for the dynamics of dinoflagellate populations, the main hosts of the new species, in addition to shedding light on chytrid interactions with other organisms in aquatic ecosystems.

4.3 Materials and methods

Sampling, isolation and cultivation of chytrids

On March 10th and April 1st 2021, samples were obtained from two harbours along the Catalan coast (NW Mediterranean Sea), Arenys de Mar (41°34'45.5" N, 2°33'19.8"E) and Vilanova i la Geltrú (41°12'54.9" N, 1°43'57.9"E) (Fernández-Valero et al., 2022, during blooms of the dinoflagellate *A. minutum* (Table 4.1). Sampling was performed when dinoflagellate abundance in the water column exceeded 10⁵ cells L⁻¹. At each location, integrated water column sampling using a tube connected to an electric pump allowed the collection of a 5 L composite sample from a depth range of 2.5-4.0 m, extending from the water surface to the bottom layer. An additional 5 L sample was collected exclusively from the bottom layer. The temperature and salinity of the water samples were measured in situ with a conductivity meter (WTW model Cond 3301), and all laboratory measurements were conducted with the same equipment. Surface sediments were obtained using a sediment corer attached to a pole, with ~30 g of sediment collected in a 50 mL Falcon tube using a scoop.

In the laboratory, the seawater samples were filtered through an 80 µm mesh to remove metazoans and large particles. Subsequently, 1 L of each seawater sample was filtered through a 10 µm mesh to retain dinoflagellate cells as well as potential dinoflagellate infections. The 10 to 80 µm fractions were concentrated to 10 mL and incubated in 6-well plates. For sediment samples, portions were placed in double-bottom tubes separated by an 8 µm mesh, with *A. minutum* added on top to promote the vertical swimming of zoospores through the mesh to separate

the parasite from the sediment. Incubations were performed in culture chambers at a constant temperature of 20 ± 1 °C with a 12:12 h light:dark photoperiod, with illumination of ~ 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ provided by a LED fluorescent tube. The presence of chytrid infections in the incubations was monitored for up to two weeks. Infected dinoflagellate cells were manually isolated to establish clonal chytrid cultures.

Table 4.1: **Origin and characterization of the studied chytrid strains.**

Taxonomic identification	<i>Dinomyces gilberthii</i> sp. nov.	<i>Paradinomyces evelyniae</i> sp. nov.
Culture code	ICMB1106	ICMB1107
Isolation date	1 April	10 March 2021
Location	Vilanova i la Geltrú	Arenys de Mar
Sampling type	Bottom layer	Integrated water column
Host species in field	<i>Alexandrium minutum</i>	<i>Alexandrium minutum</i>
Host abundance (cells L ⁻¹)	1.1×10^5	9.5×10^4
Salinity	35.4	24.4
Temperature (°C)	15.1	16.3
Host in culture	<i>A. minutum</i>	<i>A. minutum</i> , <i>Ostreopsis</i> sp.
Culture code of the host	ICMB292	ICMB292; ICMB293
18S rDNA gene	ON081639; ON081640	ON081641; ON081642
Length (base pairs; bp)	1325; 1337	964; 1117
ITS rDNA gene	OQ865234; OQ865235	OQ865236
Length (bp)	643; 607	526
28S rDNA gene	OQ865232; OQ865233	OQ865231
Length (bp)	900; 892	904

Chytrid cultures were maintained in 24-well plates by transferring a 1 mL aliquot of mature sporangia to a new well containing 1 mL of an exponentially growing, uninfected host stock culture every three or four days. Host cultures were maintained under non-axenic conditions in L1 medium with a salinity of 36. Chytrid and dinoflagellate host cultures were maintained under the conditions described above. The dinoflagellate host strains used in this study were obtained from the culture collection of the Institut de Ciències del Mar (ICM-CSIC). *Alexandrium minutum* strain ICMB292 served as a host in the maintenance of newly isolated strain ICMB1106, but the latter also survived with *Ostreopsis* sp. strain ICMB293 as a host. *Ostreopsis* sp. (ICMB293) mixed in equal proportions with *A. minutum* (ICMB292) was used to maintain the newly isolated strain ICMB1107. *Ostreopsis* sp. was chosen because the time required for its consumption by the parasite was longer than that of other dinoflagellates, due to its larger biovolume, thus prolonging the maintenance period of the cultures in the lab.

Light microscopy

The life cycle of strains ICMB1106 and ICMB1107 was investigated by light microscopy. Regular observations of newly produced infections were conducted to identify the different life-cycle stages and sporangium development. A drop of each chytrid culture was placed on a glass slide and observed with a Nikon Eclipse Ts2R-FL inverted microscope. Photographs were taken with a Nikon DS-10 digital camera, using a PLAN APO 100X magnification objective and differential interference contrast. NIS Elements v5.3 software was used to capture the images. Time-lapse 40× photographs from an aliquot of each chytrid strain placed on glass-bottom plates were taken every 10 min for four days using a Zeiss Axio Observer Z1 microscope, the Axiocam 503 mono camera, and Zen pro 2012 software.

Scanning electron microscopy (SEM)

The structure of the sporangia was examined by SEM. Five mL of ICMB1106 and ICMB1107 cultures containing mature sporangia were fixed in 2% glutaraldehyde for 15 min at room temperature. The ICMB1107 sample was post-fixed with osmium tetroxide with a final concentration of 1% for 15 min at room temperature. The pH was not adjusted. The fixed sporangia were then filtered through a Nucleopore polycarbonate filter (Pleasanton, California, USA) with a diameter of 13 mm and a pore size of 3 μm and washed in distilled water. After dehydration in an ethanol series (25, 50, 75, 90, 96, and 3 $\times$ 100%, 10 min each), the filters were critical-point-dried using a Bal-Tec CPD030 apparatus (Leica Microsystems, Vienna, Austria), mounted in holders, and sputter-coated with gold on a Quorum Q150R S (Quorum Technologies, Ltd, Laughton, UK). They were then examined with a variable pressure scanning electron microscope working at 5 kV (Hitachi S-3500N, Hitachi High Technologies Corp., Japan) at the Servei de Microscòpia Electrònica (ICM-CSIC).

Transmission electron microscopy (TEM)

The ultrastructure of the zoospores was analyzed by TEM. Strains ICMB1106 and ICMB1107 were grown in 6-well plates until a large number of free-living zoospores were observed in the cultures. The supernatant from each well was collected in a 50-mL Falcon tube and centrifuged at 3893 rcf for 6 min at room temperature to concentrate the sample to 2 mL. A threshold concentration of 2.5×10^5 zoospores mL^{-1} was established for initiating block preparation for TEM analysis. The 1.5 mL sample was fixed in 2% glutaraldehyde, postfixed with osmium tetroxide, dehydrated with acetone, and embedded in Spurr resin. Ultrathin serial sections (50-70 nm) obtained using a Leica ultramicrotome UC7 (Leica Microsystems) were stained with 2% uranyl acetate for 10 min and lead-staining solution for 5 min before they were observed with a JEOL JEM-1010 transmission electron microscope fitted with a Gatan Orius SC1000 (model 832) digital camera at the TEM-SEM Electron Microscopy Unit, Scientific and Technological Centers of the University of Barcelona (CCiTUB).

Molecular procedures and phylogenetic analyses

Individual mature sporangia were isolated from the cultures using glass micropipettes and the single-cell PCR protocol was implemented as described in Reñé and Hoppenrath (2019) and Reñé et al. (2021). A first PCR was performed using the primers EK82F and 28S-1611R (López-García et al., 2001; Moreira et al., 2007) to amplify the entire ribosomal operon followed by (semi-) nested PCR. The region corresponding to the 18S rDNA was amplified using the specific primers CRYPTO22F and EUK1520R (Lazarus and James, 2015; López-García et al., 2003) as reported in Fernández-Valero et al. (2022). The ITS rDNA region was amplified using ITS1 and ITS4 primers (White et al., 1990), and the 28S rDNA region using primers D1R and D3B (Nunn et al., 1996; Scholin and Anderson, 1994). Both ITS and 28S rDNA were amplified in 25 μL reactions containing 1 μL of the first PCR product as template, 2.5 μL of 10 $\times$ buffer, 1.5 mM MgCl_2 , 0.2 mM of each dNTP, 0.4 μM of forward and reverse primers, and 2 U of Taq DNA polymerase. The PCR program was as follows: initial denaturation at 94 $^{\circ}\text{C}$ for 2 min, 35 cycles including denaturation at 94 $^{\circ}\text{C}$ for 15 s, polymerization at 55 $^{\circ}\text{C}$ for 30 s, and elongation at 72 $^{\circ}\text{C}$ for 60 s, followed by a final elongation at 72 $^{\circ}\text{C}$ for 60 s (Reñé et al., 2021). Sanger sequencing with the aforementioned forward and reverse primers was performed by an external service (Genoscreen, France). The molecular sequences obtained were deposited in GenBank with accession numbers OQ865231–OQ865233 (28S rDNA) and OQ865234–OQ865236 (ITS rDNA).

A concatenated 18S rDNA + ITS + partial 28S rDNA dataset covering the diversity of Rhizophydiales Letcher was constructed following that used in Reñé et al. (2022). It was aligned using MAFFT v7 (Kato and Standley, 2013) and the resulting alignment was manually trimmed to remove poorly aligned positions. The final alignment comprised 52 sequences and 4113 positions. The best-fit substitution model was determined using jModeltest v2.1 (Darriba et al., 2012), resulting in GTR+I+GAMMA under both the Akaike information criterion and the Bayesian information criterion. A maximum likelihood (ML) phylogenetic tree was constructed with RAxML v8.2.12 (Stamatakis, 2014), inferring the best tree from 1000 different starting trees. Bootstrap statistical support was evaluated using 1000 pseudoreplicates. The ML analyses were run at the Marine Bioinformatics Service (MARBITS) of the Institut de Ciències del Mar (ICM-CSIC). A Bayesian reconstruction was conducted using MrBayes v3.2.1 (Ronquist et al., 2012), based on the GTR+I+GAMMA model, four MCMC chains, and 1,000,000 generations. Convergence of the MCMC analyses was confirmed in that the average standard deviation of split frequencies was below 0.01, the potential scale reduction factor was close to 1, effective sample sizes were >200, and no trends were observed in the plots of generations versus log probability. The first 10% of trees were then considered as burned-in and the Bayesian posterior probabilities (BPP) were determined for the majority rule consensus tree.

Exploration of metabarcoding datasets

The presence and global distribution of the two species were explored in public V4 18S rDNA metabarcoding datasets, specifically, metaPR2 v2.0.1 (Vaulot et al., 2022), a compilation of 59 published metabarcoding datasets from different surveys that employed environmental metabarcoding targeting the V4 region of the 18S rRNA gene. The search was performed using the 18S rDNA sequences obtained from cultures of ICMB1106 (ON081639) and ICMB1107 (ON081641). These two sequences were also queried for sequence similarity using NCBI-BLASTN against different metabarcoding datasets available from the Catalan coast. Spatial distributions were assessed using the COPAS BioProject PRJNA630546 (Fernández-Valero et al., 2022; Reñé et al., 2021) and temporal distributions using SMART Bioprojects PRJNA1023727 and PRJNA891539. A detection was considered positive when the query coverage was >80% and the sequence identity >99%.

4.4 Results

Chytrids infecting the dinoflagellate *Alexandrium minutum* were detected in samples from the harbours of Vilanova i la Geltrú and Arenys de Mar (Catalan Coast, NW Mediterranean Sea) obtained during blooms of that species (Table 4.1). In both cases, samples were collected during spring, when the water temperature was 15-16 °C and host abundance was $\sim 10^{15}$ cells L⁻¹. The salinity values differed significantly between the two sites, i.e., 35.4 and 24.4, respectively.

Phylogeny

Molecular analyses were conducted to ascertain the phylogenetic relationships of the two chytrid strains (Fig. 4.1). Both were determined to belong to the family Dinomycetaceae (order Rhizophydiales). This cluster (99% bootstrap /1.00 BPP) comprised two sister clades, one including *Dinomyces arenysensis* Karpov & Guillou, strain ICMB1106, and *Dinomyces* sp. P242 - RCC3408, and the other including *Paradinomyces triforaminorum* and strain ICMB1107,

with maximum statistical support for both. The 28S rDNA (OQ865232OQ865233) and ITS (OQ865234–OQ865235) sequences of ICMB1106 showed 100% identity with *Dinomyces* sp. P242 - RCC3408 (KJ027549 and KJ027550) sequences, respectively (Table 9.2: Suppl. Table 1). The 18S rDNA sequence of *Dinomyces* sp. P242 - RCC3408 was not available. The sequence identity between ICMB1106 and *D. arenysensis* was 98.2% for 18S rDNA, 77.3% for ITS, and 91.6% for 28S rDNA. The sequence identity of ICMB1107 and *P. triforaminorum* was 98.6%, 83.8%, and 94.5%, respectively. These results justified the designation of strains ICMB1106 and ICMB1107 as representing two new species belonging to the family Dinomycetaceae, namely, *Dinomyces gilberthii* sp. nov. and *Paradinomyces evelyniae* sp. nov., respectively.

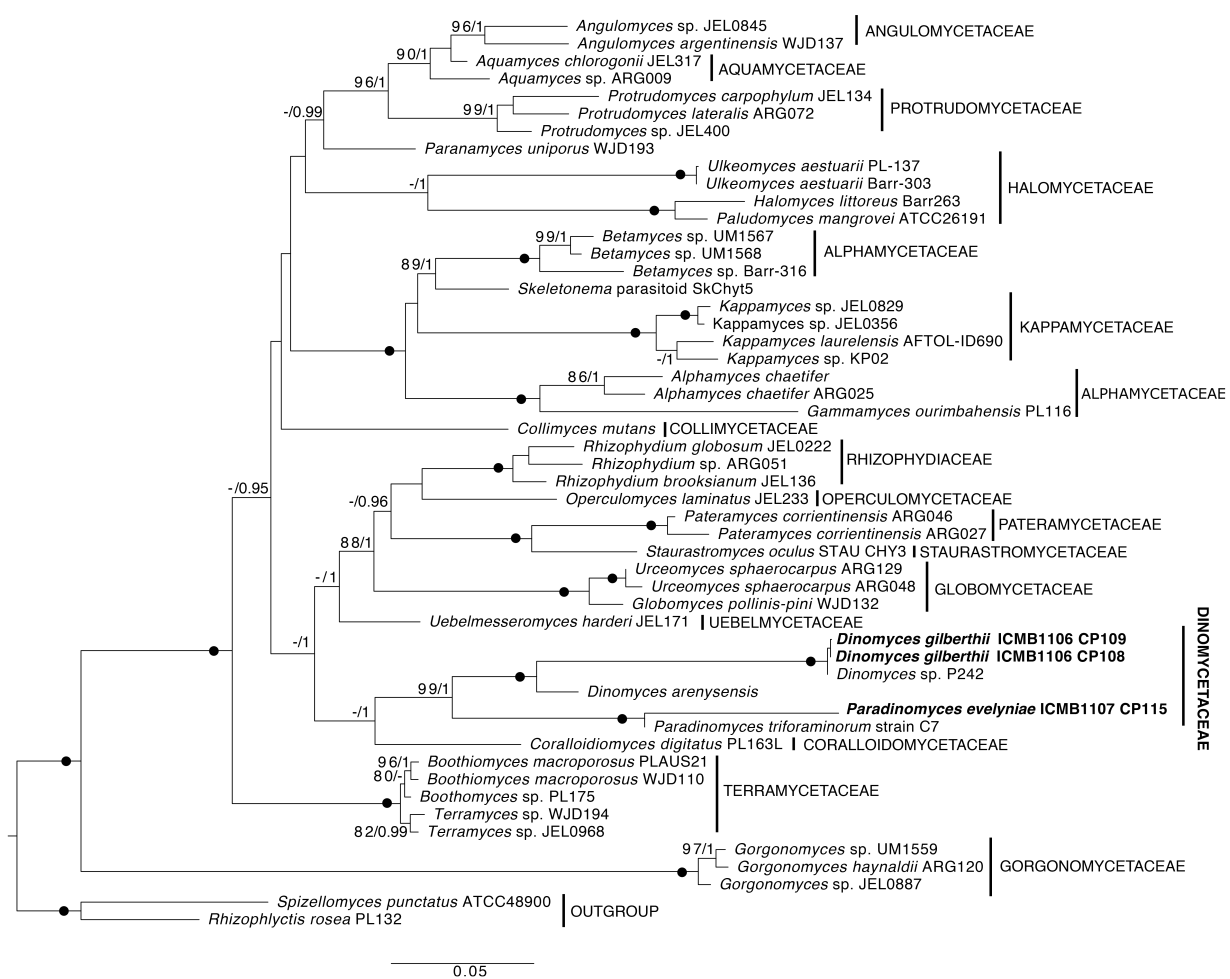


Figure 4.1: **Phylogenetic tree of Rhizophydiales based on concatenated 18S + ITS + 28S rDNA sequences.** Sequences in bold were obtained in this study. *Spizellomyces punctatus* (Spizellomycetales) and *Rhizophlyctis rosea* (Rhizophlyctidales) sequences were used as an outgroup. The numbers above branches indicate the statistical support (bootstrap/Bayesian posterior probability); only values >80% and 0.95, respectively, are shown.

Taxonomy

***Dinomyces gilberthii* sp. nov.** Karpov, Fernández-Valero & Reñé

Order Rhizophydiales Letcher, 2006

Family Dinomycetaceae Karpov & Guillou, 2014

Genus *Dinomyces* Karpov & Guillou, 2014

Index Fungorum: IF 900632.

GenBank accession numbers: ON081639-ON081640 (18S rDNA), OQ865234-OQ865235 (ITS), and OQ865232-OQ865233 (28S rDNA).

Diagnosis: Parasitoid of dinoflagellates with a likely host preference for *Alexandrium minutum*. Drop-shaped epibiotic sporangium 15-25 µm in length on the host *Alexandrium minutum*, with one or two papillae and connected to the host through a rhizoid with an apophysis. Spherical zoospores 2.0-2.6 µm in diameter, with a posterior lipid globule and a flagellum 8-11 µm long. Infects the dinoflagellates *Alexandrium minutum* and *Ostreopsis* sp.

Etymology: The species is named in honour of Gilberth Fernández, the father of the first author of this study.

Type: Figure 4.2B.

Type strain: ICMB1106, isolated infecting the dinoflagellate *Alexandrium minutum* in Vilanova i la Geltrú harbour (41°12'54.9" N, 1°43'57.9" E), Catalonia (NW Mediterranean Sea), in April 2021.

Hapantotype: Fixed specimen of zoospores and sporangia derived from strain ICMB1106 embedded in a resin block and a stub for electron microscopy, deposited in the Marine Biological Reference Collections (CBMR) at the Institut de Ciències del Mar (ICM-CSIC, Barcelona, Catalonia, Spain) under the accession number ICMCBMR000651 (Guerrero et al., 2023).

Habitat: Marine coastal waters.

Morphological and ultrastructural description of *Dinomyces gilberthii* life-cycle stages

Zoospores of this chytrid species are spherical, with a diameter of 2.0-2.6 µm. They have a posterior flagellum 8-11 µm long and a posterior lipid globule (Fig. 4.2I). Once attached to the host, the zoospore encysts and a rhizoid with an apophysis develops inside the host cell (Fig. 4.2B, D, F). The rhizoid branches feed the chytrid and allow the growth of the sporangium (Fig. 2A, B, C, D), which develops one or two papillae (Fig. 4.2J). Multiple infections are common for this strain, as several zoospores were seen to settle on the host, encyst, and germinate within the dinoflagellate (Fig. 4.2A, B, G). Furthermore, multiple infections may not occur simultaneously (Fig. 4.2E, G). The number of infections in a single host cell determines the size of the sporangium and thus the number of zoospores produced per sporangium. If a host cell is infected by a single zoospore, the final biovolume of the sporangium will be similar to that of the host cell (Fig. 4.2D, F). However, if there are multiple infections of the same cell, the biovolume of each sporangium will be lower, and so will the number of zoospores produced per sporangium (Fig. 4.2 E, G). Mature sporangia are drop-shaped, with a length of 15-25 µm when a single infection of an *A. minutum* cell occurs, and typically develop one to two, rarely three, discharge papillae. After reaching its maximum size, sporangia show a gradual compartmentalization of the lipid globule (Fig. 2E, F, G), zoospores begin to individualize, and they are eventually released through the papillae once mature (Fig. 4.2H). The presence of sporangia with two discharge papillae is common, observed in ~29% of sporangia (Fig. 4.2J), while the development of three discharge papillae is rare (<0.1%, not shown). The complete development of the parasitoid, from the cyst to the mature sporangium and zoospore release, takes ~25 h (Figure 9.2: Suppl Video 1).

The ultrastructural characters of zoospores are key to the determination of their taxonomic affiliation. The general ultrastructural organization of *Dinomyces gilberthii* zoospores is shown

in Figure 4.3A-D. The nucleus lies near the center of the cell, slightly shifted towards one side (Fig. 4.3B-D). The ribosomal core occupies the central part of the zoospores, separating the nucleus from the mitochondrion and the microbody-lipid globule complex. A large lipid globule is present, showing a prominent fenestrated cisterna (rumposome) facing the cell surface and covered with a flat microbody (Fig. 4.3A, B). One mitochondrial profile with flat cristae is attached to the ribosomal core outlined by the endoplasmic reticulum (Fig. 4.3A-C). Small dense bodies are scattered throughout the dense cytoplasm (Fig. 4.3A-C) and a vesiculated zone in the cytoplasm is present in the ribosomal core cavity near the kinetid (Fig. 4.3B, D). At the cell posterior, a paracrystalline inclusion was observed (Fig. 4.3D). The zoospores also contain a rather prominent vacuole with amorphous contents (Fig. 4.3A). The kinetid is composed of a flagellar kinetosome and a short non-flagellar kinetosome (here called a centriole) together forming a 90° angle and connected by a short fibrillary bridge (Fig. 4.3A). The kinetosome has an anterior root of five microtubules, which is typical for Rhizophydiales (Fig. 4.3B).

***Paradinomyces evelyniae* sp. nov. Karpov, Fernández-Valero & Reñé**

Order Rhizophydiales Letcher, 2006

Family Dinomycetaceae Karpov & Guillou, 2014

Genus *Paradinomyces* Reñé & Karpov, 2022

Index Fungorum: IF 900676.

GenBank accession numbers: ON081641–ON081642 (18S rDNA), OQ865236 (ITS), and OQ865231 (28S rDNA).

Diagnosis: Parasitoid of dinoflagellates, with a likely host preference for *Alexandrium minutum* and *Ostreopsis* sp. Pear-shaped epibiotic mature sporangium 30–35 µm in length when infecting an *Alexandrium minutum* cell, with a pointed end producing a rhizoid, without papillae. Spherical zoospores 2.1–2.8 µm in diameter, with a single posterior lipid globule and a flagellum 11–13 µm long. Zoospores released from the sporangium through 1–3 discharge pores.

Etymology: The species is named in honour of Evelyn Valero, the mother of the first author of this study.

Hapantotype: Fixed specimen of zoospores and sporangia derived from strain ICMB1107 embedded in a resin block and a stub for electron microscopy, deposited in the Marine Biological Reference Collections (CBMR) at the Institut de Ciències del Mar (ICM-CSIC, Barcelona, Catalonia, Spain) under the accession number ICMCBMR000652 (Guerrero et al., 2023).

Type: Figure 4.4D.

Type strain: ICMB1107 isolated infecting the dinoflagellate *Alexandrium minutum* in Arenys de Mar harbour (41°34'45.5"N, 2°33'19.8"E), Catalonia (NW Mediterranean Sea), in March 2021.

Habitat: Marine coastal waters.

Morphological and ultrastructural description of *Paradinomyces evelyniae* life-cycle stages

Free-living zoospores of this chytrid species are spherical, with a diameter of 2.1–2.8 µm. They have an 11 to 13 µm-long posterior flagellum and a posterior lipid globule (Fig. 4.4I). Once the zoospore attaches to a host cell, it encysts. The early trophic phase begins when the cyst produces a rhizoid, which penetrates the host cell. Young sporangia are spherical to pear-shaped, with a prominent central lipid globule (Fig. 4.4B–E). Mature sporangia are pear-shaped, 30–35 µm in length when derived from a single infection of an *A. minutum* cell, and with a pointed end producing the rhizoid (Fig. 4.4H). Subsequent division of the plasmodium results in the formation of numerous small zoospores (Fig. 4.4H, I; (Figure 9.3: Suppl Video 2). The sporangia do not have visible structures related to zoospore discharge. The release occurs through discharge pores instead (Fig. 4.4J, L, M). The number of infections in the same host cell and the host

species biovolume determine the final size of the sporangium (Fig. 4.4F, H) and the number of zoospores. As infections do not occur synchronously, different stages of infection were observed among sporangia within the same host. The development of the parasitoid, from the cyst to the mature sporangium and release of the zoospores, takes approximately 30 h (Figure 9.3: Suppl Video 2).

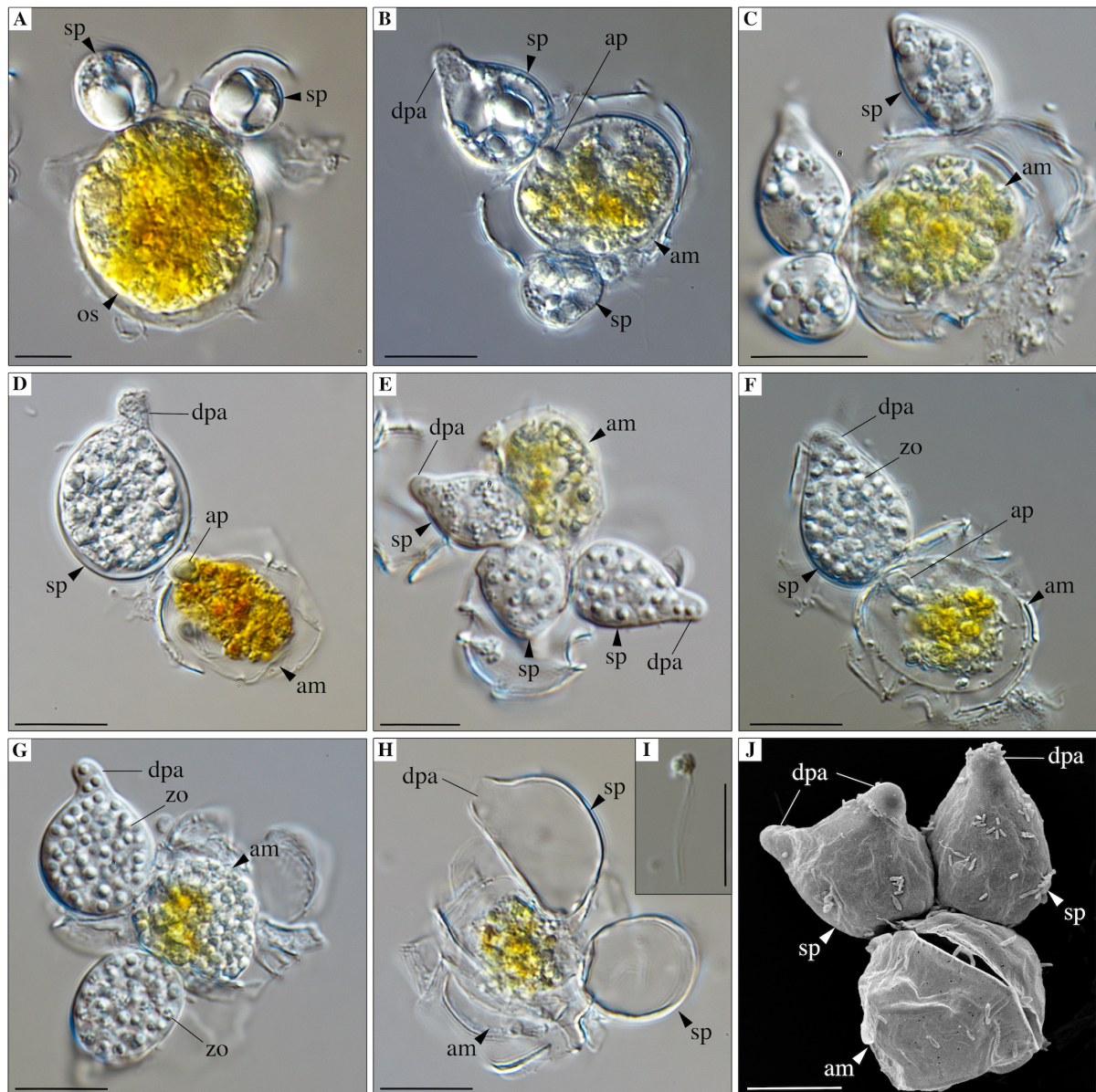


Figure 4.2: **Life-cycle stages of *Dinomyces gilberthii* sp. nov. in the light microscope (A-I) and in the SEM (J).** (A) *A. minutum* infected by two young sporangia. (B, C) Young sporangium with a developed discharge papilla. (D) Sporangium before zoospore formation. (E) Sporangia showing the lipid globule compartmentalization during zoospore formation. (F, G) Mature sporangium with a discharge papilla. (H) Empty sporangium with a discharge papilla. (I) Zoospore. (J) Two mature sporangia with one and two discharge papillae. Abbreviations: am, *A. minutum*; ap, apophysis; dpa, discharge papilla; os, *Ostreopsis* sp.; sp, sporangium; zo, zoospores. Scale bars: 10 μ m.

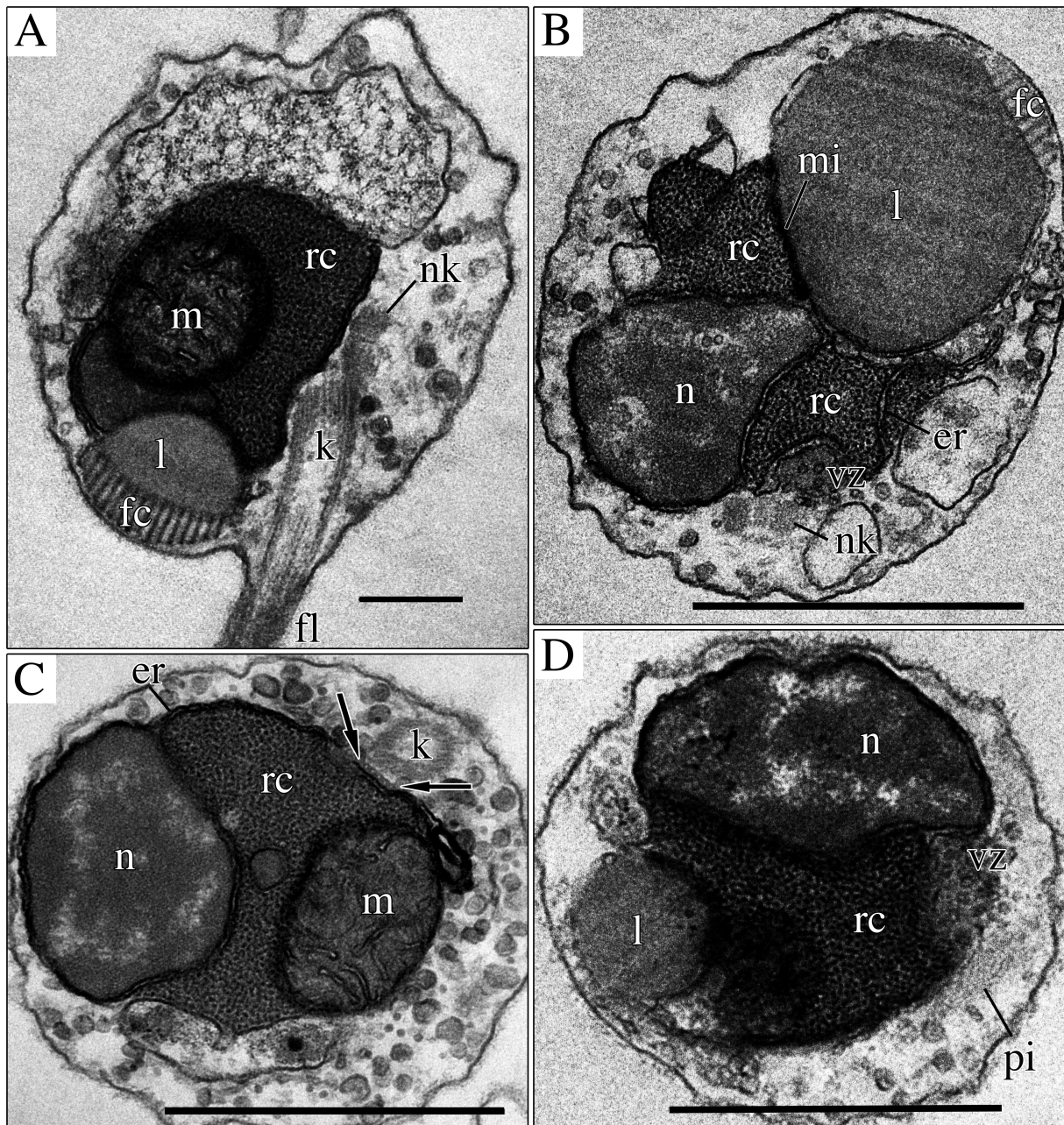


Figure 4.3: **Ultrastructure of *Dinomyces gilberthii* sp. nov. zoospores.** (A) Longitudinal section showing the posterior flagellum. (B-D) General organelle disposition and cytoskeletal structures in different planes: cross-section of the flagellar kinetosome and five-microtubular root (D, **arrows**), longitudinal section of non-flagellar kinetosome (C), paracrystalline inclusion (D). Abbreviations: er, endoplasmic reticulum; fc, fenestrated cistern (botrosome); fl, flagellum; k, flagellar kinetosome; l, lipid globule; m, mitochondrion; mi, microbody; n, nucleus; nk, centriole (non flagellar kinetosome); pi, paracrystalline inclusion; rc, ribosomal core; vz, vesiculated zone. Scale bars: 400 nm (A) and 1 µm (B-D).

The general ultrastructural organization of *P. evelyniae* zoospores is shown in Figure 4.5. The nucleus lies near the center of the cell, slightly shifted towards one side (Fig. 4.5A). The ribosomal core occupies the central part of the zoospore, separating the nucleus from the mitochondria and the microbody-lipid globule complex. A large lipid globule is covered with a flat microbody and has no fenestrated cisterna (Fig. 4.5B). Two mitochondrial profiles with flat cristae are attached to the ribosomal core outlined by the endoplasmic reticulum (Fig. 4.5A, B, E). Small dense bodies are scattered throughout the dense cytoplasm, with a vesiculated zone seen in the ribosomal core cavity near the kinetid (Fig. 4.5A, C). A paracrystalline inclusion lies in the cytoplasm near the nucleus (Fig. 4.5A, D). The kinetid is composed of the flagellar kinetosome (Fig. 4.5B, C) and a short non-flagellar kinetosome (not shown).

Geographical and temporal distribution of the novel fungal species

The Chytridiomycota species described in this study were detected in available metabarcoding datasets representing various locations along the Catalan coast (Table 4.2; Reñé et al., 2021). Examination of the spatial distribution of chytrids along the Catalan coast revealed the presence of *Dinomyces gilberthii* in other harbours (Cambrils, El Masnou and Barcelona) besides Vilanova i la Geltrú (from where it was isolated) during blooms of the dinoflagellate *A. minutum*. It was detected at the beach of Pals during a bloom of the dinoflagellate *Gymnodinium litoralis* Reñé and at the beach at the mouth of the La Muga river, concurrent with a bloom of mixed dinoflagellates dominated by *Prorocentrum micans* Ehrenberg (Reñé et al., 2021) as well. In a study examining the temporal patterns of parasites (unpublished) during coastal dinoflagellate blooms, *D. gilberthii* was detected at El Grau beach during the summer months (late June, July, August) and at L'Estartit beach (late June, July). The dinoflagellate communities in those samples were dominated by *G. litoralis*, but other dinoflagellate species, such as *A. taylori* Balech and *Levanderina fissa* (Levander) Moestrup, Hakanen, Gert Hansen, Daugbjerg & M.Ellegaard, were present as well. In a monthly sampling at Port Olímpic, *D. gilberthii* was detected in the water column during January and December 2022, when the dinoflagellate community was dominated by *A. minutum* and *Prorocentrum triestinum* J.Schiller, respectively. *Dinomyces gilberthii* was also found in sediment samples from January, April, and July 2021. The dominant dinoflagellate in parallel water column samples was *Biecheleria* spp. Seawater temperature and salinity ranges during the detections were 11.8-27.1 °C and 28.6-38.2, respectively (Table 4.2).

Paradinomyces evelyniae, isolated during an *A. minutum* bloom at Arenys de Mar, was also detected in the water column from El Masnou harbour during *A. minutum* blooms and in sediments from Cambrils harbour. During monthly sampling at Port Olímpic, *P. evelyniae* was detected in seawater during December 2021 and January and December 2022, when the dominant dinoflagellate in the water column was *A. minutum*. The chytrid was also identified in sediment samples obtained in January, February, and May 2022, but was not detected again until December 2022, when *Biecheleria* sp. dominated the water column though other dinoflagellates were present as well. The seawater temperature and salinity ranges of the sampling sites were 11.8-21.9 °C and 36.4-38.0, respectively. *Paradinomyces evelyniae* was also occasionally found in samples from El Grau beach.

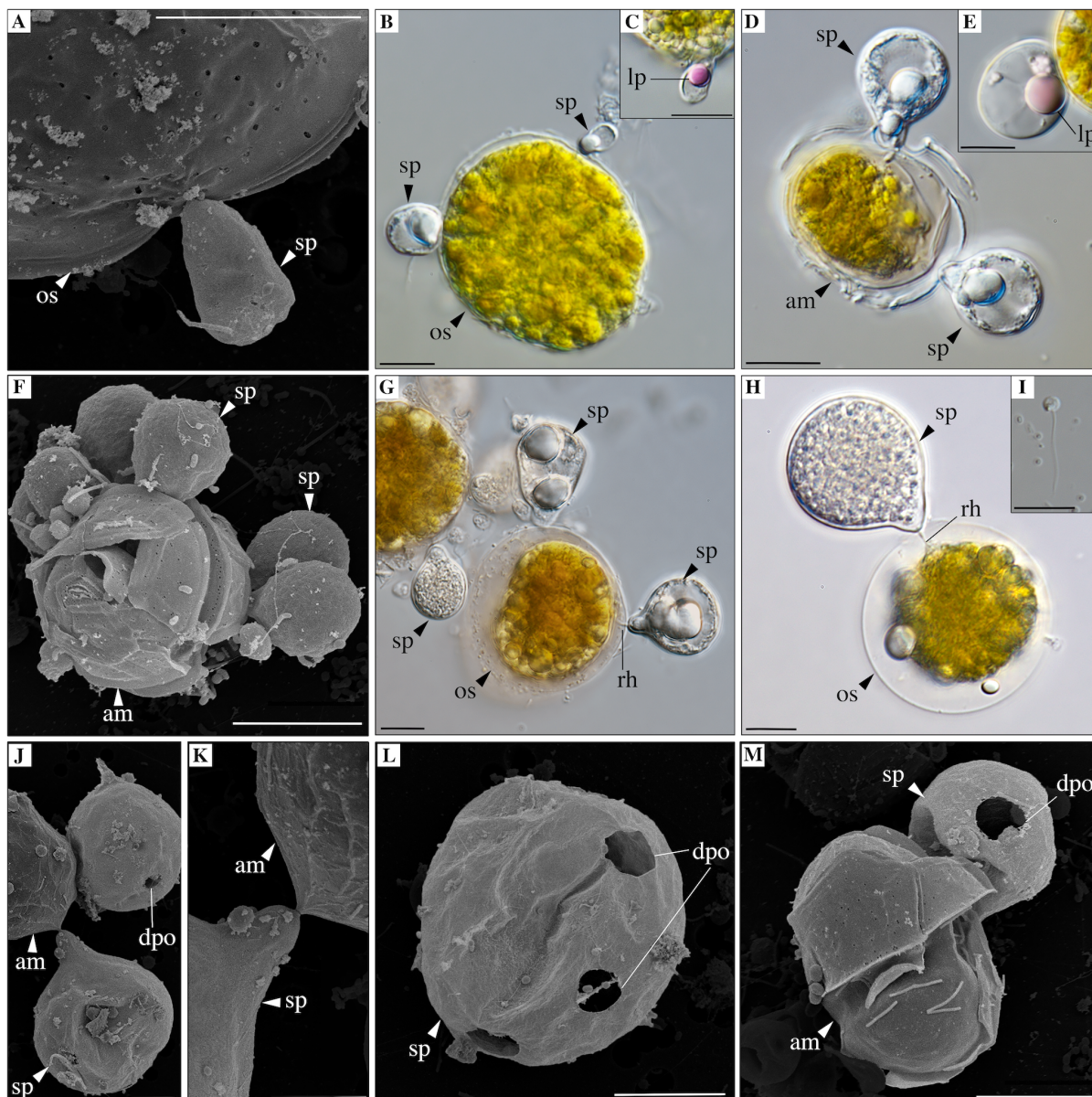


Figure 4.4: **Life-cycle stages of *Paradinomyces evelyniae* sp. nov. in the light microscope (B, C, D, E, G, H, I) and in the SEM (A, F, J, K, L, M).** (A) Early sporangium. (B) Two young sporangia infecting an *Ostreopsis* sp. cell. (C) Nile Red staining of a lipid globule from a young sporangium. (D) Development of the infection of two sporangia in a cell of *A. minutum*. (E) Growth of the lipid droplet in a Nile Red-stained sporangium. (F) Multiple infections in a cell of *A. minutum*. (G) Division of the lipid droplet and growth of the sporangium in a cell of *Ostreopsis* sp. (H) Mature sporangium containing zoospores. (I) Zoospore. (J, K) Base of the sporangium connected to a host cell. (L) Three open discharge pores of an empty sporangium. (M) Empty sporangium with a discharge pore. Abbreviations: am, *A. minutum*; dpo, discharge pore; lp, lipid globule; rh, rhizoid; sp, sporangium; os, *Ostreopsis* sp. Scales bars: 10 µm.

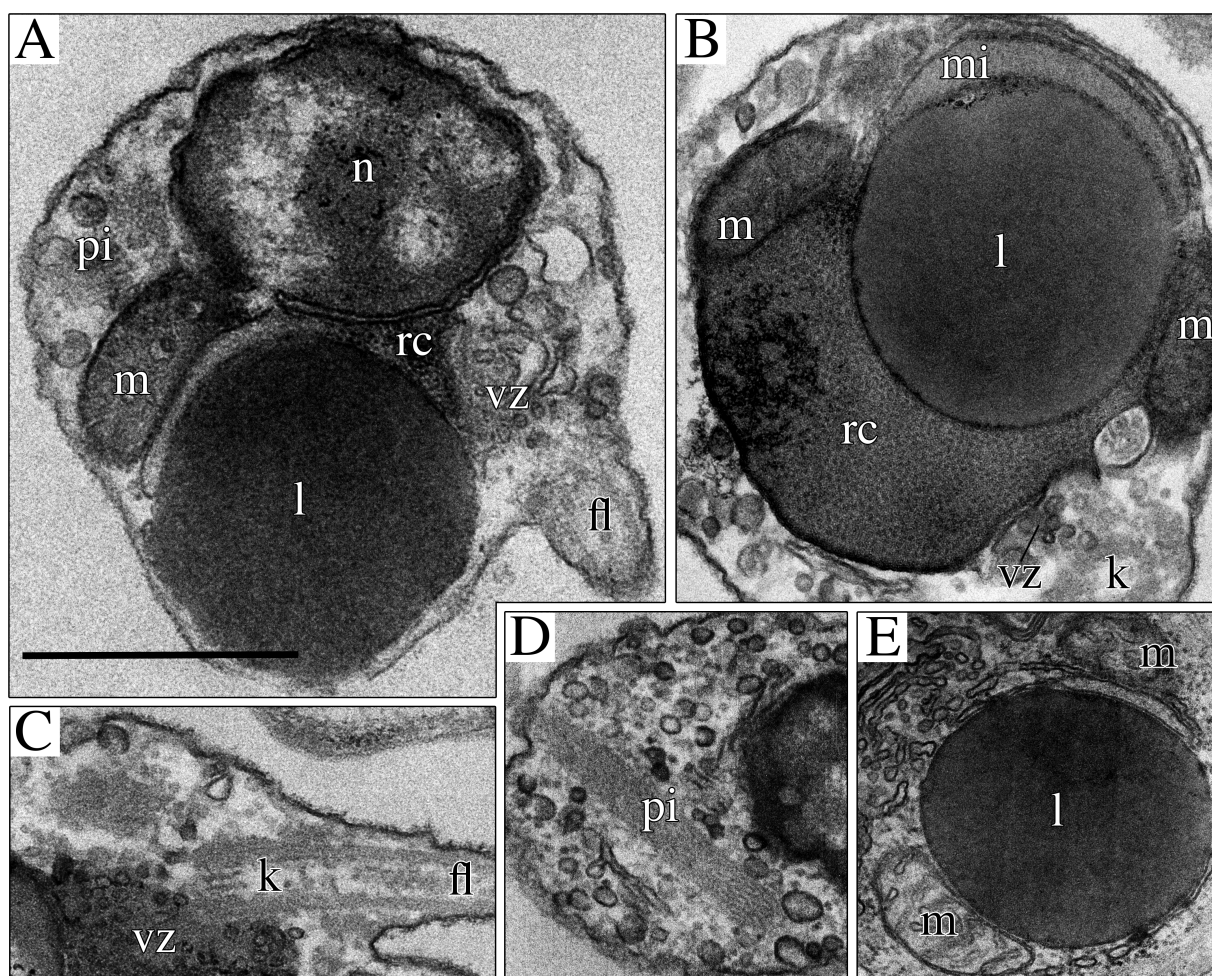


Figure 4.5: **Ultrastructure of *Paradinomyces evelyniae* sp. nov. zoospores.** (A, B, E) Nucleus and general organelle disposition. (C) Longitudinal section of the kinetosome and flagellum. (D) Longitudinal section of paracrystalline inclusion. Abbreviations: cy, cyst; fl, flagellum; k, flagellar kinetosome; l, lipid globule; m, mitochondrion; mi, microbody; n, nucleus; pi, paracrystalline inclusion; rc, ribosomal core; vz, vesiculated zone. Scale bars: 500 nm.

Consultation of the metaPR2 database for records of the two species revealed only the presence of *P. evelyniae* in the Baltic Sea. In that case, sampling had been conducted at the Tvärminne zoological station (entrance to the Gulf of Finland, Storfjärden) and in the coastal region of Krogarviken. The species was found in both the surface and the euphotic layer (down to ~15 m in depth) at those locations, as well as in the bottom water of Storfjärden, at depths ranging from 15 to 30 m. The temperature and salinity ranges at the sampling sites were 7.3–11.5 °C and 5.4–6.2, respectively (Table 4.2).

4.5 Discussion

Identity and characteristics of the newly described species

The present phylogenetic tree based on 18S, ITS, and 28S rDNA sequences showed two main clades within Dinomycetaceae. One included *Dinomyces arenysensis* and the new species *D. gilberthii*,

while the other comprised *Paradinomyces triforminorum* and the new species *P. evelyniae*. The identity between sequences belonging to the same clade was >98% for 18S rDNA, >70% for ITS, and >90% for 28S rDNA, while the identity between sequences belonging to different clades was much lower. As mentioned above, *D. gilberthii* corresponds to the species previously recorded as *Dinomyces* sp. P242 - RCC3408 (Lepelletier et al., 2014). Morphological or ultrastructural information was not provided in that report, and the species was tentatively classified as a *Dinomyces* representative based on the low divergence between its ITS and 28S rDNA sequences and those of *D. arenysensis*. Our results are in agreement with that interpretation.

The morphological, ultrastructural, and molecular results obtained in this study justify the description of the species included in each clade as belonging to the genera *Dinomyces* and *Paradinomyces*. A morphological distinction between them is in that the sporangia of *D. gilberthii* and *D. arenysensis* have an apophysis, whereas those of *P. evelyniae* and *P. triforminorum* have branched rhizoids. Ultrastructural differences were also observed between the respective zoospores. Zoospores of *D. gilberthii* have fenestrated cisternae and kinetid characteristics similar to those of *D. arenysensis*, whereas the zoospores of *Paradinomyces triforminorum* and *P. evelyniae* lack fenestrated cisternae. Although the kinetid was not studied in detail, that of *D. gilberthii* has the same anterior five-microtubular root seen in kinetids of other Dinomycetaceae, and an inclined kinetosome orientation was found in both *D. gilberthii* and *P. evelyniae*. Zoospores of the two strains contain a paracrystalline inclusion, as also found in *P. triforminorum* (Reñé et al., 2022). A paracrystalline inclusion was not detected in zoospores of *D. arenysensis* (Lepelletier et al., 2014), but the reinvestigation of its original TEM images revealed a bundle of microtubules at the zoospore's anterior side, as visible in Figure 4.4D of Lepelletier et al. (2014). The paracrystalline inclusion consists of a pool of tubulins that may also assume a microtubular appearance, as observed in zoospores of *P. evelyniae* (Fig. 4.5). Given that zoospores of all described members of Dinomycetaceae contain a paracrystalline inclusion, it can be regarded as a character of the whole family.

Regarding interspecies differences, the two strains differ genetically and morphologically from the previously described species of *Dinomyces* and *Paradinomyces*. In the case of *Dinomyces*, the sporangia of *D. gilberthii* exhibit a drop-shaped growth derived from the development of discharge papillae for zoospore release. This structure is lacking in *D. arenysensis*, whose sporangia are more spherical. Furthermore, zoospores of these two species differ in their dimensions. In the case of *Paradinomyces*, early sporangium development in *P. evelyniae* is marked by a prominent lipid globule. In addition, the sporangia of *P. triforminorum* have three openings for zoospore release, whereas *P. evelyniae* has a variable number (1-3) of discharge pores. However, zoospores of the two *Paradinomyces* species are of the same dimensions and have the same general ultrastructure.

Table 4.2: Origin of amplicon sequence variants (ASVs) detected by metabarcoding and identified as *D. gilberthii* sp. nov. & *P. evelyniae* sp. nov., and characteristics of corresponding samples.

Species	Type	Location	Sampling date	Compartment	Dominant dinoflagellate	Temp. (°C)	Salinity	ASV match and references
<i>Dinomyces gilberthii</i>	Harbour	Cambrils ¹	15 May 2019	seawater, sediment	<i>Alexandrium minutum</i>	18.7	36.9	ASV84;
		Barcelona ¹	28 Mar 2019	seawater, sediment		15.3	38.2	Reñé et al. (2021)
		El Masnou ¹	2 Mar 2018	seawater		11.8	37.7	
			7 Mar 2018	seawater, sediment		13.2	38.0	
	Beach	La Muga ¹	5 Jun 2019	seawater	mixed dinoflagellates dominated by <i>Prorocentrum micans</i>	26.4	37.4	
		Pals ¹	4 Jul 2019	seawater, sediment	<i>Gymnodinium litoralis</i>	21.5	35.7	
		Port Olímpic ¹	11 Jan 2022	seawater	<i>A. minutum</i>	12.9	36.6	ASV4101;
	Harbour		11 Jan 2022	sediment	<i>Biecheleira</i> spp.	12.9	36.6	Unpublished results
			26 Jan 2022	sediment		12.7	37.0	
			5 Apr 2022	sediment	<i>Biecheleira</i> sp., <i>Alexandrium ostenfeldii</i>	13.2	36.4	
			29 Jul 2022	sediment	<i>Pyrophacus steinii</i> , <i>Biecheleira</i> sp.	27.1	37.6	
			15 Dec 2022	seawater	<i>Prorocentrum triestinum</i>	14.7	36.4	
			21 Jun 2022	seawater	<i>G. litoralis</i>	21.7	37.0	
	Beach	L'Estartit ¹	29 Jun 2022	seawater	<i>G. litoralis</i> , <i>Alexandrium taylori</i> , <i>Levanderina fissa</i>	24.4	37.2	
			8 Jul 2022	seawater	<i>Heterocapsa</i> spp.	22.8	37.3	
			21 Jun 2022	seawater	<i>G. litoralis</i>	22.9	32.9	
			08 Jul 2022	seawater		23.0	35.9	
			18 Jul 2022	sediment	<i>A. taylori</i>	26.9	30.3	
			27 Jul 2022	seawater	<i>G. litoralis</i>	26.3	28.6	
		El Grau ¹	19 Aug 2022	seawater	<i>Heterocapsa</i> sp.	24.6	37.5	

Continue to the next page.

Species	Type	Location	Sampling date	Compartment	Dominant dinoflagellate	Temp. (°C)	Salinity	ASV match and references
<i>Paradinomyces evelyniae</i>	Harbour	Cambrils ¹	15 May 2019	sediment	<i>A. minutum</i>	18.7	36.9	ASV613;
		El Masnou ¹	2 Mar 2018	seawater		11.8	37.7	Reñé et al. (2021)
		Port Olímpic ¹	7 Mar 2018	seawater, sediment		13.2	38.0	ASV5858;
	Beach		14 Dec 2021	seawater	<i>A. minutum</i>	14.3	36.5	Unpublished results
			11 Jan 2022	seawater		12.9	36.6	
			11 Jan 2022	sediment	<i>Biecheleira</i> sp.	12.9	36.6	
			26 Jan 2022	sediment	<i>Biecheleira</i> sp., <i>Pyrophacus steinii</i>	12.7	37.0	
			23 Feb 2022	sediment	<i>Biecheleira</i> sp.	14.0	36.9	
			19 May 2022	sediment	<i>Heterocapsa</i> sp., Suessiaceae	21.9	36.8	
			15 Dec 2022	seawater	<i>Prorocentrum triestinum</i>	14.7	36.4	
		Grau ¹	19 Aug 2022	sediment	<i>Heterocapsa</i> sp.	24.6	37.5	
		Krogarviken ²	8 Oct 2012	water	<i>Heterocapsa triqueta</i> , <i>Biecheleira baltica</i> , Dinophyceae sp.	11.6	6.1	ASV New1456; Enberg et al. (2018)
		semi-enclosed bay						
	Deep bay	Sforfjärden ²	8 Oct 2012	surface water	<i>Heterocapsa triqueta</i> , <i>Biecheleira baltica</i>	11.9	6.0	
			8 Oct 2012	bottom water	<i>Heterocapsa triqueta</i> , <i>Biecheleira baltica</i>	11.9	6.0	
			19 Oct 2012	bottom water	<i>Biecheleira baltica</i>	7.3	6.3	
			29 Oct 2012	surface water	<i>Heterocapsa triqueta</i> , <i>Biecheleira baltica</i>	9.0	5.4	
			29 Oct 2012	bottom water	<i>Gymnodinium corollarium</i>	10.5	5.6	

¹ NW Mediterranean Sea, ² Baltic Sea

Factors influencing the distribution and behaviour of *Dinomyces* and *Paradinomyces* species in marine environments

Both the establishment of chytrid-dinoflagellate interactions and the characterization of host preferences can be challenging when based only on metabarcoding information. However, it is clear that chytrid occurrence is influenced by the presence of preferred protist hosts, as demonstrated in the present study and that of Fernández-Valero et al. (2022). *Dinomyces gilberthii* was detected in various locations along the Catalan coast, both on beaches during summer and in harbour sediments throughout the year. This widespread coastal distribution of *D. gilberthii* highlights its adaptability. The role of this species in the dynamics of coastal dinoflagellate blooms could be similar to that studied in *D. arenysensis*, which varies from host-parasite coexistence (observed in the field and laboratory experiments) to near extinction of the host population (observed in laboratory experiments) (Fernández-Valero et al., 2023).

The persistence of *D. gilberthii* in the water column during spring and summer coincided with high biomass blooms of the dinoflagellates *A. minutum* and *G. litoralis* or mixed communities dominated by these dinoflagellates. However, despite the co-occurrence of *G. litoralis* blooms and *D. gilberthii* indicated by metabarcoding datasets, attempts to induce infections of *G. litoralis* strain ICMB235 in the laboratory were unsuccessful (data not shown). In previous research, the inability of *Dinomyces* sp. P242 - RCC3408 (= *D. gilberthii*) to infect *G. litoralis* (strain ICMB226) was observed in cross-infection experiments (Lepelletier et al., 2014). Co-occurrence in the field but failure infections in the laboratory suggest strain-strain incompatibilities or *D. gilberthii*'s preference for alternative species during *G. litoralis* blooms. The need for further exploration of the chytrid's host range, with early indications of specificity for members of the genera *Alexandrium* Halim, *Scrippsiella* Balech, and *Ostreopsis* J.Schmidt (Lepelletier et al., 2014), is a reminder of the caution required when interpreting the simultaneous presence of two species in a metabarcoding dataset, as it may not represent a definitive relationship between them.

In contrast to *D. gilberthii*, *P. evelyniae*, with a more restricted occurrence in the water column, was mainly detected in harbour sediments, during winter and early spring, despite an exceptional detection in beach sediments in summer. The presence of *A. minutum*, one of the *P. evelyniae*'s dinoflagellate hosts, in the water column may have influenced the occurrence of the chytrid. Its more limited distribution along the Catalan coast, i.e., only during *A. minutum* blooms, compared to that of *D. gilberthii*, suggests a higher host specificity.

The frequent detection of chytrid–dinoflagellate pairs in coastal ecosystems implies greater importance of biotic factors, especially the host presence, than abiotic factors in the parasite development. On the other hand, the detection of chytrids in sediments points to this compartment as a species reservoir, when primary hosts are absent from the water column. Nonetheless, abiotic factors may also influence species distribution. The presence of *D. gilberthii* and *P. evelyniae* in Mediterranean samples and the occurrence of *Paradinomyces* spp. in the Baltic Sea question previous conclusions regarding the abiotic preferences of chytrids with respect to salinity. Notably, *P. triforaminorum* was previously described in low-salinity waters from the Baltic Sea during a bloom of the dinoflagellate *Kryptoperidinium foliaceum* (F.Stein) Lindemann (Reñé et al., 2022), and *P. evelyniae* was detected in Northern Baltic Sea waters (Enberg et al., 2018). Further studies on how salinity modulates the geographical distribution of chytrids could provide deeper insights into their ecological dynamics within diverse aquatic ecosystems, especially for species able to adapt and survive in marine ecosystems, which thus far are assumed to include only a few (Rojas-Jimenez et al., 2019).

Indeed, zoosporic fungi have been described as being less resistant to marine environments than other fungal groups, such as Basidiomycota and Ascomycota (Jones et al., 2022) and they show optimal growth under low salinity conditions (Guo et al., 2023). This may explain why locations, such as the Baltic Sea or sea ice, host a relatively high proportion of chytrids (Hassett et al., 2019). However, the same study also mentions locations of the Mediterranean Sea and Red Sea, both of which are characterized by high salinity and the presence of a high richness of Chytridiomycota. Therefore, whether salinity is a key factor in the chytrid biogeography is still an open debate.

Our study offers perspectives on the spatial and temporal distribution of two newly identified marine chytrids, including their recurrent presence in specific localities. Repetitive occurrences may be mainly influenced by the presence of the species in sediments along the year and host availability in the water column. The description of these species expands our understanding of the biological components that interact directly with dinoflagellate species, revealing added complexity in the dynamics of phytoplankton blooms. In the absence of primary hosts, parasites able to infect secondary hosts or enter dormant states (in the sediments) would have strong survival advantages. Both survival mechanisms and the persistence of *D. gilberthii* and *P. evelyniae* in sediment reservoirs remain to be examined in order to elucidate the direct role of these chytrid species in coastal ecosystems.

Assessing the global presence of Chytridiomycota species in metabarcoding datasets from marine regions is challenging due to limited detections in global molecular datasets. The scarcity of these detections can be attributed to inadequate sampling efforts, particularly in regions where these fungi occur prominently, such as in benthic and epiphytic compartments (Fernández-Valero et al., 2022). Additionally, limitations may arise from the lack of sampling during conditions favorable for chytrid growth, such as microalgal blooms (Fernández-Valero et al., 2023). Undetected taxa are not proof of absence because a rare biosphere might not be revealed by metabarcoding due to insufficient sequencing depth. This study underscores the need to employ diverse sampling methods in diverse habitats and to integrate molecular and morphological techniques to gain a thorough understanding of the lesser-studied fungal diversity within marine ecosystems.

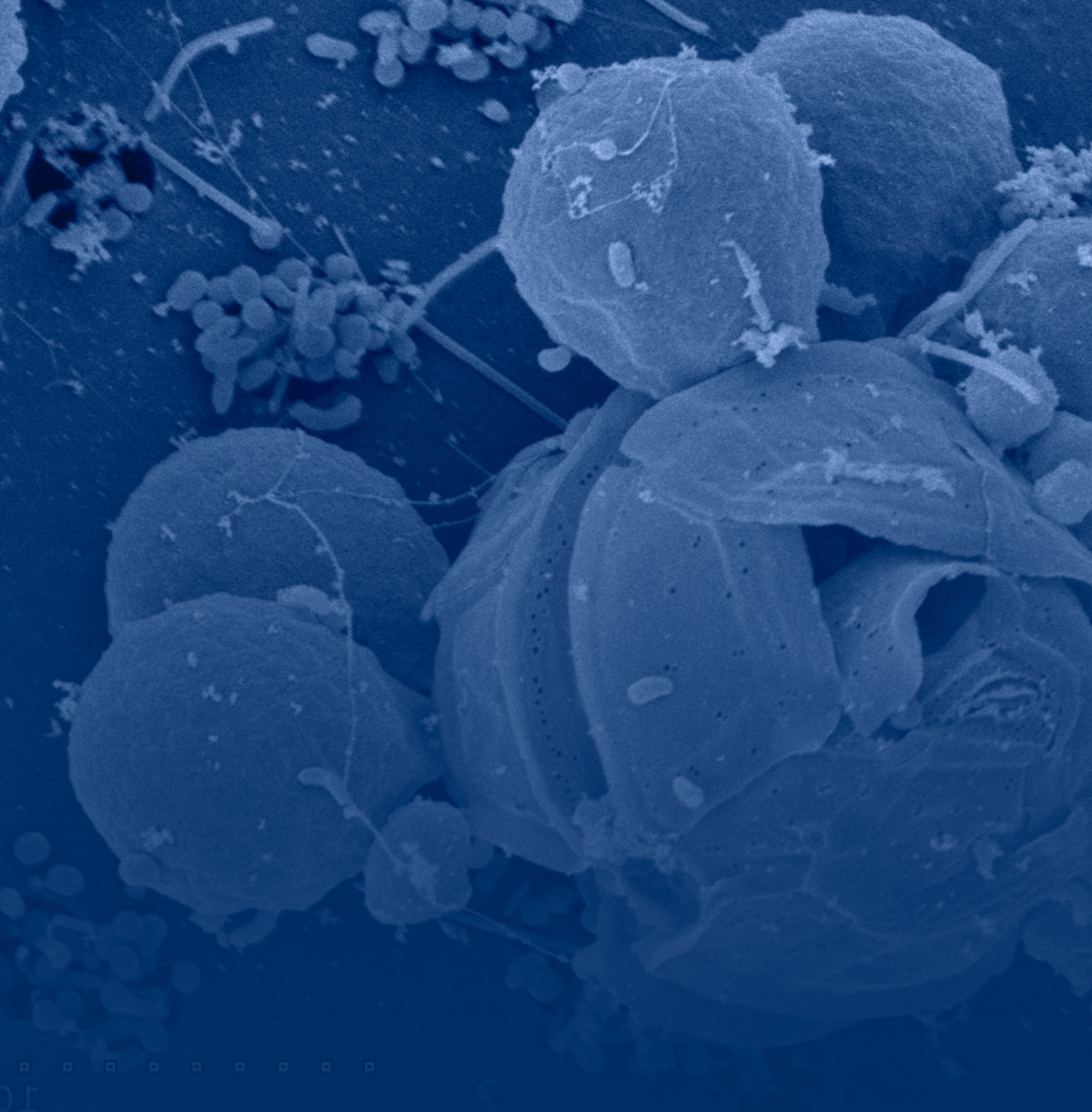
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5. Summary of results

5 Summary of results

This thesis provides valuable insights into the diversity and dynamics of Chytridiomycota in relation to their phytoplankton hosts. Studies conducted in the NW Mediterranean Sea demonstrate both spatial and temporal variations in chytrid populations and their interactions with dinoflagellates. Notably, specific chytrid species exhibit differing seasonal patterns and ecological adaptations, further enriching our understanding of these understudied marine fungal communities. The thesis specifically contributes to the understanding of parasitic fungi in marine environments in three key aspects:

Chapter 2- Spatial variation of chytrids during blooming dinoflagellates

Challenge: The interactions of parasitic fungi with their phytoplankton hosts in the marine environment are not well established. We investigated the diversity and interactions of Chytridiomycota fungi with dinoflagellate-dominated communities in the NW Mediterranean Sea. Differences were observed between the protist communities in seawater and sediment, the latter having more diverse and abundant chytrid populations. A total of 77 unique chytrid sequences were identified, 70 of which were host-specific. Chytrid abundance was higher in samples corresponding to *Ostreopsis* sp. and *Alexandrium minutum* blooms. Three Chytridiomycota species were identified through co-culture and molecular methods, including *Dinomyces arenysensis* and two undescribed species.

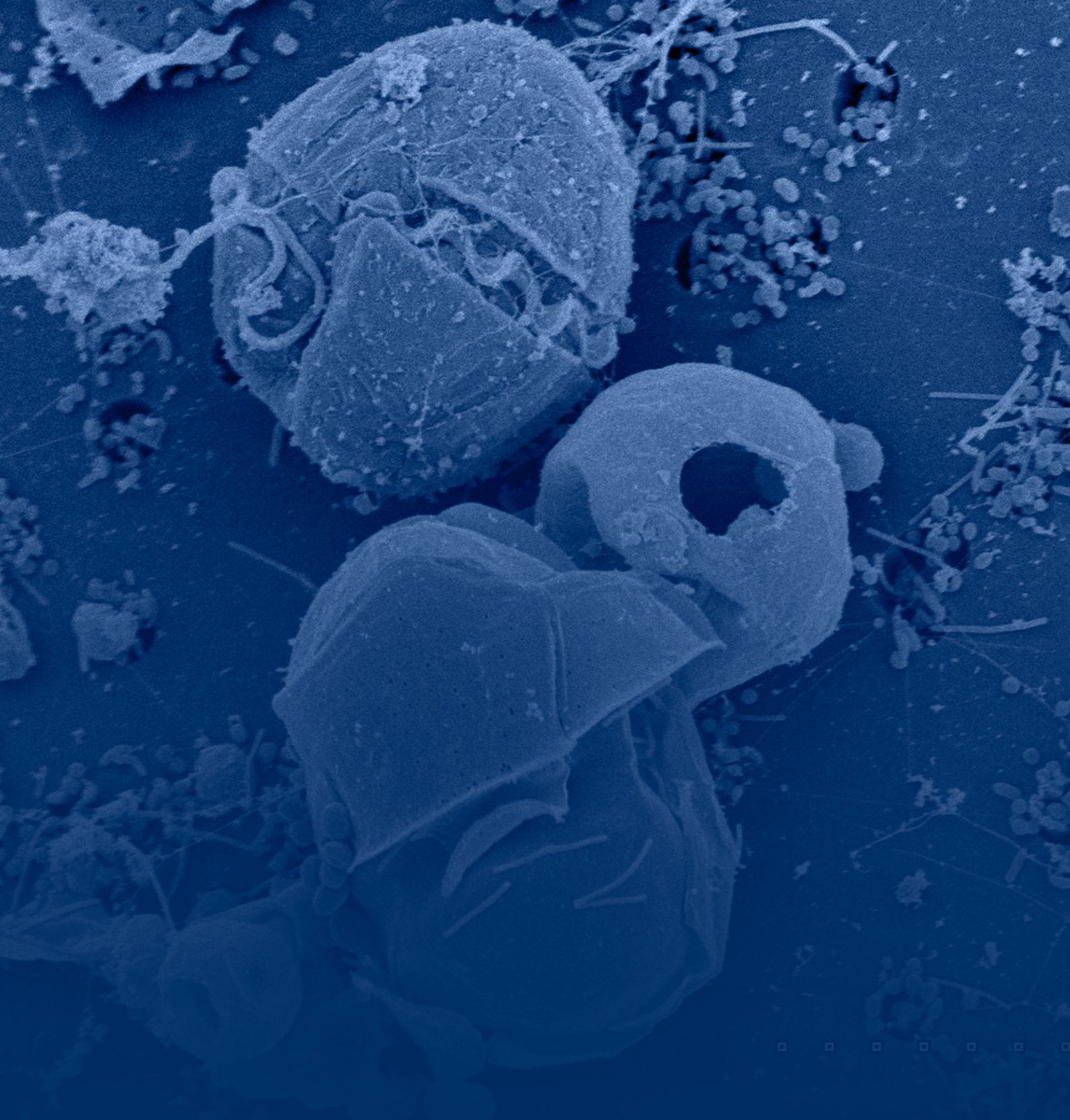
Chapter 3 - Temporal dynamics of chytrids during epiphyte bloom on macroalgae

Challenge: The temporal dynamics of parasitic fungi with their hosts in the marine environment is poorly characterised. We examined the temporal dynamics of epiphytic protists on macroalgae biofilms, focusing on the role of fungi, particularly chytrids, in relation to a dinoflagellate proliferation. Fungi represented less than 1% of the protist community, but increased to 30% due to the emergence of Chytridiomycota, coinciding with a rise in dinoflagellate abundance. The dominant chytrid was *D. arenysensis*, with a peak infection rate of 2%. Laboratory experiments revealed intraspecific variability in *D. arenysensis* - *Ostreopsis* interactions, ranging from coexistence to host near-extinction. One chytrid strain was found to be a facultative parasite, being able to use pollen as an organic matter source.

Chapter 4 - New chytrids species in the coastal area

Challenge: The diversity of parasitic fungi in marine environments is mostly unknown. Two new species of Chytridiomycota, *Dinomyces gilberthii* and *Paradinomyces evelyniae*, both belonging to the Dinomycetaceae family, were identified in Mediterranean samples during *Alexandrium minutum* blooms, and they were formally described. Distinct morphological and molecular features differentiate these species from previously known relatives. *D. gilberthii* is widespread in Mediterranean coastal ecosystems, particularly during summer blooms, indicating high adaptability. *P. evelyniae* has a more limited distribution, generally found in harbour sediments during winter and early spring. *P. evelyniae* is also present in the Baltic Sea at low salinity conditions.

These results contribute to filling gaps in our current knowledge of marine fungal communities, parasitic fungi diversity, and their interactions with phytoplankton hosts in coastal environments.



6. General discussion

6 General discussion

6.1 Assessing Chytridiomycota molecular diversity in marine environments: methodological challenges

To date, knowledge about basal fungi in marine environments, especially in relation to dinoflagellates, has been limited. This thesis contributes to exploring the diversity of this group, employing two main approaches: environmental sequencing analysis, used to describe fungal molecular diversity (defined as the richness of taxonomic units) and the isolation and cultivation of strains of the predominant species from field samples.

Comparing the molecular diversity of Chytridiomycota across various marine studies presents challenges due to the lack of standardised methodologies. These challenges stem from differences in the sequencing regions employed (e.g., 18S rDNA, ITS, or 28S rDNA) and the strategies used to define taxonomic units, namely ASVs (Amplicon Sequence Variants) or OTUs (Operational Taxonomic Units). Such discrepancies significantly influence the diversity indices reported across studies. Consequently, this thesis examines the molecular diversity of Chytridiomycota as reported in the existing literature, with a focus on the total number of taxonomic units identified within eukaryotic and/or fungal groups, as detailed in Table 6.1 This table presents the taxonomic richness of the Chytridiomycota fungal groups through environmental sequencing and positions this group in comparison to other fungal and eukaryotic taxonomic groups. It presents the genetic sequence regions analysed, providing insights on their importance for taxonomic differentiation and information on the geographic and ecological contexts of the collected data.

The attempt to compare the molecular diversity with other studies highlights some common characteristics. The results on the diversity of chytrids obtained in this thesis during dinoflagellate proliferations are similar with findings from other Mediterranean Sea studies. For instance, a study conducted in the Gulf of Naples, Italy, identified up to 700 eukaryote ASVs with Chytridiomycota accounting for 2.5% in sediments and 0.3% in the water column (Reñé et al., 2023). Comparable studies conducted in the eastern Mediterranean coastal areas and the Ionian Sea's submarine canyons (central Mediterranean) reported approximately 4% Chytridiomycota in relation to total fungal diversity (Barone et al., 2018; Rubin-Blum et al., 2022). In the English Channel, UK, a study on coastal planktonic fungi found that Chytridiomycota represented an average of 8.6% of total fungal taxa. This group demonstrated a distinct association with diatom blooms over several years (Taylor and Cunliffe, 2016). In the Arctic, Chytridiomycota comprised 1.2% of total eukaryotic taxa in layers with the highest chlorophyll concentration (in samples with a minimum of 100 ASVs) (Kiliyas et al., 2020). Notably, areas of lower salinity, such as those influenced by sea ice melting, showed increased Chytridiomycota diversity and an average relative abundance of 16%. These geographically focused studies reveal that Chytridiomycota represent a modest portion of the molecular diversity, less than 3% of the total eukaryotic community (Table 6.1). Yet, broader research encompassing numerous locations shows Chytridiomycota as a significant clade in molecular diversity and relative abundance, comparable to Dikarya (Hassett et al., 2019; Debeljak and Baltar, 2023), accounting for about 30% of the fungal community

(Table 6.1). Nonetheless, Chytridiomycota’s molecular diversity appears non-uniform across marine environments, being more pronounced in coastal areas.

The integration of these findings with data from various studies indicates that Chytridiomycota have limited molecular diversity compared to other fungal or eukaryotic communities. However, their specific composition varies markedly depending on the host species present (see Chapter 2). Most sampling conducted in this thesis and available literature corresponds to conditions of relatively low phytoplankton diversity. As a hypothesis for future research, it is plausible that in coastal environments with greater phytoplankton diversity and thus a broader range of potential hosts, the molecular diversity of associated chytrids could be higher than reported one.

Environmental sequencing has revealed the largely unknown molecular diversity of the Chytridiomycota. Key orders of Chytridiomycota such as Chytridiales, Rhizophydiales, Mesochytriales, Gromochytriales, and Lobulomycetales, have been identified by this method, in accordance with studies by Hassett et al. (2017, 2019); Kiliyas et al. (2020); Tibell et al. (2020). To date, 15 diatom-parasitic chytrids species have been formally described in marine and brackish environments (Danz and Quandt, 2023) classified only in two orders: eight in Chytridiales and seven in Rhizophydiales. In terms of chytrids parasitoids on dinoflagellates, only three species have been described in marine and brackish habitats: *Dinomyces arenysensis* (Lepelletier et al., 2014) in the Mediterranean Sea, and *Paradinomyces triforaminorum* (Reñé et al., 2022) and *Ericiomyces syringoforeus* (Karpov et al., 2021) in the Baltic Sea. This limited number of described species is likely due to the challenges in cultivating these organisms, including a lack of knowledge about the hosts that facilitate their development to their inherently slow growth.

This thesis presents the isolation of 12 strains of parasitoid chytrids, as detailed in Table 2.1 of Chapter 2 and Table 3.1 of Chapter 3. Taxonomic analysis of these strains has revealed they correspond to four species of chytrids, three of them being new to science (two belonging to the order Rhizophydiales and one to Lobulomycetales). Two of these species have been formally described, and the third one represents the first successfully isolated species of Lobulomycetales from marine environments. This achievement was facilitated through isolating and culturing chytrid species based on observed preferential host relationships in the field. Detailed analysis of host-parasite coexistence provides valuable insights into how to culture these parasites.

6.2 Coastal habitats harbour heterogeneous diversity of Chytridiomycota: insights from the Mediterranean Sea.

The thesis contributes to the broader understanding that coastal environments serve as hotspots for Chytridiomycota diversity, compared to other oceanic environments. The diversity within the coastal habitat is substantially varied, covering planktonic, benthic, and epiphytic habitats, as previously indicated (Picard, 2017). This study reported Chytridiomycota constituting 20% of fungal taxa across different sedimentary environments, such as intertidal sand flats, wetland sediments, with approximately 80% of these taxa being habitat-specific. Chapters 2 and 3 detail the molecular diversity of Chytridiomycota across coastal habitats, highlighting sediments as richer in diversity compared to the water column. These results reinforce coastal sediments as reservoirs of diversity for zoospore parasites, including fungi. Analyses of sediment samples, such as those from the southern Tyrrhenian Sea’s coastal boundary zone (Mediterranean Sea) (Reñé et al., 2023), support the findings of a higher molecular diversity in sediments than in the water column.

Table 6.1: **Taxonomic richness of Chytridiomycota obtained from environmental sequencing studies.** Cultured chytrid strains, sampling date and location, dinoflagellate host, and the molecular sequences obtained. The last column reports the match between strain sequences and ASVs obtained by metabarcoding.

Location	Marine area	Sequence region	Taxonomic units of eukaryotes	Taxonomic units of fungi	Taxonomic units of Chytridiomycota	Habitat	Reference
Arctic Region	Coastal zone	V4-18S rDNA	1677 ASVs (100%)	-	20 ASVs (1.2%)	Water column	Kilias et al., (2020)
English Channel, Celtic Sea	Coastal zone	ITS	-	23 OTUs (100%)	2 OTUs (8.6%)	Water column	Taylor and Cunliffe, (2016)
Baltic Sea	Coastal zone	18S rRNA	2176992 seq. (100%)	91128 seq. (5.91%)	-	Surface water	Rojas-Jimenez et al., (2019)
Oslofjord, Baltic Sea	Coastal zone	18S rDNA	236 OTUs (100%)	21 OTUs (8.9%)	7 OTUs (3%)	Macroalgae surface	Markussen Bjorbækmo et al., (2023)
Thuval, Red Sea	Brine pool	18S rRNA	100% (13074 reads)	68-99% (reads)	-	Sediment	Wang et al., (2014)
Bohai Sea and North Yellow Sea	Coastal zone	18S rRNA	-	100% OTUs (71842 reads)	32% OTUs	Sediment	Wang et al., (2017)
South Shetlands Islands, Antarctica	Coastal zone	ITS	-	133 ASVs (100%)	2 ASVs (1.5%)	Sediment	da Silva et al., (2022)
Carteret County, Atlantic	Coastal zone	28S	-	100% OTUs	21.3% OTUs	Sediment	Picard, (2017)
Ionian Sea	Deep Sea	ITS	-	100% OTUs	2.1% OTUs	Surface water	
			-	1742 OTUs (100%)	69 OTUs (4%)	Sediment	Barone et al., (2018)
Eastern Mediterranean Sea	Coastal - offshore transect	ITS	-	100% (reads)	1.7 - 3.9% (reads)	Sediment	Rubin-Blum et al., (2022)

Continue to the next page.

Location	Marine area	Sequence region	Taxonomic units of eukaryotes	Taxonomic units of fungi	Taxonomic units of Chytridiomycota	Habitat	Reference
Mediterranean Sea, Arctic, Atlantic and Pacific Oceans	Global transects	V9-18S rDNA	39391 ASVs (100%)	713 ASVs (1.8%)	127 ASVs (0.3%)	Sediment	Cordier et al., (2022)
Gulf of Naples, Tyrrhenian Sea	Coastal zone	V4-18S rDNA	833 ASVs (100%)	31 ASVs (3.72%)	15 ASVs (1.8%)	Sediment	Reñé et al., (2023)
NW Mediterranean Sea	Coastal zone	V4-18S rDNA	2540 ASVs (100%)	58 ASVs (2.28%)	21 ASVs (0.82%)	Water column	Chapter 1
			2729 ASVs (100%)	119 ASVs(4.36%)	35 ASVs (1.28%)	Water column	
NW Mediterranean Sea	Coastal zone	V4-18S rDNA	4367 ASVs (100%)	165 ASVs (3.77%)	63 ASVs(1.44%)	Sediment	Chapter 2
			2153 ASVs (100%)	203 ASVs (9.4%)	28 ASVs (1.3%)	Macroalgae surface	
		ITS		466 ASVs (100%)	19 ASVs (4.5%)		

Moreover, chapter 3 examines fungal community variations in a benthic niche over three months, during a dinoflagellate proliferation. The observed shifts in fungal diversity correlate with changes in the composition of the eukaryotic epibiont community. These patterns resemble those in studies of fungal molecular diversity in different brown algal species (Bonthond et al., 2022; Markussen Bjorbækmo et al., 2023). While overall fungal diversity (based on OTUs) was similar among macroalgal species, relative abundance varied with the host algae (Markussen Bjorbækmo et al., 2023). Bonthond et al. (2022) also noted differences in fungal diversity among algal species, algal tissues and in life cycle stages of the same host species. The results of this thesis, together with other research on marine epibionts, reveal that the molecular diversity of the Chytridiomycota varies as a function of the substrate, associated epibiont community and undergoes significant changes over time. These variations are likely influenced by factors such as the vegetal substrate stage, benthic proliferation events, or the development of biofilms that act as nutrient traps, enhancing the growth and reproduction of resident organisms. Thus, these benthic niches emerge as niches of increased diversity, potentially hosting more interactions than planktonic environments.

As revealed, the factors influencing Chytridiomycota diversity in coastal environments remain largely unknown. While comprehensive knowledge is emerging, coastal habitats might be perceived as junctions linking terrestrial, freshwater, and marine ecosystems. Generally, fungal diversity diminishes from terrestrial, freshwater, and then to marine habitats. Notably, rivers and freshwater systems predominantly harbour basal Chytridiomycota fungi, as indicated by Debeljak and Baltar (2023). Therefore, the fungal diversity in coastal areas may be attributed to terrestrial and freshwater sources flowing into the sea, such as river runoff or fluvial discharges to the coast. This hypothesis could further explain the role of salinity in shaping Chytridiomycota communities, as discussed by Scholz et al. (2017), and the occurrence of typically terrestrial and freshwater taxa in coastal habitats. These observations provide a robust foundation for future ecological and evolutionary research in this domain.

In summary, the observed variation in taxonomic composition among different coastal habitats underscores the significance of acknowledging the heterogeneity in Chytridiomycota distribution. It is plausible to propose that Chytridiomycota diversity is influenced by geographic and spatial factors, including habitat type (e.g. water column or sediment), the characteristics of sedimentary habitats (e.g. benthic niche) and the influence from the continent and freshwater systems. Biotic factors such as the type of benthic substrate, the composition of the eukaryotic community, and particularly the nature of host organisms, are also crucial factors as well.

6.3 Parasitic interactions between chytrids and dinoflagellates

Understanding the parasitic interactions between chytrids and dinoflagellates is complex, particularly due to the transient nature of these species. However, laboratory cultures offer valuable opportunities to study their interactions and dynamics. Existing research indicates that chytrid species can infect multiple dinoflagellate hosts, as demonstrated by studies such as those by Lepelletier et al. (2014) and Karpov et al. (2021). An exception to this pattern is *Paradinomyces triforamini* which has been observed to specifically infect *Kryptoperidinium foliaceum* (Reñé et al., 2022). In any case, it is worth mentioning that experiments with *Paradinomyces* have been limited to a small selection of potential hosts including (*A. minutum*, *A. ostenfeldii* and *P. cordatum*). This limitation prevents an accurate assessment of its host specificity.

Through molecular sequencing of chytrid species we also can explore ecological aspects such as host preference through co-occurrence and competition for resources among different parasite

species. This approach provides a broad perspective of spatial and temporal distribution of species, allowing to infer co-occurrence networks among different taxonomic groups. However, it is important to note that these co-occurrences merely suggest potential interactions. Cultures allow to confirm interactions, but there is a bias regarding the ability to demonstrate all interactions in the laboratory that actually occur in the ecosystem. Techniques such as fluorescent in situ hybridisation (FISH) with specific molecular probes can also be used to observe by epifluorescence microscopy the presence of parasitic interactions in natural samples and to confirm these interactions (Salomon et al., 2009; Chambouvet et al., 2019). Other techniques such as fluorescent labelling, like DAPI staining of the nuclei, can help to identify these interactions, as was done to determine the percentage of chytrid infection on dinoflagellates in Chapter 3. Therefore, combining these methodologies would provide a more accurate approach to describing the dynamics and interactions between chytrids and dinoflagellates.

Environmental sequencing data obtained in this thesis provides additional information on host preferences, showing certain chytrid species predominate depending on the dinoflagellate host. Chapters 2 and 4 indicate the dominant chytrid species in *Ostreopsis* sp. and *A. minutum* proliferations are *Dinomyces arenysensis* and *Dinomyces gilberthii*, respectively, the latter being described as a new species in this thesis. Laboratory cultures confirmed that both species can infect *Ostreopsis* sp. and *A. minutum*. In fact, *D. arenysensis* was first identified during a proliferation of *A. minutum* (Lepelletier et al., 2014). Laboratory data suggest that these two chytrids might compete for the same resource. However, metabarcoding analyses presented in Chapter 2 indicate that *D. gilberthii* sequences were not found in the samples of *Ostreopsis* sp. proliferations, and vice versa for *D. arenysensis* in *A. minutum* proliferations. This suggests that both chytrid species may have developed preferences for their respective hosts through coevolution. The observed dynamics between these parasitic species aligns with the theories of Gandon (2004), which suggest that parasites develop a preference for one host, compromising their virulence capacity towards other potential hosts. In this context, *D. gilberthii* could have reduced its virulence on alternative hosts by preferring *A. minutum*, and *D. arenysensis* would have done the same with *Ostreopsis*. In any case, our results show members of the genus *Dinomyces* as the main contributors to chytrid-dinoflagellate parasitic systems in the NW Mediterranean Sea.

Two other new species, *Paradinomyces evelyniae*, and *Lobulomycetales* sp. (corresponding to strain ICMB1108/ASV83 from Chapter 3, pending description), were isolated during *A. minutum* and *Ostreopsis* sp. proliferations, respectively. Although *P. evelyniae* can infect *Ostreopsis* sp. and *A. minutum*, similarly to the dominant chytrid species, no clear patterns were observed to determine their primary or preferred hosts because they were rarely detected. Therefore, further experiments are needed to determine its infection strategy and whether it is a generalist species with low virulence, or has high affinity and virulence towards a yet unidentified preferred host.

In this thesis, efforts were conducted to determine the distribution of target species across different oceans using global environmental sequencing datasets, and to infer potential interactions with known or additional hosts. The analyses showed that parasitic species observed in this thesis are rarely detected in those datasets and not correlated with putative preferred hosts, potentially due to their temporal dynamics. Molecular data suggest that chytrids have low relative abundance but can become significant when their preferred host is present in abundance. This aspect is particularly observed in coastal environments, where phytoplanktonic species (potential hosts) often produce recurrent high biomass blooms. The low detection rate of these parasitic species in the molecular datasets consulted may result from a sampling bias, as sampling generally occurs in oceanic waters with low phytoplankton biomass. Thus, it cannot be ruled out that these species would have been detected if samples had been taken in coastal areas and/or under conditions of high phytoplankton biomass. In any case, it has been possible to detect the presence of some of

the species discovered in this thesis in other environments. Specifically, *Paradinomyces evelyniae*, a species detected during the proliferation of *A. minutum* in harbours of the Catalan coast, was also detected in localities of the Baltic Sea under the presence of other dinoflagellate species like *Heterocapsa triquetra* or *Biecheleria baltica*. This supports the hypothesis that this species has an affinity for certain host species.

Finally, another important ecological aspect in the dynamics of organisms interactions is the competition with other co-occurring zoosporic parasites, such as Perkinsids. Perkinsids are known for wide coastal distribution and specific parasite-host interactions. Chapter 2 and Reñé et al. (2021) illustrate the coexistence of Perkinsids and Chytridiomycota in *Ostreopsis* sp. proliferations. In Cubelles, the dominance among zoosporic parasites was mainly *Dinovorax pyriformis*, followed by *Dinomyces arenysensis*, while in Sitges, *D. arenysensis* dominated, with a lower presence of *D. pyriformis*. These results not only show the coexistence of zoosporic parasitoids belonging to different groups, but also highlight the possible competition between them. Collectively, these findings involving host preference, infectivity, host competition, and the observation of these organisms on a global scale underscore the complexity and dynamics of interactions within eukaryotic communities.

6.4 Brief methodological notes, and current and future perspectives for the study of marine fungal communities

Here, we intend to offer some methodological advice (though not exhaustive) on the use of specific rDNA regions for enhancing our understanding of fungal diversity, while also stressing the significance of preserving cultures for research.

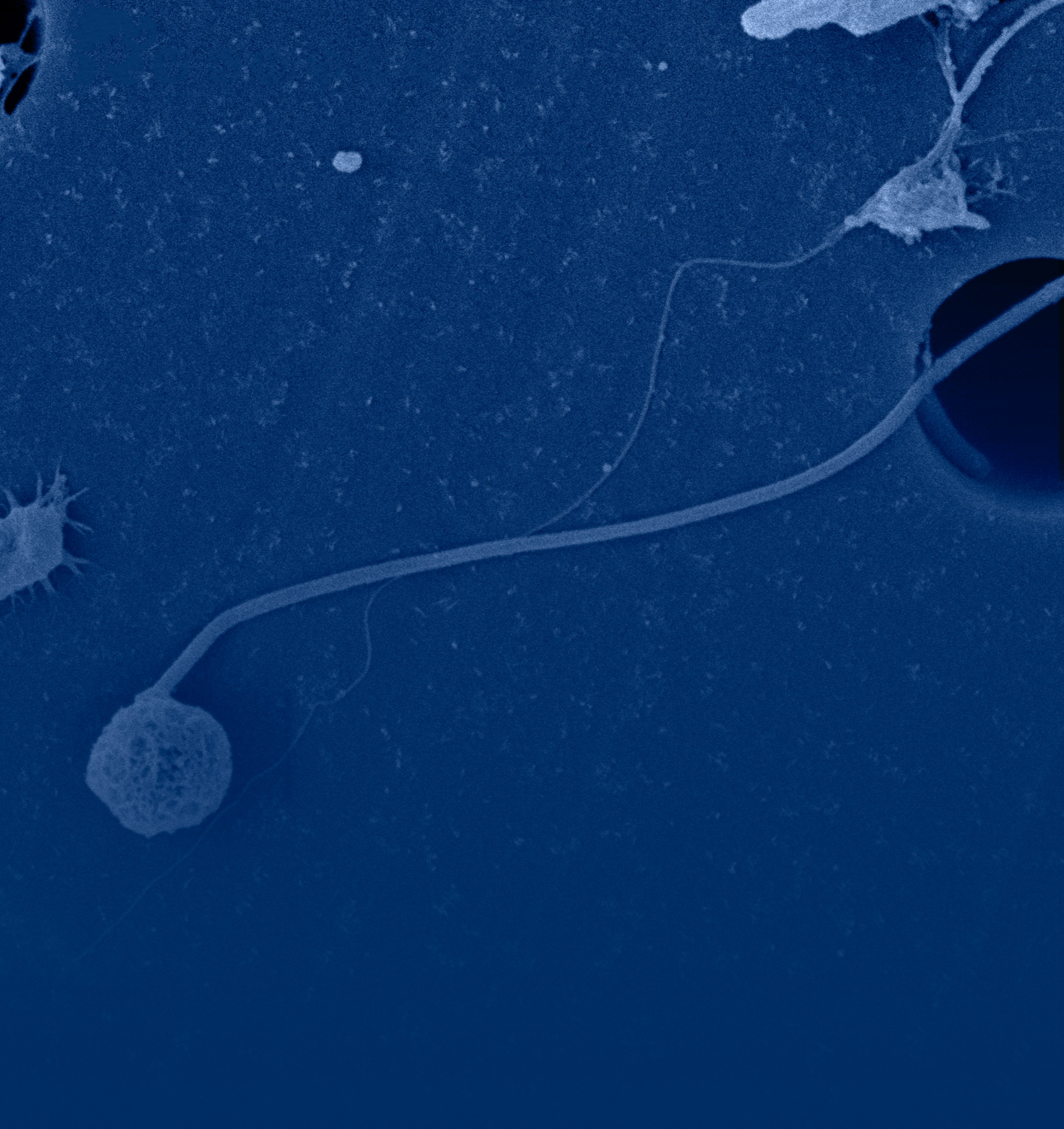
Nowadays, high-throughput sequencing tools have greatly improved our understanding of fungal diversity distribution patterns (Nilsson et al., 2019). This thesis used two rDNA regions to determine community composition: the V4 region of the small ribosomal subunit (18S rDNA) was employed to examine eukaryotic community composition, while the internal transcribed spacer (ITS) was used specifically for studying the fungal community composition. Each region provides different levels of taxonomic resolution (Schoch et al., 2012). The ITS and other regions, such as the large ribosomal subunit (28S rDNA) provide higher species-level resolution. In contrast, 18S rDNA, due to its more conserved characteristic, is more suitable for broader taxonomic levels, albeit with lower species-level resolution in fungal taxonomy. An important aspect to consider is the uneven number of reference sequences available for each of the ribosomal subunits. The ITS region, although limited in fungal sequences and biased towards terrestrial and freshwater organisms, might not define sequences at the phylum level (Peng and Valentine, 2021). Conversely, the 18S rDNA region, commonly used for microorganism identification and classification, has a more extensive database of reference sequences (Panzer et al., 2015). Hence, this thesis and recent studies on fungal communities have employed both ITS (or 28S) and 18S rDNA regions for more comprehensive taxonomic classification as suggested by diverse authors (Hassett et al., 2017; Banos et al., 2018; Sen et al., 2021; Van den Wyngaert et al., 2022).

Future research into fungal diversity is trending towards obtaining complete genomes of these organisms for a deeper understanding of fungal phylogeny, function and evolution, an area of constant change and rediscovery (Bass et al., 2018; Tedersoo et al., 2018; Strasser and Monaghan, 2022; Thomé et al., 2023). By acquiring genomic data, researchers can obtain more complete molecular information of studied taxa, moving beyond the limitations of using specific genes for classification purposes (Tedersoo et al., 2015). Techniques like shotgun sequencing, which involves

direct sequencing of the sample, have shown promising results in determining fungal community composition (Hassett et al., 2019; Morales et al., 2019). However, the challenge lies in the scarcity of reference genomes and comprehensive fungal genomic databases to interpret genomic information. Current databases are more focused on terrestrial fungi and human health pathogens (Sharma, 2016) leading to significant uncertainties for other taxonomic groups (Tedersoo et al., 2015). This gap also poses difficulties in comparing enzyme activities or interpreting transcriptome analyses, especially when contrasting data from databases dominated by terrestrial fungi with those derived from marine fungi (Hagestad et al., 2021). Despite the challenges, the ongoing efforts in this field are directed to fill these gaps.

Regarding the preservation of the cultures, it is imperative to maintain species after their isolation for detailed analyses of parasite-host interactions and host preferences. Currently, the cultures of this thesis are accessible at the ICM culture facilities. Depositing species in culture collections is crucial, yet it involves considerable time and resources. Therefore, promoting methodologies for the maintenance and conservation of species in these biobanks, such as cryopreservation, a method proven successful for some species (Rad-Menéndez et al., 2018), is essential. During the course of this thesis, attempts were made to cryopreserve the new chytrid strains, yielding negative results. This failure highlights the complex and potentially unique requirements for the cryopreservation of marine Chytridiomycota, suggesting that these organisms may necessitate specialised protocols or conditions beyond the standard procedures applied to other fungi or microbial groups. The challenges encountered underscore the necessity for further research into tailored techniques that can accommodate the specific physiological and metabolic characteristics of marine Chytridiomycota. Indeed, comprehensive information on the species, including, host range, life cycle, and other aspects, must be gathered to ensure efficient management of cultured species and their successful long term preservation. The availability of cultures belonging to new chytrid species will allow to significantly enrich the research on marine fungi.

In summary, advances in marine fungal research are inherently subject to the study techniques employed, each of them having their own biases and limitations. It underscores the need for a plurimethodological approach, integrating multiple techniques to gather complementary information from different perspectives. As demonstrated in this thesis, the combination of high-throughput sequencing and culture techniques facilitates the identification and characterisation of fungal species as well as the enhancement of our understanding of specific interactions. Molecular and traditional methodologies should be viewed as complementary, offering a more comprehensive approach to determine organisms identity as baseline knowledge and particularly in communities with largely unknown members, such as marine fungal ecosystems.



7. Conclusions

7 Conclusions

1. In coastal areas, a distinct chytrid community exists, closely associated with its preferred host.
2. The diversity and abundance of the chytrid community is higher in the sediments than in the water column.
3. The epiphytic fungal community, and chytrid representatives on macroalgae, change during the succession and proliferation of phytobenthos, alternating dominance among the different fungal groups.
4. Parasitoid-host co-cultures of chytrids and dinoflagellates allowed the discovery of a new species of Lobulomycetales and the description of two new species *Dinomyces gilberthii* and *Paradinomyces evelyniae*.
5. Most abundant and widespread species corresponded to *Dinomyces arenysensis* and *Dinomyces gilberthii*.
6. *Dinomyces arenysensis* displays intraspecific variability in virulence enabling their coexistence with the host *Ostreopsis* sp. Furthermore, it has the ability to use pollen as a resource, suggesting it is a facultative parasite that does not depend on dinoflagellates for completing its life cycle and survival.
7. *Dinomyces gilberthii* shows a wide distribution in Mediterranean habitats and is particularly prevalent during summer dinoflagellate blooms. The absence of *D. gilberthii* in global sequence databases suggests that this species is endemic to the coastal zone of the NW Mediterranean Sea.
8. *Paradinomyces evelyniae* has a limited distribution on the NW Mediterranean Sea coast, occurring mainly in harbour sediments and sporadically in the water column in winter and early spring. Sequences of *P. evelyniae* have been detected in the Baltic sea, in a wide salinity gradient, demonstrating the tolerance of this species in diverse coastal-marine habitats.



8. General references

8 General references

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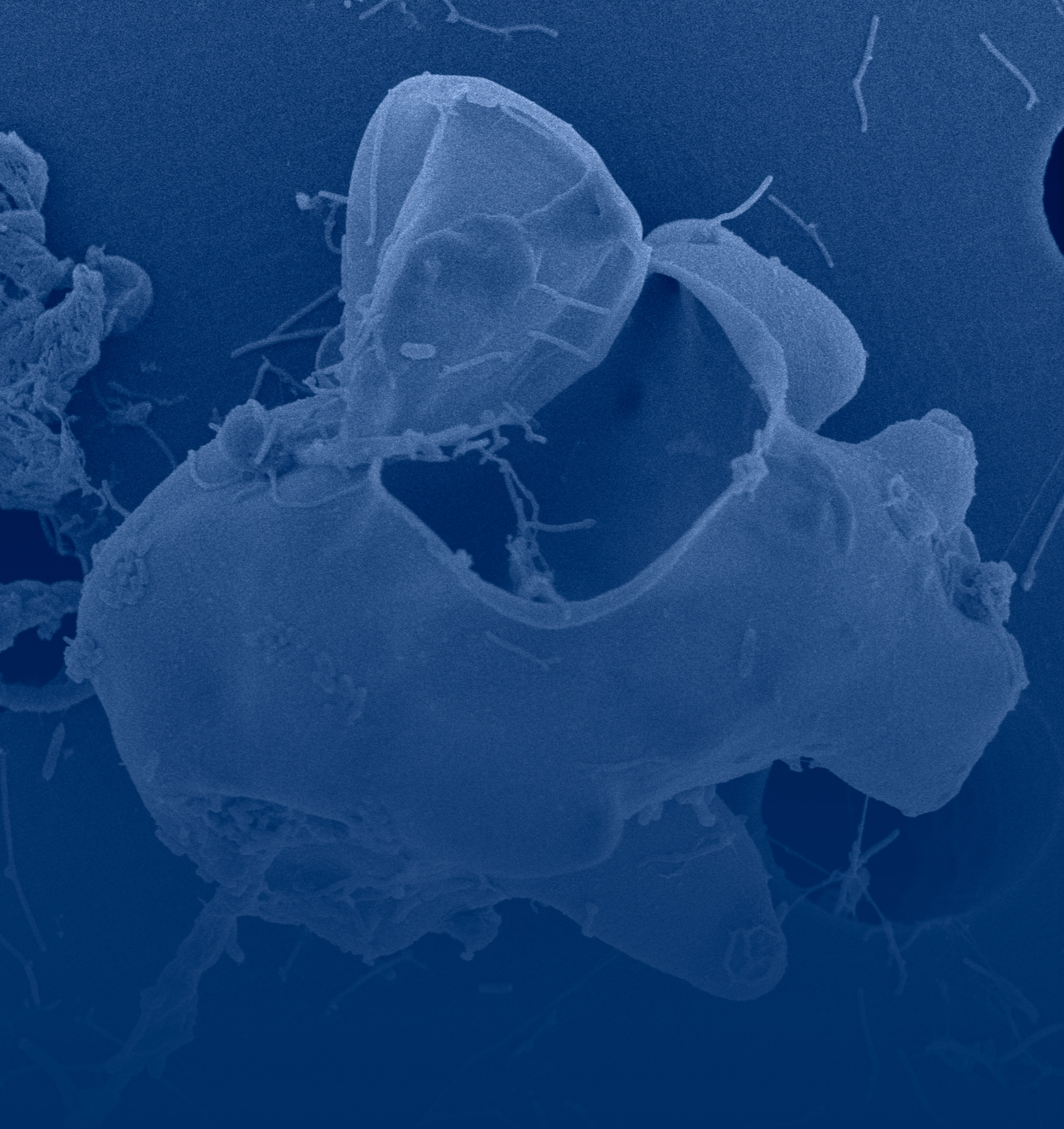
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9. Annex

9 Annex

9.1 Original article of chapter 2

Dinoflagellate hosts determine the community structure of marine Chytridiomycota: demonstration of their prominent interactions

Fernández-Valero, A.D., Reñé, A., Timoneda, N., Sampedro, N. & Garcés, E. (2022) Dinoflagellate hosts determine the community structure of marine Chytridiomycota: Demonstration of their prominent interactions. *Environmental Microbiology*, 24(12), 5951–5965. Available from: <https://doi.org/10.1111/1462-2920.16182>

RESEARCH ARTICLE



Dinoflagellate hosts determine the community structure of marine Chytridiomycota: Demonstration of their prominent interactions

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Funding information

Consejo Superior de Investigaciones
Científicas, Grant/Award Number: Project
COPAS CTM2017-86121-R; Ministerio de
Ciencia e Innovación, Grant/Award Numbers:
Project SMART PID2020-112978GB-I00,
Severo Ochoa Centre of Excellence
CEX2019-0000928S; Ministerio de Ciencia,
Innovación y Universidades, Grant/Award
Number: Formación de doctores
PRE2018-084893

Abstract

The interactions of parasitic fungi with their phytoplankton hosts in the marine environment are mostly unknown. In this study, we evaluated the diversity of Chytridiomycota in phytoplankton communities dominated by dinoflagellates at several coastal locations in the NW Mediterranean Sea and demonstrated the most prominent interactions of these parasites with their hosts. The protist community in seawater differed from that in sediment, with the latter characterized by a greater heterogeneity of putative hosts, such as dinoflagellates and diatoms, as well as a chytrid community more diverse in its composition and with a higher relative abundance. Chytrids accounted for 77 amplicon sequence variants, of which 70 were found exclusively among different blooming host species. The relative abundance of chytrids was highest in samples dominated by the dinoflagellate genera *Ostreopsis* and *Alexandrium*, clearly indicating the presence of specific chytrid communities. The establishment of parasitoid-host co-cultures of chytrids and dinoflagellates allowed the morphological identification and molecular characterization of three species of Chytridiomycota, including *Dinomyces arenysensis*, as one of the most abundant environmental sequences, and the discovery of two other species not yet described.

INTRODUCTION

Members of the basal fungal division Chytridiomycota, informally called chytrids, are found in soil, freshwater, brackish, and marine habitats. They exist in a free-living zoospore stage until they come into contact with a host/substrate resource, with which they physically interact using a rhizoid system (Laundon et al., 2020). Chytrids can be saprophytic, parasitic, or both and have a wide host range (Dunne et al., 2013; Hyde et al., 2020; Rasconi et al., 2011, 2014; Sime- Ngando, 2012; Thompson et al., 2005). Chytrid zoospores, produced as the result of a successful infection, are also a food source for grazing predators (Kagami

et al., 2004, 2014). Consequently, chytrids not only act as a food web driver, they also define a particular trophic link (Agha et al., 2016; Gerphagnon et al., 2019; Rasconi et al., 2014, 2020; Sánchez Barranco et al., 2020). They contribute to shaping host populations as well as interspecific competition between their hosts and other species while also fully participating in trophic interactions (Kagami et al., 2014; Rasconi et al., 2014; Sime- Ngando, 2012).

The chytrid–host relationship has been studied mainly in freshwater models, which evaluated the dynamics between chytrids and phytoplankton (Kagami et al., 2014). Chytrid infections have been reported in diatoms (Canter, 1969; Seto & Degawa, 2018),

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chlorophytes (Karpov et al., 2010; Vélez et al., 2011), cyanobacteria (Agha et al., 2016), and dinoflagellates (Leshem et al., 2016). In marine ecosystems, the few studies conducted on chytrids showed that chytrid biomass correlates positively with phytoplankton (Gutiérrez et al., 2016; Lepère et al., 2016). Environmental studies based on metabarcoding of the SSU rRNA and rDNA of marine microbial eukaryotes have revealed an unexpected and uncharacterized diversity of chytrid-like fungal sequences (Comeau et al., 2016; Richards et al., 2012, 2015), detected in ecosystems ranging from the Arctic and Antarctic (Kiliass et al., 2020; López-García et al., 2001; Picard, 2017; Scholz et al., 2016), to tropical zones (Hassett et al., 2017, 2020) and in habitats ranging from sediments to deep as well as shallow waters (Banos et al., 2020; Hassett & Gradinger, 2016; Käse et al., 2021; Priest et al., 2021; Scholz et al., 2016; Yang et al., 2021; Zäncker et al., 2021). A greater fungal diversity was detected in sediments than in the water column, and in the epipelagic than in the mesopelagic zone (Guo et al., 2015; Li et al., 2019; Xu et al., 2014).

Studies of the parasitic relationships between marine chytrids and diatoms (Scholz et al., 2014; Worden et al., 2015) have shown that chytrid parasitism affects the dynamics of marine diatom blooms (Gutiérrez et al., 2016). In situ experiments on the susceptibility of diatoms to chytrid infections demonstrated the influence of light stress and host abundance, as well as the preferential infection of different host species (Scholz et al., 2017). However, far less is known about the interactions of chytrids with dinoflagellates in the marine environment and very few species of dinoflagellate-associated marine chytrids have been described. The parasitic chytrid *Dinomyces arenysensis* was isolated from a bloom of *Alexandrium minutum* and in laboratory studies was shown to infect different species of dinoflagellates, most of them belonging to the genus *Alexandrium* (Lepelletier et al., 2014). Other species of marine Chytridiomycota isolated, cultured, and formally described so far include members of *Thalassochytrium* (Nyvall et al., 1999), *Rhizophydium* (Amon, 1984) and *Chytridium* (Küpper & Müller, 1999). Recently, species belonging to the *Ericiomyces* and *Paradinomyces* were isolated from a brackish environment in the Baltic Sea (Karpov et al., 2021; Reñé et al., unpublished).

Despite the growing number of chytrid species identifications and insights into host–parasite interactions, the impact and prevalence of chytrid infections of marine dinoflagellates and the role of chytrids in the marine food web are largely unknown, mainly due to the scarcity of field studies at the community level, including those examining biological interactions within consortiums. Such studies have also been hindered by the lack of annotated reference sequences of marine

chytrids in DNA databases, which has made it difficult to determine the identity, ecological role (parasitic or saprophytic), and host interactions of marine chytrids. Moreover, correlations inferred from environmental DNA data cannot be systematically interpreted as indicators of biotic interactions (Blanchet et al., 2020). Therefore, studies that combine cultivation, host range determinations, single-cell PCR techniques, and genome sequencing, among others (Garvetto et al., 2019; Grossart et al., 2016; Kagami et al., 2021) are needed to fully understand the structure and function of the chytrid community, the parasitic and competitive strategies of chytrids, as well as the ecological or temporal niches of chytrids (Van den Wyngaert et al., 2022).

Therefore, in this study, we examined the diversity of Chytridiomycota during dinoflagellate blooms at several coastal locations of the NW Mediterranean Sea and then used a combination of metabarcoding, phylogenetic identification, and laboratory cultivation approaches to characterize the pairwise interactions of Chytridiomycota and their hosts.

EXPERIMENTAL PROCEDURES

Procedures for metabarcoding sequencing and analyses

Nineteen samplings were carried out along the Catalan coast (NW Mediterranean Sea) in 2018 and 2019, including during high-biomass blooms of five dinoflagellate species (*Alexandrium minutum*, *Ostreopsis* sp., *Alexandrium taylori*, *Gymnodinium litoralis*, and *Kryptoperidinium foliaceum*) and under no-bloom conditions. Sediment and pre-filtered (>200 µm) seawater samples were collected at each coastal location (Table S1). Detailed information about the sampling procedures was provided in a previous study (Reñé et al., 2021). Briefly, two replicates of each seawater sample were immediately filtered by serial filtration through 10-µm and 0.8-µm polycarbonate filters, resulting in two fractions differing in size: small (0.8–10 µm) and large (10–200 µm). The filters and sediment samples were stored frozen at –80°C until processed.

Metabarcoding analyses were performed by extracting genomic DNA from the environmental sample filters and from 10 g of each sediment sample using the DNeasy PowerMax soil Dneasy kit (Qiagen) according to the manufacturer's instructions. After DNA extraction, a first PCR was performed using universal eukaryotic primers designed against metazoan EK-565F (Simon et al., 2014) and 18S-EUK-1134-UnonMet (Bower et al., 2004), resulting in an amplicon of about 560 base pairs. This was followed by metabarcoding sequencing of the V4 region of SSU rDNA on an Illumina PE300, using the primers EK-565F and E1009R

(Comeau et al., 2011), to determine the protist community, which yielded an amplicon of 346 base pairs. Raw molecular data were processed using DADA2 (Callahan et al., 2016), and the obtained ASVs were taxonomically assigned using the PR2 version 4.12 database (Guillou et al., 2013). Raw metagenomic reads were available at the NCBI Sequence Read Archive under Bioproject number PRJNA630546. Detailed information was provided in a previous study (Reñé et al., 2021).

Differences in richness and diversity according to the size fraction were assessed by testing the significance of the Chao1 and Shannon indexes using Wilcoxon tests. Potential host–parasite interactions were assessed by analysing the correlations between Chytridiomycota and dinoflagellates using a relative abundance matrix constructed independently of both phylogenetic groups. Only data from the large seawater fraction were used for this analysis, in order to consider potentially active chytrid sequences. Correlations were calculated using the cor function of the Rcran stats package, applying the Pearson correlation coefficient. Only correlations with a *p* value < 0.05 were further examined.

Isolation and culture of chytrids during dinoflagellate blooms

Additional coastal locations were sampled from 2019 to 2021, focusing on blooms of the dinoflagellates *Ostreopsis* sp. and *A. minutum*. Samplings were performed when dinoflagellate abundances in the water column were >10⁴ cells L⁻¹. Details of the sampled locations can be found in Table 1. *Ostreopsis* sp. was sampled at four different locations: Cubelles, Llavanes, Tossa, and La Fosca. Since *Ostreopsis* sp. is an epiphytic dinoflagellate, macroalgal samples were collected at shallow depths in bottles containing 1 L of 0.2-µm-filtered seawater. Additionally, 2.5 L of seawater was collected at a depth of 1–1.5 m. *A. minutum* proliferations were sampled in the harbours of Arenys, Vilanova, and L'Estartit. At each location, a tube connected to an electric pump was used to collect 5 L of integrated seawater from the water column (2.5- to 4-m depth) and 5 L from the deeper layer. Surface sediments were obtained using a sediment corer connected to a pole, with ~30 g of sediment placed in a 50-ml Falcon tube using a scoop.

In the laboratory, seawater samples from both blooms were pre-filtered through an 80-µm mesh to remove metazoans and large particles. One litre of each seawater sample was concentrated to 10 ml and aliquots of the concentrate were incubated in six-well plates. For the macroalgal samples from the *Ostreopsis* blooms, the macroalgae were washed in the flask three times with 1 L of seawater to resuspend the epiphytic

TABLE 1 Cultured chytrid strains, sampling date and location, dinoflagellate host, and the molecular sequences obtained.

Cultured chytrid	Strain name	Location	Coordinates	Sampling date	Host	18S rDNA gene	ASV match
<i>Dinomyces arenysensis</i>	ICMB1101	Cubelles	41°11'51.8" N, 1°40'23.0" E	27 August 2019	<i>Ostreopsis</i> sp.	ON081632; ON081633	ASV46; ASV66
<i>D. arenysensis</i>	ICMB1102	Llavaneres	41°33'07.5" N, 2°29'31.6" E	22 July 2020	<i>Ostreopsis</i> sp.	ON081634; ON081635	ASV46; ASV66
<i>D. arenysensis</i>	ICMB1103	Tossa	41°42'55.9" N, 2°55'56.4" E	11 August 2020	<i>Ostreopsis</i> sp.	ON081636	ASV46; ASV66
<i>D. arenysensis</i>	ICMB1104	La Fosca	41°51'27" N, 3°8'38" E	20 August 2020	<i>Ostreopsis</i> sp.	ON081637	ASV46; ASV66
<i>D. arenysensis</i>	ICMB1105	Estartit harbour	42°3'5" N, 3°12'9" E	10 March 2021	<i>Alexandrium minutum</i>	ON081638	ASV46; ASV66
<i>Rhizophydiales</i> sp.	ICMB1106	Vilanova harbour	41°12'54.9" N, 1°43'57.9" E	01 April 2021	<i>A. minutum</i>	ON081639; ON081640	ASV84
<i>Rhizophydiales</i> sp.	ICMB1107	Arenys harbour	41°34'45.5" N, 2°33'19.8" E	10 March 2021	<i>A. minutum</i>	ON081641; ON081642	ASV613

Note: The last column reports the match between strain sequences and ASVs obtained by metabarcoding.

community. The resuspended samples were filtered through an 80- μm mesh, and 5-ml aliquots of each supernatant were then transferred to six-well plates for incubation. Lastly, portions of the surface sediment sampled were placed in double-bottomed tubes separated by an 8- μm mesh. *A. minutum*, as the host, was placed on the top to promote vertical swimming of the zoospores through the mesh and thus separate the parasite from the sediment.

All incubations were maintained in chambers at $20 \pm 1^\circ\text{C}$ with a 12:12 h (light: dark) photoperiod. Illumination was provided by LED fluorescent tubes with a photon irradiance of $\sim 40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The incubations were periodically checked for the presence of chytrid infections for up to 2 weeks. Infected dinoflagellate cells were manually isolated to establish monoclonal chytrid strains. Chytrids were cultured in 24-well plates under the same conditions as the incubations. Cultures were established by transferring a 1-ml aliquot of mature sporangia every 3–4 days to a well containing 1 ml of an exponentially growing uninfected host stock culture. The stock cultures of *A. minutum* (ICMB292) and *Ostreopsis* sp. (ICMB293) used to maintain the respective chytrid strains (Table 1) were obtained from the culture collection of the Institut de Ciències del Mar, Barcelona, Spain. A drop taken from each chytrid culture was placed on a glass slide and observed using a Leica DMIRB inverted microscope. Photographs were taken at $40\times$ magnification using a Jenoptik ProgRes C10 Plus camera; ProgRes Capture-Pro v2.8.8 software was used to capture the images.

DNA extraction from cultures and single-cell PCRs

Molecular information on the parasitoid strains was obtained by isolating individual mature sporangia. Glass micropipettes were used to physically separate the sporangium from the host dinoflagellate cell. The single-cell protocol was implemented following Reñé and Hoppenrath (2019) and Reñé et al. (2021). A first PCR was performed using the primers EK82F and 28S-1611R to amplify the entire ribosomal operon (López-García et al., 2001; Moreira et al., 2007). The PCR product was then used as a template to amplify the partial SSU rDNA gene, using the specific primers CRYPTO2-2F and EUK-1520R (Lazarus & James, 2015; López-García et al., 2003). The 4 μl of each product was electrophoresed on a 1.2% agarose gel. The gel was stained with SYBR Safe (Invitrogen) to visualize the bands and confirm the correct amplicon length. For samples with poorly visualizable bands, a third semi-nested PCR was performed using the specific primers CRYPTO2-2F and AU4v2 (Lazarus & James, 2015) and the product of the previous PCR as template. Additionally, the product of the first PCR was

used to amplify the LSU rDNA region, using the primers D1R and D3B (Nunn et al., 1996; Scholin & Anderson, 1994). The specific SSU and LSU rDNA regions were amplified in 25- μl reactions containing 1 μl of template DNA, 2.5 μl of buffer 10, 1.5 mM MgCl_2 , 0.2 mM of each dNTP, 0.4 μM of direct and reverse primers, and 2 U of Taq DNA polymerase. The PCR programme was as follows: initial denaturation at 94°C for 2 min, 35 cycles including denaturation at 94°C for 15 s, polymerization at 55°C for 30 s, and elongation at 72°C for 60 s, followed by a final elongation at 72°C for 60 s (Reñé et al., 2021). Finally, Sanger sequencing with forward and reverse primers was performed by an external service (Genoscreen, France). The obtained molecular sequences were deposited in GenBank under the accession numbers ON081632–ON081642 (SSU rDNA) and ON113503–ON113504 (LSU rDNA *Dinomyces arenysensis*).

Phylogenetic analysis

SSU rDNA sequences from the cultures were queried for sequence similarity using NCBI-BLAST against all ASVs from the metabarcoding dataset (Altschul et al., 1990). A dataset was constructed using the SSU rDNA sequences recovered from GenBank and covering the diversity of Chytridiomycota orders. The dataset was aligned using MAFFT v.7 (Katoh & Standley, 2013) and the –auto option and then trimmed with trimAL v.1.2 (Capella-Gutiérrez et al., 2009), using the –automated option to remove poorly aligned regions, resulting in 85 sequences and 1685 positions. The best substitution model was tested using jModelTest 2 (Darriba et al., 2012) and a maximum-likelihood phylogenetic tree was inferred using RAxML v.8 (Stamatakis, 2014), based on all parameters estimated by the programme and the best tree inferred from 1000 different starting trees. Once the best ML tree was obtained, the ASV sequences were added to the previous alignment using the –add option from MAFFT and trimmed again to remove poorly aligned positions in the V4 region. A maximum-likelihood phylogenetic tree was inferred again using the previously obtained tree, with reference sequences as a constrained tree. Bootstrap statistical support was evaluated using 1000 replicates.

RESULTS

Diversity and relative abundance of the protist community

Metabarcoding analyses evidenced differences in protist community composition between seawater and sediment. While the sediment was characterized by a greater heterogeneity that included dinoflagellates

(class Dinophyceae), diatoms, and other groups, such as Perkinsida and Spirotrichea (Figure 1), the seawater fraction was dominated by dinoflagellates, with a relative abundance per sample of 21.3%–97% versus 1%–69.3% in the sediment. The dominant dinoflagellate species identified in seawater coincided with those responsible for blooms and confirmed by microscopy (*Alexandrium minutum*, *Ostreopsis* sp., *Alexandrium taylori*, *Gymnodinium litoralis*, and *Kryptoperidinium foliaceum*). The dinoflagellate species composition in samples obtained during no-bloom conditions was heterogeneous, dominated by *Prorocentrum triestinum*, *Scrippsiella* sp., *Ansanella* sp., and *Levanderina fissa*.

A previous study compared the community composition of dinoflagellates based on microscopy and metabarcoding findings (Reñé et al., 2021). It showed that, after dinoflagellates, diatoms (class Bacillariophyta) were the second most abundant group, comprising 0.04%–54.2% of the seawater samples and 1.7%–56.8% of the sediment samples. Like dinoflagellates, diatoms were present in all studied samples. Other phytoplankton groups, such as Chlorophyta (class Chlorodendrophyceae), were poorly represented, as their relative abundance was generally <7.5% in both seawater and sediment and their distribution was much more localized (Figure 1).

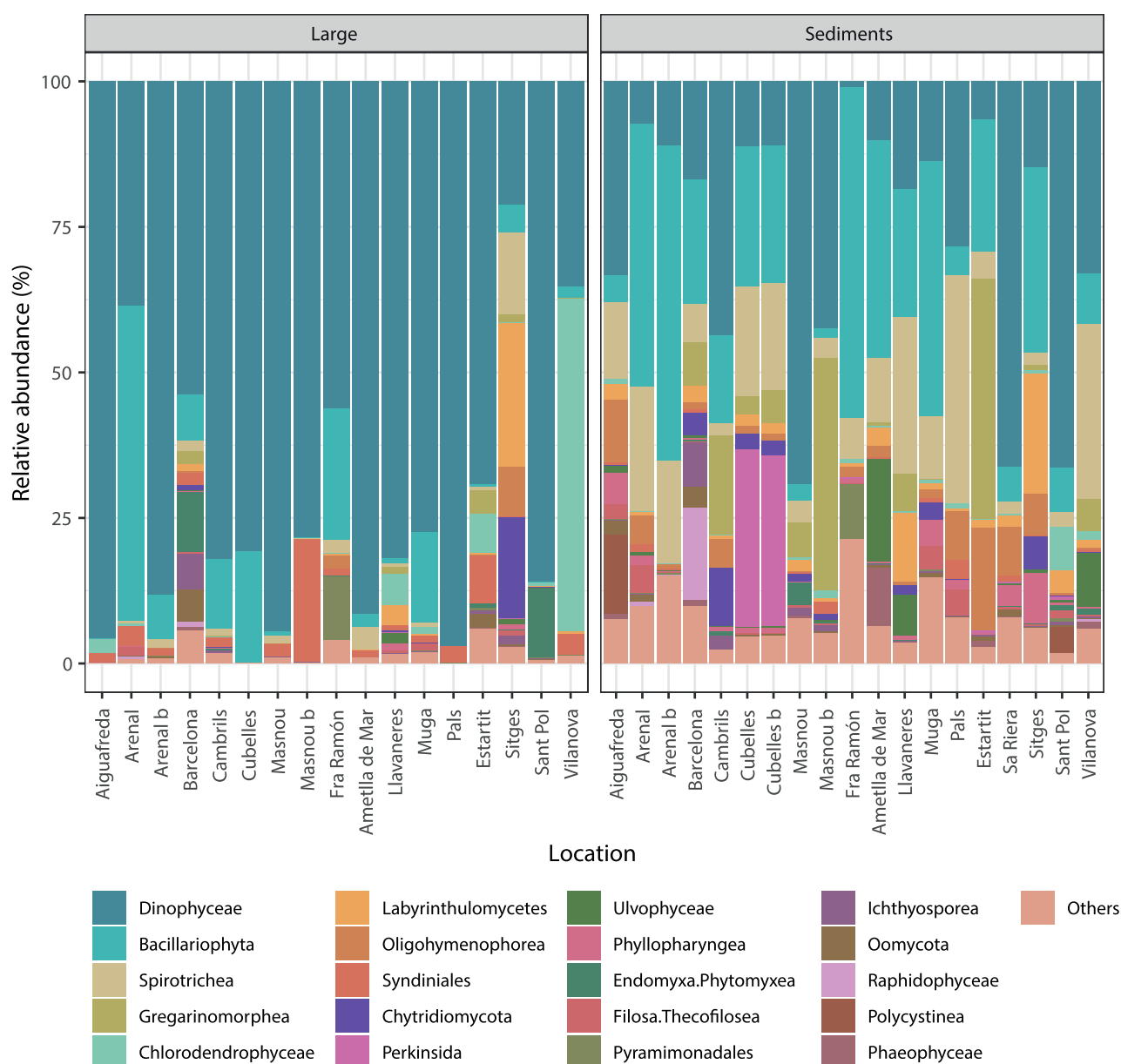


FIGURE 1 Barplot showing the relative abundance of the protist community in each sample as determined by metabarcoding. On the x-axis, samples from each location are grouped according to the fraction type: seawater >10 µm (left) and sediment (right). Replicates from the same location were standardized and averaged. The bars are divided according to the taxonomic class.

Diversity and relative abundance of the chytrid community

Metabarcoding analyses showed that the fungal community was dominated by members of the Chytridiomycota (chytrids), representing 1.04% of the total eukaryotic sequences (Figure 1). Chytrid sequences were detected in 72 of the 101 samples from all studied locations and accounted for a median 0.07% of the total relative abundance of the eukaryote community in seawater and a median 0.75% of that in sediment. In the

large seawater fraction, the highest relative abundance of chytrids was in the sample from Sitges (14.48%) whereas in the small seawater fraction, low percentages were registered in all locations. Among the sediment samples, chytrid relative abundance was highest (>10%) in the samples from Cambrils (Figure 2).

Alpha-diversity analyses (Chao1 richness and Shannon indices) of the chytrid community showed a higher diversity of Chytridiomycota sequences in sediment samples than in samples collected from the water column ($p < 0.05$ for both indices) (Figure 3).

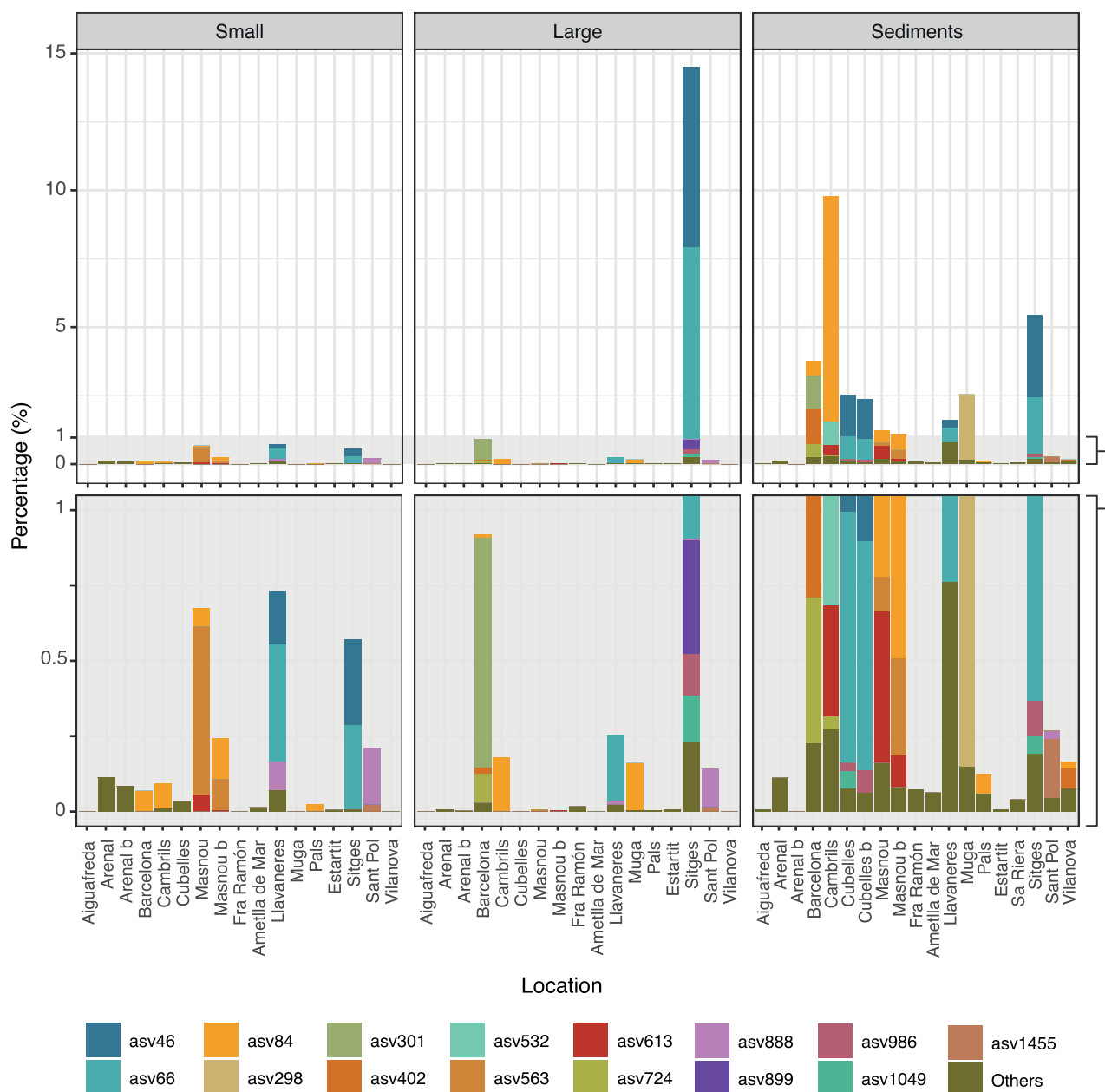


FIGURE 2 Relative abundance of the Chytridiomycota community obtained from 19 locations and the corresponding fraction: seawater small (0.8–10 µm), seawater large (>10 µm), and sediments. The relative abundances of the 15 most abundant ASVs are shown. The remaining 62 chytrid ASVs are grouped into the 'Others' category. Replicates from the same location were standardized and averaged. The lower graph shows a zoom of the relative abundance between 0% and 1% (grey area).

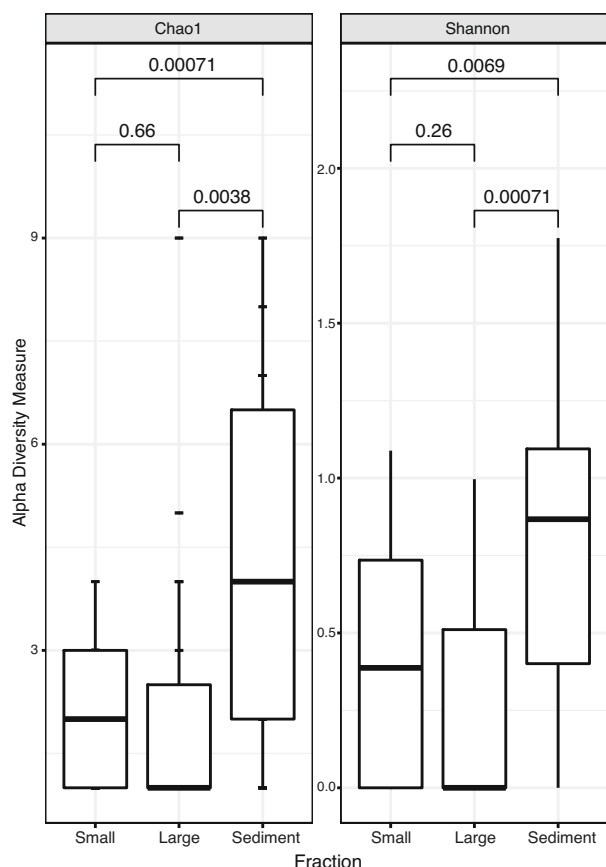


FIGURE 3 Bar-plot of the Chao1 and Shannon alpha-diversity indices, comparing the ASVs of sediment and seawater (small and large fraction) samples.

Chytrids accounted for a total of 77 ASVs. Among the 63 ASVs in sediment, 42 were exclusively detected therein. The large seawater fraction contained 25 ASVs and the small seawater fraction 19 ASVs, including 7 unique ASVs in each fraction. The majority of the reads belonging to chytrids corresponded to only three ASVs (Figure 2): ASV46, with relative abundances of up to 6.7% (seawater) and 3.0% (sediment); ASV66, with relative abundances of up to 7.0% (seawater) and 2.0% (sediment); and ASV84, with relative abundances of up to 0.3% (seawater) and 8.2% (sediment). The presence of chytrid ASVs varied depending on the locality and therefore the blooming dinoflagellate species in the sample (Figure 4). During *A. minutum* blooms, 27 chytrid ASVs were detected, 25 of which were unique to this bloom type. During *Ostreopsis* sp. blooms, 19 chytrid ASVs were obtained, of which 17 were unique. The non-bloom samples contained 17 chytrid ASVs, of which 11 were found only in these samples. During blooms of the dinoflagellates *Alexandrium taylori*, *Gymnodinium litoralis*, and *Kryptoperidinium foliaceum*, fewer than seven chytrid ASVs were obtained from each one. These results showed that most chytrid ASVs were exclusive to a particular bloom type.

A. minutum and *Ostreopsis* sp. blooms had the highest relative abundances of chytrids (Figure 4). ASV46 and ASV66 dominated the chytrid communities in all samples collected from *Ostreopsis* sp. blooms and were detected exclusively in those samples. Among the samples corresponding to *A. minutum* blooms, ASV84 had the highest relative abundance and was present in three of the five studied locations. However, this chytrid sequence was not exclusive to *A. minutum* as it was also present in blooms of *G. litoralis* and in the no-bloom samples (Figure 4).

Demonstration of chytrid–dinoflagellate interactions

The potential associations between dinoflagellate hosts and the chytrid sequences obtained in this study were evaluated in a correlation analysis. Figure 5 shows the 23 chytrid ASVs that correlated significantly with at least one dinoflagellate in the large seawater fraction. Among the dominant chytrid sequences, ASV46 co-occurred with *Amphidinium* sp. (0.91), *Scrippsiella* sp. (0.89), *Coolia* sp. (0.89), *Cryptothecodinium* sp. (0.98), and *Ostreopsis* sp. (0.49); ASV66 with *Ostreopsis* sp. (0.9), *Zooxanthellae nutricula* (0.81) and *Fragilidium* sp. (0.8); and ASV84 with *Alexandrium tamarens* (0.77) and *Polykrikos tanit* (0.79) (Figure 5).

Based on analyses of diversity, relative abundance, and host–parasite co-occurrences with respect to the Chytridiomycota community, *Ostreopsis* sp. and *A. minutum* blooms were selected as the best candidates to investigate the chytrid–dinoflagellate interactions at the studied locations. Additional samplings performed during *Ostreopsis* sp. and *A. minutum* blooms allowed the establishment of seven chytrid strains in culture, with four strains isolated from *Ostreopsis* and three from *A. minutum* (Table 1).

Identification of cultured strains and the phylogenetic classification of chytrid sequences detected by metabarcoding

Light microscopy revealed three distinct morphologies among the isolated strains. The first, exemplified by strain ICMB1106, consisted of mature sporangia with papillae, which allow zoospore discharge (Figure 6A). The second, exemplified by strain ICMB1107, contained a single lipid globule that was present during the long period of early sporangium development (Figure 6B). The third, exemplified by strains ICMB1101, ICMB1102, ICMB1103, ICMB1104, and ICMB1105, formed mature spherical sporangia without papillae (Figure 6C). The SSU rDNA sequences obtained from strains ICMB1101–1105 were identical

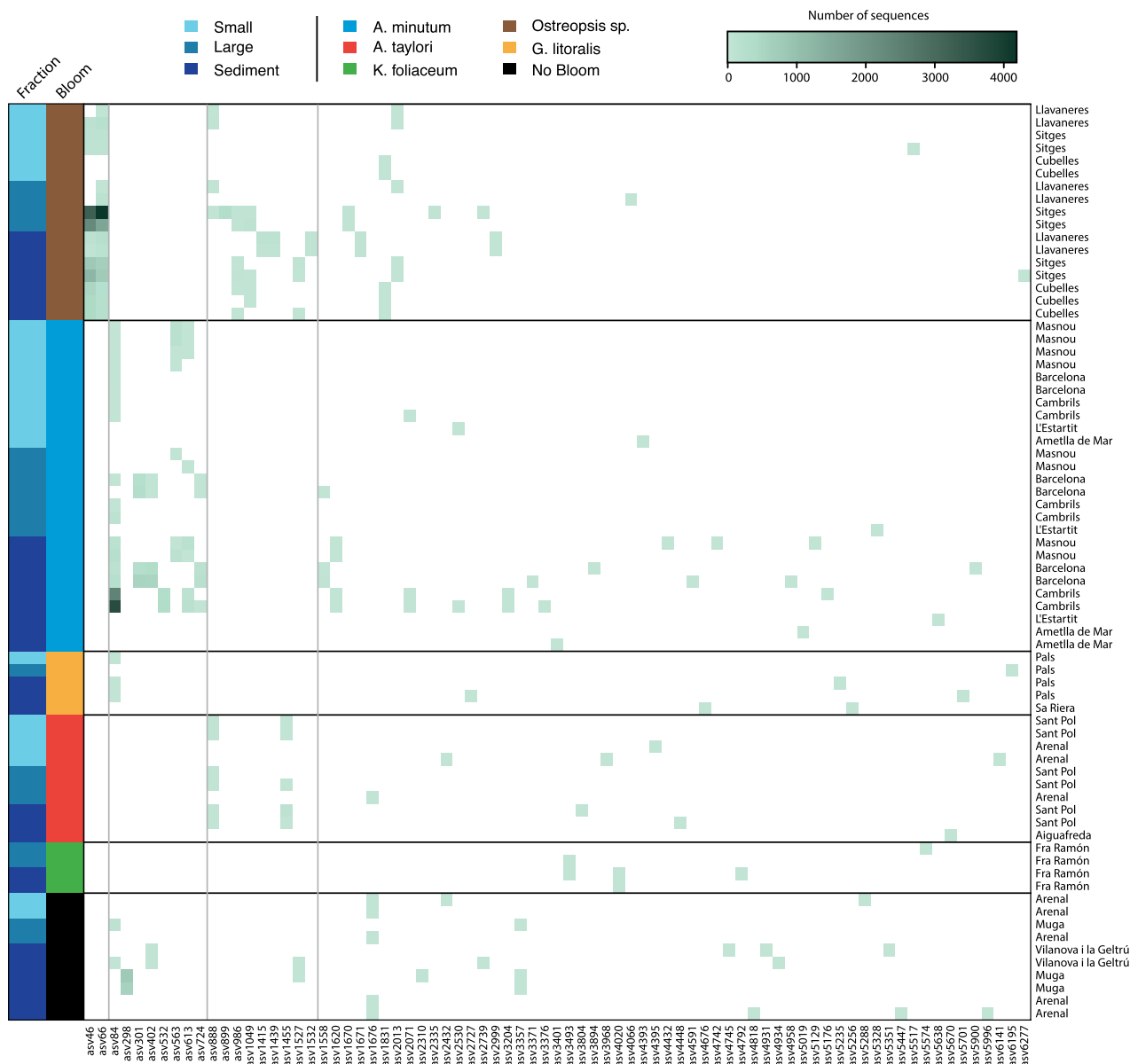


FIGURE 4 Heatmap of the chytrid ASVs (x-axis) identified in all samples (y axis). The left bars report the fraction and bloom type. The green colour gradient represents the number of sequences in the protist community, ranging from 0 (white) to 4183 (dark green).

and had 99.72% and 100% identity with ASV46 and ASV66, respectively. The analysis of the V4 SSU rDNA barcodes of ASV46 and ASV66 showed that the sequences were homologous, differing by only one nucleotide. This finding and the detection of a polymorphic position in sequences obtained from the cultures indicated that the two ASVs represent intraspecific variability of the same species (Table 1). Strains ICMB1101-ICMB1105 morphologically resembled *Dinomyces arenysensis*, a species previously detected in the study area. However, only the LSU rDNA and ITS sequences of *Dinomyces arenysensis*, not its SSU rDNA sequences, are publicly available. The LSU rDNA sequences of strains ICMB1101 and ICMB1102 were therefore obtained for comparison

with the sequences available in GenBank. The sequences of both strains showed 100% identity with the LSU rDNA sequence of *Dinomyces arenysensis* (KJ027548). Consequently, these cultured strains, and thus ASV46 and ASV66, were assigned to *Dinomyces arenysensis*.

The correspondence analysis showed that the SSU rDNA fragment of strain ICMB1106 matched ASV84 (100% identity) and that of strain ICMB1107 matched ASV613 (100% identity). The latter ASV had a relative abundance of <0.5% and was found in only two of the five *A. minutum* blooms sampled. Both strain ICMB1106 and strain ICMB1107 corresponded to newly detected marine chytrid species that interact parasitically with *A. minutum*. Detailed morphological and

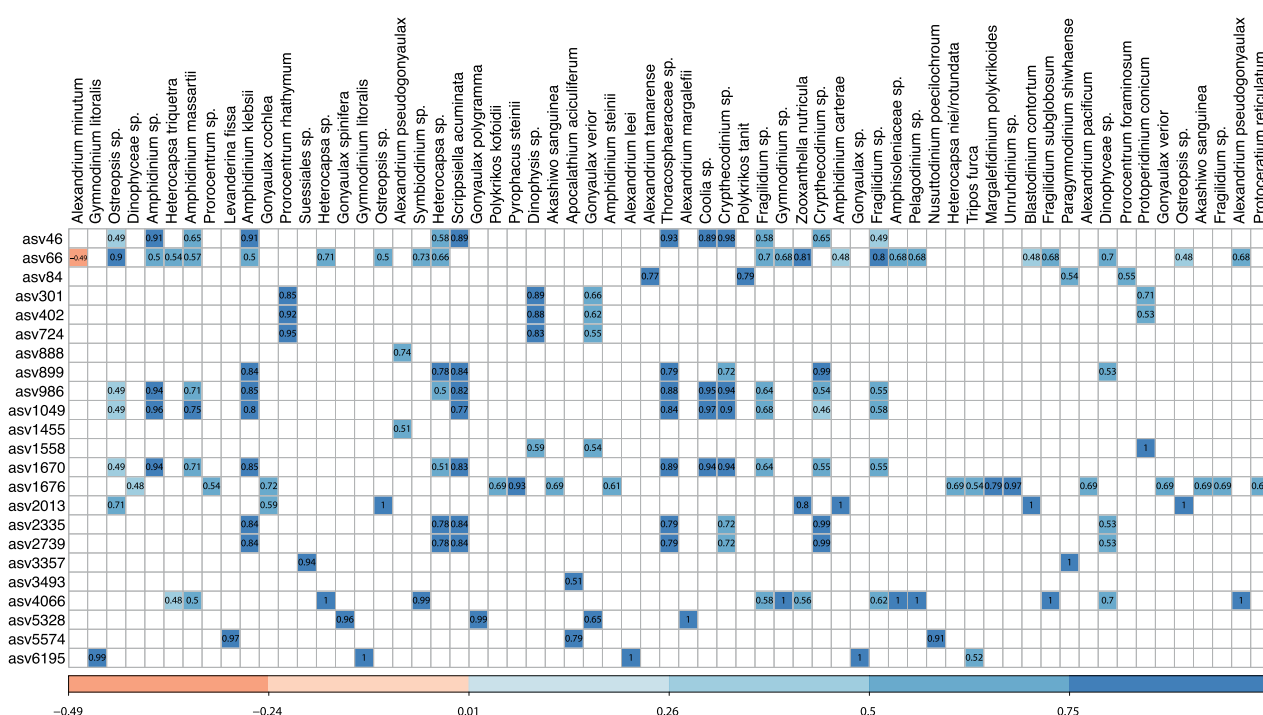


FIGURE 5 Evidence of potential host-chytrid co-associations based on correlations between dinoflagellate and chytrid ASVs. The Spearman correlation coefficients for the dinoflagellate and Chytridiomycota sequences, considering samples corresponding to the large seawater fraction and sediment, are shown. A significance threshold of 0.05 was applied. The correlation value is shown as a gradient ranging from dark blue (1) to orange (−0.5).

molecular information as well as formal descriptions will be provided in a future study.

The phylogenetic positions of the 77 chytrid ASVs in the Chytridiomycota phylum were analysed (Figure 7). Overall, most of the sequences were included in Rhizophydiales (23 ASVs, 30,866 reads) and Lobulomycetales (25 ASVs, 2348 reads). The three species isolated in this study clustered within Rhizophydiales. Eight of the ASVs corresponding to Rhizophydiales belonged to *Dinomyces arenysensis*, specifically ribotypes ASV46 (10,867 reads) and ASV66 (8936 reads), which, as noted above, differed in a single position. Five ASVs could be assigned to Rhizophydiales sp.1 Vilanova (ICMB1106), mainly represented by ASV84 (7388 reads), while ASV613 (532 reads) corresponded to Rhizophydiales sp.2 Arenys (ICMB1107). The sequences of *D. arenysensis* and Rhizophydiales sp. 1 Vilanova were closely related, but the position of Rhizophydiales sp. 2 Arenys was more distant. Nine additional ASVs could not be assigned to any of those species. Among Lobulomycetales, two ASVs clustered at the base of the clade, with the remaining 23 ASVs clustering together and forming a sister group with sequence EF443138. Representatives of other orders were also determined, including Zygothryctales (1 ASV, 5 reads) and Rhizophyctiales (6 ASVs, 38 reads). The phylogenetic position of some ASVs could not be assigned with certainty. A clade of four ASVs (952 reads) formed a sister clade

with Mesochytriales and Gromochytriales; three ASVs (72 reads) clustered in the clade *incertae sedis* 1 sensu (Seto et al., 2020), and eight ASVs (1503 reads) formed a sister clade with Neocallimastigomycota. Some ASVs did not show a clear relationship with known orders. Three ASVs, represented by 24 reads, clustered at the base of the Chytridiomycota.

DISCUSSION

In this study, the structure of Chytridiomycota communities under conditions of dinoflagellate blooms in coastal environments was characterized. Among the sequences obtained from the eukaryotic community, 1.04% belonged to Chytridiomycota. In studies of other marine environments, the relative abundance of this group similarly accounted for 1%–2% of the eukaryotic community (Celussi et al., 2018; Edgcomb et al., 2011; Enberg et al., 2018; Hassett et al., 2017, 2020; Picard, 2017; Stern et al., 2015). However, in our samples, the relative abundance of Chytridiomycota greatly varied depending on the location, the dinoflagellate bloom, and the sample source (small and large seawater and sediment fractions), ranging from a lack of detections to chytrids abundances that accounted for 14.48% of the total eukaryote sequences. A large variation in relative abundance was also observed in previous studies of coastal waters. For example, in a study

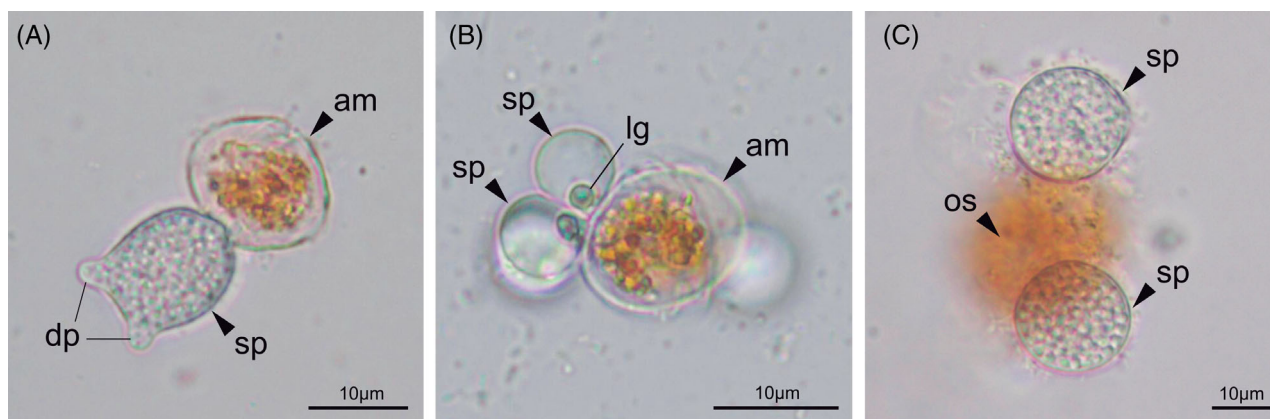


FIGURE 6 Microscopy images of chytrids on dinoflagellates. (A) Strain ICMB1106 infecting *Alexandrium minutum*. (B) Strain ICMB1107 infecting *A. minutum*. (C) Strain ICMB1104 (*Dinomyces arenysensis*) infecting *Ostreopsis* sp. am, *Alexandrium minutum*; dp, discharge papillae; lg, lipid globule; os, *Ostreopsis* sp.; sp, sporangium.

of microbial community structure in the Arctic Ocean, fungal environmental sequences accounted for a mean 1.35% of eukaryotic sequences, but they ranged from 0.01% to 59%, with Chytridiomycota and Chytridiomycota-like sequences constituting >50% of the fungal fraction (Comeau et al., 2016). A large variation in chytrid relative abundance was also found in the Plymouth coastal region (UK), where a specific chytrid sequence ranged from non-detectable to comprising 100% of the fungal community (Taylor & Cunliffe, 2016). The large oscillations in relative abundance may be primarily due to the availability and abundance of the chytrid host, as both influence the community composition not only of Chytridiomycota (Gutiérrez et al., 2016; Ibelings et al., 2004) but also that of other marine zoospore parasitic groups (Alacid et al., 2017). Other factors, such as predator grazing on free-living zoospores (Johansson & Coats, 2002) and the genetic diversity of the host and parasite populations (Blanquart et al., 2016), may also lead to oscillations in the relative abundances of chytrids in marine environments.

The results also showed the unequal distribution of chytrids between the water column and sediment in the same location, as in all cases the diversity Chytridiomycota sequences was higher in sediment than in planktonic samples. These results are in agreement with other studies in which Chytridiomycota, as well as other zoospore parasitic groups such as Perkinsea, were consistently detected in the sediment and their community structure was stable over time (Chambouvet et al., 2011; Orsi et al., 2013; Picard, 2017; Reñé et al., 2021; Xu et al., 2014). These observations support the hypothesis that sediments are a reservoir of diversity of zoospore parasites, which remain in a latent phase until favourable conditions enable their activation (Picard, 2017; Reñé et al., 2021).

The community structure of Chytridiomycota in all samples was represented by 77 different ASVs, but

only ASV46, ASV66, and ASV84, were highly abundant; the rest were either rarely detected or of low relative abundance. The dominant ASVs belonged to Rhizophydiales, consistent with its inclusion of several known parasites of dinoflagellates (Karpov et al., 2021; Lepelletier et al., 2014; Reñé et al., 2021). ASVs belonging to other orders, such as Lobulomycetales, were also obtained. Consistent with previous studies, our results suggest that the dominance of certain chytrid species is determined by host abundance (Picard, 2017; Scholz et al., 2017) and by the presence of different niches, which in turn depends on the available host/substrate resource, as demonstrated for freshwater chytrids (Van den Wyngaert et al., 2022). In our study, despite its temporal and geographical bias, the chytrid community associated with proliferations of *A. minutum* highly differed from that associated with proliferations of *Ostreopsis* sp., as >80% of the chytrid taxa were exclusive to each bloom type. This difference suggests that the structure of the chytrid community is driven by specific chytrid–host interactions. This was also observed for the marine fungal community under Arctic ice, in which members of the Lobulomycetes were dominant and parasitized diatoms, with a preference for *Pleurosigma elongatum* (Hassett et al., 2017). In a study in the Solthörn tidal flat, parasitic interactions between different chytrid strains infecting specific taxa of benthic diatoms were likewise observed (Scholz et al., 2016).

The chytrid–host co-occurrence determined by metabarcoding was further investigated in culture, and specifically with the host species *A. minutum* and *Ostreopsis* sp. From the *A. minutum* bloom samples, a new, as yet undescribed chytrid species (ICMB1106) was isolated and cultured. This species corresponded to the most representative chytrid sequence detected (ASV84) in that bloom. However, the sequence correlated with the dinoflagellates *Polykrikos tanit*, *Paragymnodinium* sp., *Prorocentrum* sp., and *A. tamarense* but

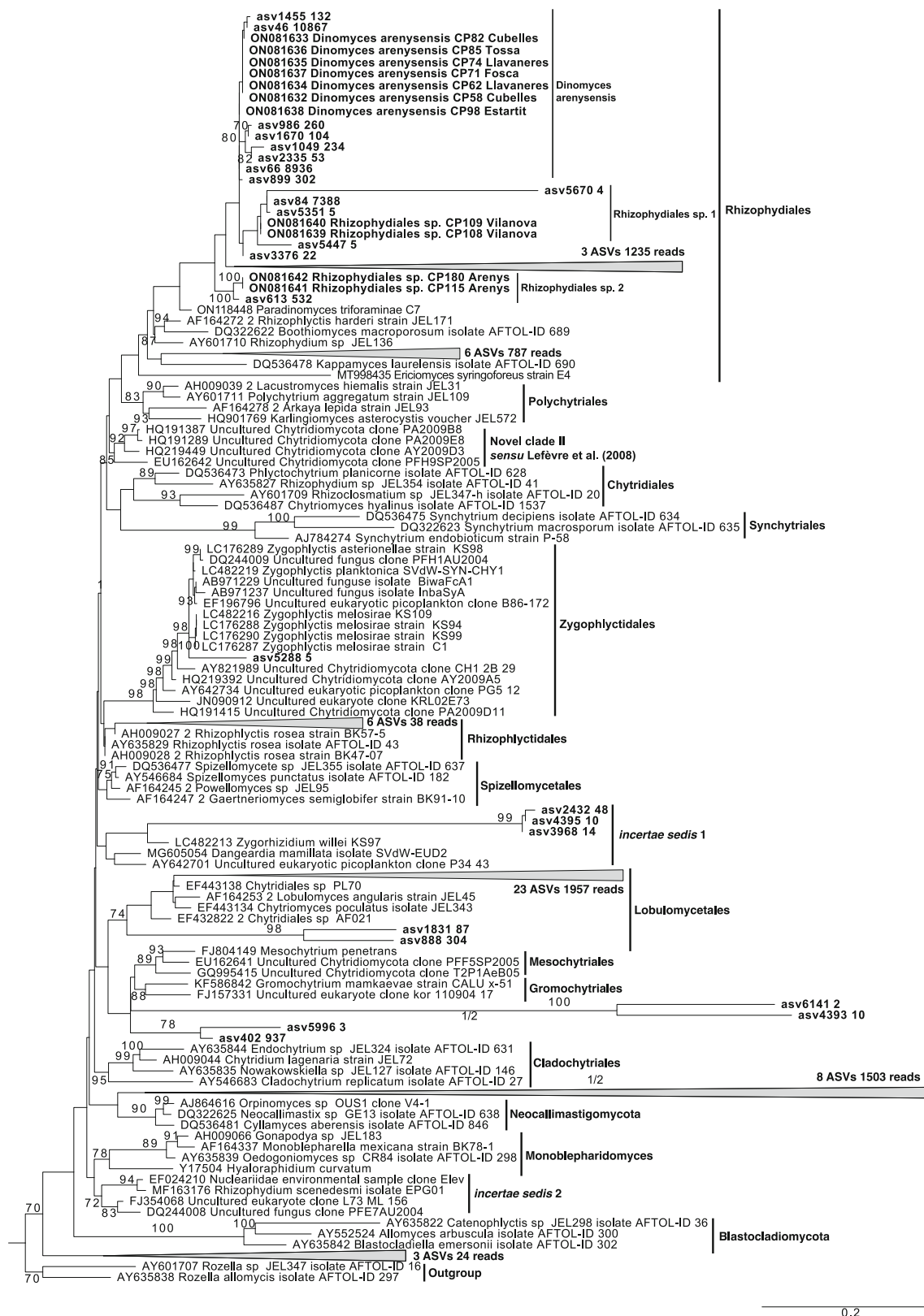


FIGURE 7 Maximum-likelihood phylogenetic tree of SSU rDNA sequences, including representatives of most orders belonging to Chytridiomycota. New sequences obtained from cultured strains and the ASVs from metabarcoding are shown in bold. The numbers following the ASV identifiers correspond to the reads obtained for the ASVs. Values in the nodes represent bootstrap (%) statistical support. Only values >70% are shown.

not with its putative host *A. minutum*. This may be due to the fact ASV84 was not always present in blooms of *A. minutum*, including those at L'Estartit and L'Ametlla de Mar (Figure 4), as other specific biotics factors may not have favoured its presence.

The cultured strain ICMB1107, linked to ASV613, was also isolated from *A. minutum* bloom samples. The low relative abundance of this chytrid, the absence of ASV613 in other bloom samples, and the lack of correlation with, at least, dinoflagellate sequences suggest that ASV613 represents a rare species whose interactions are thus far unknown. Establishing a link between interactions and co-occurrence is often challenging, especially for rare or transient species, independent of the method used (Calatayud et al., 2020). However, the establishment of laboratory cultures allows the interactions of these species and thus their dynamics in the marine environment to be studied in detail.

Among the ASVs assigned to *Dinomyces arenysensis*, both the metabarcoding sequences and the four strains obtained at different blooms of *Ostreopsis* sp. provide evidence of a prominent interaction between the chytrid parasite and this dinoflagellate. This finding also supports a previous lab host-range study, in which a moderate level of *Ostreopsis* sp. infection was demonstrated, although in that study *D. arenysensis* was isolated from an *A. minutum* bloom and was shown to infect that species at a high level (Lepelletier et al., 2014). The metabarcoding sequences of *D. arenysensis* also correlated with *Heterocapsa* sp., and *Scrippsiella* sp., thus supporting the same lab host-range experiment, which additionally found that the sensitivity of infection of these dinoflagellates was, respectively, moderate and high (Lepelletier et al., 2014). Correlations of *D. arenysensis* with *Amphidinium* sp., *Coolia* sp., and other dinoflagellates based on metabarcoding sequences have also been reported. However, neither culturing efforts nor host-range studies capture the interactions in nature (Blanchet et al., 2020). Thus, whether the correlations between *D. arenysensis* and these species were due simply to the usual appearance of the species in this epiphytic assemblage or to a true parasitic interaction with the dinoflagellate is unclear.

Our study showed that the community structure of Chytridiomycota in coastal environments is highly dependent on the blooming dinoflagellate species. It also demonstrated that insights into chytrid–dinoflagellate interactions can be gained through a combination of environmental sequencing, such as metabarcoding, and detailed laboratory studies of confirmed host (dinoflagellate)–parasite (chytrid) interactions. This combined approach can contribute to providing an accurate picture of the chytrid community and therefore of its host preferences as well as the consequences for the marine eukaryotic community.

AUTHOR CONTRIBUTIONS

Albert Reñé and Esther Garcés designed the study; all authors participated in sampling, laboratory procedures and data analysis; Alan Denis Fernández-Valero, Albert Reñé and Esther Garcés wrote the manuscript, with contributions from all co-authors.

ACKNOWLEDGEMENTS

This study was funded by the Spanish MICINN Project SMART PID2020-112978GB-I00, the MINECO Project COPAS CTM2017-86121-R. The authors acknowledge the institutional support of the 'Severo Ochoa Centre of Excellence' accreditation (CEX2019-000928-S). Alan Denis Fernández-Valero is funded by the MICIU grant 'Ayudas para contratos predoctorales para la formación de doctores 2018 (PRE2018-084893)'. Jordina Gordi is thanked for her dedication in maintaining the cultures.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. All the authors approve its submission and no-one entitled to authorship has been excluded. All prevailing local, national and international regulations and conventions, and normal scientific ethical practices, have been respected.

DATA AVAILABILITY STATEMENT

DNA sequences data from cultures have been submitted to the GenBank databases under accession number ON081632-ON081642 (SSU rDNA) and ON113503-ON113504 (LSU rDNA *Dinomyces arenysensis*). Raw data used in the study are available in NCBI SRA PRJNA630546.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Fernández-Valero, A.D., Reñé, A., Timoneda, N., Sampedro, N. & Garcés, E. (2022) Dinoflagellate hosts determine the community structure of marine Chytridiomycota: Demonstration of their prominent interactions. *Environmental Microbiology*, 24(12), 5951–5965. Available from: <https://doi.org/10.1111/1462-2920.16182>

9.2 Supplementary material of chapter 2

Table 9.1: **Supplementary table 1.** Characteristics of sampling sites and dates, adding information to Table 1 from Reñé et al. 2021. Asterisks indicate that only sediment was processed..

Location	Type	Date	Coordinates	Main blooming dinoflagellate
El Masnou	Harbour	02-03-2018	41°28'31.7"N; 2°18'26.1"E	<i>Alexandrium minutum</i>
El Masnou	Harbour	07-03-2018	41°28'31.7"N; 2°18'26.1"E	<i>A. minutum</i>
Barcelona	Harbour	28-03-2019	41°22'45.1"N; 2°10'56.2"E	<i>A. minutum</i>
Cambrils	Harbour	15-05-2019	41°03'47.3"N; 1°03'35.5"E	<i>A. minutum</i>
L'Estartit	Harbour	05-06-2019	42°03'05"N; 03°12'09"E	<i>A. minutum</i>
L'Ametlla de Mar	Harbour	27-06-2019	40°52'54.4"N; 0°48'10.6"E	<i>A. minutum</i>
Sant Pol	Beach	25-07-2018	41°47'25.1"N; 03°02'58.2"E	<i>Alexandrium taylorii</i>
L'Arenal	Beach	18-07-2019	40°47'31.3"N; 00°42'16.6"E	<i>A. taylorii</i>
Aiguafreda	Beach	04-07-2019	41°57'52.1"N; 03°13'37.4"E	<i>A. taylorii</i>
Sa Riera*	Beach	04-07-2019	41°58'22.7"N; 03°12'43.3"E	<i>Gymnodinium litoralis</i>
Pals	Beach	04-07-2019	41°59'45.2"N 03°12'00.5"E	<i>G. litoralis</i>
Llavaneres	Beach	25-07-2018	41°33'07.5"N; 02°29'31.6"E	<i>Ostreopsis</i> sp.
Sitges	Beach	07-08-2018	41°13'12.0"N; 01°46'48.0"E	<i>Ostreopsis</i> sp.
Cubelles*	Beach	21-08-2019	41°11'51.8"N; 01°40'23.0"E	<i>Ostreopsis</i> sp.
Cubelles	Beach	27-08-2019	41°11'51.8"N; 01°40'23.0"E	<i>Ostreopsis</i> sp.
Fra Ramon	Coastal lagoon	18-06-2019	42°01'12.0"N 3°11'24.0"E	<i>Kryptoperidinium foliaceum</i>
Vilanova i la Geltrú	Harbour	05-06-2018	41°12'54.9"N; 1°43'57.9"E	No Bloom of target host
La Muga	Beach	05-06-2019	42°13'48.0"N; 3°07'12.0"E	No Bloom of target host
L'Arenal	Beach	27-06-2019	40°47'31.3"N; 0°42'16.6"E	No Bloom of target host

9.3 Original article of chapter 3

The succession of epiphytic microalgae conditions fungal community composition: how chytrids respond to blooms of dinoflagellates.

Fernández-Valero, A.D., Reñé, A., Timoneda, N. et al. The succession of epiphytic microalgae conditions fungal community composition: how chytrids respond to blooms of dinoflagellates. ISME COMMUN. 3, 103 (2023). <https://doi.org/10.1038/s43705-023-00304-x>

ARTICLE OPEN



The succession of epiphytic microalgae conditions fungal community composition: how chytrids respond to blooms of dinoflagellates

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This study aims to investigate the temporal dynamics of the epiphytic protist community on macroalgae, during the summer months, with a specific focus on fungi, and the interactions between zoospore chytrid parasites and the proliferation of the dinoflagellates. We employed a combination of environmental sequencing techniques, incubation of natural samples, isolation of target organisms and laboratory experiments. Metabarcoding sequencing revealed changes in the dominant members of the epiphytic fungal community. Initially, fungi comprised < 1% of the protist community, mostly accounted for by Basidiomycota and Ascomycota, but with the emergence of Chytridiomycota during the mature phase of the biofilm, the fungal contribution increased to almost 30%. Chytridiomycota became dominant in parallel with an increase in the relative abundance of dinoflagellates in the community. Microscopy observations showed a general presence of chytrids following the peak proliferation of the dinoflagellate *Ostreopsis* sp., with the parasite, *D. arenysensis* as the dominant chytrid. The maximum infection prevalence was 2% indicating host-parasite coexistence. To further understand the *in-situ* prevalence of chytrids, we characterised the dynamics of the host abundance and prevalence of chytrids through co-culture. These laboratory experiments revealed intraspecific variability of *D. arenysensis* in its interaction with *Ostreopsis*, exhibiting a range from stable coexistence to the near-extinction of the host population. Moreover, while chytrids preferentially parasitized dinoflagellate cells, one of the strains examined displayed the ability to utilize pollen as a resource to maintain its viability, thus illustrating a facultative parasitic lifestyle. Our findings not only enrich our understanding of the diversity, ecology, and progression of epiphytic microalgal and fungal communities on Mediterranean coastal macroalgae, but they also shed light on the presence of zoospore parasites in less-explored benthic habitats.

ISME Communications; <https://doi.org/10.1038/s43705-023-00304-x>

INTRODUCTION

Fungi are an important component of the microeukaryotic community in aquatic environments. They engage in a wide variety of parasitic and saprophytic interactions [1] involving diverse members of the microbial community. Studies conducted in freshwater habitats have shown that fungal interactions range from competitive strategies for resource acquisition to the development of coexistence mechanisms based on niche differentiation [2]. While abiotic factors exert an impact on fungal community [3, 4], biotic factors, especially the influence of other organisms within the microbial community, also play a significant role [5]. Within the microbial community, the temporal patterns of fungal species composition range from seasonal and repetitive cycles to brief peaks in abundance. For instance, sudden increases in the abundance of zoospore fungi belonging to the Chytridiomycota (referred to as chytrids) occur nearly simultaneously with diatom blooms [2, 6]. In addition, infections by members of the Chytridiomycota during the proliferations of other microalgal groups, including cyanobacteria [7, 8] and dinoflagellates [9, 10], have also been documented.

Fungi have been identified in diverse niches across the marine environment [11], including the benthic habitat [12]. The identification of a large number of fungal sequences in the benthic habitat indicates its role as a reservoir of zoospore parasites, including chytrids, thus contributing to the overall diversity of the marine environment. In a previous study, the diversity of benthic microbial communities and the prevalence of specific fungal groups have revealed gradients spanning coastal, offshore, and deep-sea regions [13]. The authors observed that Chytridiomycota were dominant in the coastal zone, whereas Ascomycota and Basidiomycota were more prevalent on the seafloor. Another study specifically conducted within coastal area highlighted higher diversity of marine chytrid in sediment compared to the water column [9].

The characteristics of the benthic habitat, including inorganic substrate such as rocky surfaces, and organic substrates such as macroalgae and seagrass surfaces, exert significant influence on shaping the microbial community that develops into a biofilm. Notably, the organic substrates within the benthic habitat, primarily consisting of macroalgae and seagrass surfaces, create

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Received: 18 August 2023 Revised: 31 August 2023 Accepted: 5 September 2023

Published online: 26 September 2023

a nutrient-rich milieu abundant inorganic carbon. This, in turn, facilitates microbial colonization and promoting biofilm formation on available surfaces [14, 15]. The community composition of these epiphytic microorganisms is shaped by multiple biotic interactions that reflects the dynamic and complex interplay between the epiphytic community and the substrate [16, 17]. In the case of epiphytic fungi, these interactions involve colonization of the substrate through zoospores or hyphae, leading to the establishment of parasites, saprophytes, mutualists or endosymbionts [18]. An example of these interactions was observed in the study of epibiotic fungi on seaweed in the Red Sea, Egypt, wherein different available host seaweed species exerted strong selective pressure and temporal changes on the associated fungal populations [19]. Specific interaction among epiphytic species modulate the community composition on macroalgae. As an illustration, the fungus *Aspergillus versicolor*, found in the brown seaweed *Sargassum thunbergii*, generates compounds with antibacterial properties, thereby selectively limiting the growth of prokaryotes [20]. Furthermore, the epibiotic fungus *Trichoderma virens*, isolated from the red alga *Gracilaria vermiculophylla*, has been reported to secrete compounds that inhibit the growth of phytoplankton species such as *Chattonella marina*, *Heterosigma akashiwo*, *Karlodinium veneticum* and *Prorocentrum donghaiense*, while also displaying toxicity towards certain zooplankton species [21].

In our study system, the colonization of macroalgae by epiphytic protist is a well-established process [22, 23]. The initial stages of epiphytic assemblage development on Mediterranean macroalgae are typically characterized by the presence of microalgae, particularly diatoms of the *Navicula*, *Nitzschia*, and *Coscinodiscus* genera. These diatoms possess the ability to attach to both inorganic or organic substrates and withstand hydrodynamic stress [24]. Moreover, certain benthic diatom species such as *Navicula* sp. and *Proschkinia* sp. have a negative impact on the growth of other microalgae, including the dinoflagellate *Ostreopsis*. As a result, they influence the composition of the epiphytic protist assemblage [25]. In the later stages of epiphyte biofilm formation on macroalgae, dinoflagellates of the genera *Gambierdiscus*, *Coolia*, *Prorocentrum*, *Amphidinium* and the aforementioned *Ostreopsis* become dominant.

Despite having knowledge of species succession within benthic epiphytic assemblages, the succession patterns of other heterotrophic protist groups, such as fungi, remain incompletely described and understood. Similarly, the temporal dynamics and interaction between chytrids and epiphytic dinoflagellates have yet to be investigated. We hypothesized that temporal changes in the composition of the epiphytic assemblage influence the presence and abundance of co-occurring fungi. Therefore, this study combines field sampling and laboratory experiments to investigate into the temporal succession of the epiphytic assemblage. Moreover, it explores the ecological interaction between Chytridiomycota and dinoflagellates over time, focusing on the dynamics of the most abundant chytrid, *Dinomyces arenysensis*, during its infection of its host, the bloom-forming dinoflagellate *Ostreopsis* sp.

MATERIALS AND METHODS

Field sampling

Twelve weekly samplings were conducted from June to September 2021 at one station in the coastal locality of Sant Andreu de Llavaneres, NW Mediterranean Sea (41°33'07.5"N; 2°29'31.6"E) to determine the temporal dynamics of the epiphytic assemblage on macroalgae. The collection and treatment of the samples were based on the standard protocol for studying epiphytes associated with macrophytic substrates [26]. The macroalgal growing season extends from early June to late August [27]. At each sampling, samples of macroalgae were collected from the rocky surface of the study area at depths of 1–1.5 m. The sampled macroalgae represent the predominant species in the study area: *Halopteris scoparia*,

Jania rubens, *Padina pavonica*, *Dictyota dichotoma* and *Cladophora* sp., and reductions in macroalgal cover or changes in epiphyte composition were not observed throughout the sampling period. The samples were placed in flasks containing 1 L of autoclaved seawater, which was used to wash and resuspend the epiphytic protist community. The macroalgae were then separated from the epiphytic resuspension and the fresh weight of the macroalgae (gFW) was determined. The resuspended samples were vigorously shaken until all biofilm was disintegrated. The epiphytic resuspensions were filtered through a 100 µm mesh and a 150 mL aliquot was fixed with Lugol's iodine to determine the cell abundance of diatoms and dinoflagellates, by settling 10 mL of each fixed sample in Utermöhl chambers for 24 h [28]. Sedgewick-Rafter counting chambers were used when the cell abundance of certain species was too high. Cells were counted using a Leica-Leitz DMIRB inverted microscope equipped with a Jenoptik ProgRes C10 Plus camera. The dimensions of ten cells of each taxon were measured using the ProgRes CapturePro v2.8.8 software. Biovolumes were calculated using the appropriate geometric formulae [29], and measurements were taken for 15 cells per taxon. The results were expressed as cubic millimetres per gram of fresh weight of macroalgae ($\text{mm}^3 \text{gFW}^{-1}$).

Metabarcoding sequencing and analyses

Each water sample derived from resuspension of the macroalgae was immediately filtered in two replicates using non-cytotoxic Nalgene™ polysulfone bottles and 8 µm polycarbonate filters with a diameter of 47 mm. The organisms on the filters represented the epiphytic assemblage spanning the 8 to 100 µm size fraction. The filters were stored frozen at −70 °C until processing. Genomic DNA from the filtered samples was extracted using the DNeasy PowerSoil kit (Qiagen) according to the manufacturer's instructions. Metabarcoding sequencing of the V4 region of the 18S rDNA was performed using the universal primers EK-565F [30] and EUK-1134R-UnonMet (biased against metazoans [31]) to determine the protist community, defined as all eukaryotic microorganisms, including fungi, ranging from 8 µm to 100 µm. The 8 µm filter would exclude fungal zoospores and consequently result in an underestimation of fungal proportions. The fungal-specific primers ITS3-KYO2 [32] and ITS4 [33] were used to amplify the 5.8S and ITS2 fragments of the rDNA operon (hereinafter referred to ITS region). This region represents the best option for amplification and taxonomic identification of fungi [34]. Both the V4 18S rDNA and the ITS regions were sequenced on an Illumina PE300 system. The raw reads were trimmed from the adaptors using CUTADAPT [35]. The DADA2 pipeline [36] was used to filter and dereplicate the reads, trim low-quality sequences, merge paired-ends, and remove chimeras. The parameters maxEE = 4, 6 and truncLen = 270, 250 were applied to both regions (V4 18S rDNA and ITS), and minOverlap = 20 for V4 18S rDNA and minOverlap = 50 for ITS. The obtained ASVs were taxonomically assigned using a simple non-Bayesian taxonomy classifier, VSEARCH v2.8.1 [37], with the PR2 database version 4.12 [38] customized with new in-house sequences for the V4 region and the full UNITE + INSD dataset from UNITE community v.8.3 for the ITS segment [39] and with bootstrap cut-off = 0.6. Unassigned ITS sequences that could not be classified as belonging to fungal organisms were removed from the dataset and were not considered for the fungal community analysis. The amplicons were 490 bp for V4 18S rDNA and 450 bp for ITS. The data were normalized according to the relative abundance of ASVs per sample. Raw metagenomic reads were submitted to the NCBI Sequence Read Archive under Bioproject numbers PRJNA891539 and PRJNA891506, corresponding to the V4 18S rDNA and ITS region, respectively. The ASV tables, including the FASTA sequences and taxonomic classifications, are available in Supplementary File 1. Metabarcoding data were analysed using the package Phyloseq and all graphs were generated using the package ggplot2 from Rcran [40–42]. Nonmetric multidimensional scaling (NMDS) using Bray-Curtis distances was then performed to analyse the dissimilarity between samples and permutational multivariate analysis of variance using distance matrices (ADONIS) was performed to assess differences between sample groups.

Dinoflagellate and chytrid sporangium cell counting by epifluorescence microscopy

Chytrid presence, defined herein as the detection of *Ostreopsis* cells infected with *Dinomyces*, was assessed using the filters representing the temporal samplings at the coastal locality. Infections were attributed to *Dinomyces* based on previously reported metabarcoding results, isolation of the strains, the identification of single cells, and the morphological

identification of sporangia from laboratory cultures of *D. arenysensis*. A 10 mL fraction of the sample derived from the macroalgal resuspension was fixed with 10% formaldehyde (v:v) for 24 h, filtered through 10 µm polycarbonate filters, and stored at -70°C . The filters were covered with a 0.1% agarose permeable film to prevent cell loss during further manipulation. The filters were then cut into triangular sections and placed on slides with a drop of DAPI oil to stain the DNA strands. The oil solution was prepared from 1.25 µL of 0.5 mg DAPI (4',6-diamidino-2'-phenylindole dichlorohydrochloride) mL^{-1} and 125 µL of a pH 9.5 solution composed of 100 mL of glycerol, 2 mL of Vectashield, and 1 mL PBS. Stained filter sections were observed at 1000× magnification using an Olympus Bx61 epifluorescence microscope connected to an Olympus DP72 camera (Olympus America Inc.). The following fluorescence filters were used for DAPI detection: excitation BP330-385, dichroic mirror DM400, and barrier filter BA420. The infection prevalence (%) was calculated as the percentage of infected *Ostreopsis* cells with respect to the total number of cells. The determination of infection prevalence in natural samples was based solely on the presence of mature sporangia, visualized using epifluorescence microscopy.

Isolation, culture of chytrids, and identification of cultured strains by single-cell PCR

All cultured strains used in this study were obtained from the stock culture collection of the Institut de Ciències del Mar (ICM-CSIC). In the laboratory, chytrid cultures were established by transferring 5 mL aliquots of the resuspended epiphytic assemblages to 6-well plates for incubation. For samples with *Ostreopsis* abundances $< 10^3$ cells g FW^{-1} , healthy cells from an *Ostreopsis* sp. culture (strain ICMB293) were added to the incubations to increase host density and promote parasite infection. All incubations were maintained in chambers at $20 \pm 1^{\circ}\text{C}$ and subjected to a 12:12 h photoperiod (light:dark). Illumination was provided by LED fluorescent tubes with a photon irradiance of $\sim 40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The incubations were periodically checked for the presence of chytrid infections for up to 2 weeks. Infected dinoflagellate cells were manually isolated to establish clonal chytrid strains. The chytrids were grown in 24-well plates by transferring a 1 mL aliquot of mature sporangia every 3–4 days to a well containing 1 mL of an exponentially growing uninfected host stock culture. An *Ostreopsis* sp. strain (ICMB293) maintained under non-axenic conditions in F/2 medium and a salinity of 36 was used to maintain the chytrid strains. Samples from the chytrid cultures were collected and stained with Alcian blue (1% solution) to identify the polysaccharide structures constituting the mucilage of the host. The isolated strains were observed using a Leica DMIRB inverted microscope. Photographs were taken at 40–60× magnification using a Jenoptik ProgRes C10 Plus camera.

Individual mature sporangia were isolated from the parasitoid strains using glass micropipettes and then subjected to further molecular characterization. The single-cell protocol was implemented following [43] and [44]. A first PCR was performed using the primers EK82F and 28S-1611R [45, 46], to amplify the entire ribosomal operon. The PCR product served as a template to amplify the partial V4 18S rDNA gene, using the specific primers CRYPTO2-2F and EUK-1520R [47, 48]. Four µL of each product was electrophoresed on a 1.2% agarose gel. The gel was stained with SYBR Safe (Invitrogen) to visualize the bands and confirm the correct amplicon length. For samples with poorly visualizable bands, a third semi-nested PCR was performed using the specific primers CRYPTO2-2F and AU4v2 [48] and the product of the previous PCR as template. Finally, Sanger sequencing with forward and reverse primers was performed by an external service (Genoscreen, Lille, France).

Infection dynamics

The temporal dynamics of *Dinomyces arenysensis* infection were examined using strains isolated during *Ostreopsis* proliferations at three different locations of the Catalan coast. Strain ICMB1101 was isolated from Cubelles Beach ($41^{\circ}11'51.8'' \text{ N}$, $1^{\circ}40'23.0'' \text{ E}$) in September 2019; strain ICMB1102 was isolated from Llanvaneres Beach ($41^{\circ}33'07.5'' \text{ N}$, $2^{\circ}29'31.6'' \text{ E}$) in July 2020; and strain ICMB1104 was isolated from La Fosca Beach ($41^{\circ}51'27'' \text{ N}$, $3^{\circ}8'38'' \text{ E}$) in August 2020. Cell abundances of the host *Ostreopsis* sp. strain ICMB293 and the chytrid zoospores in each stock culture were determined. Culture flasks were inoculated in triplicate with healthy host cells and zoospores from each chytrid strain (ICMB1101, ICMB1102, ICMB1104), at a host:parasite ratio of 1:10 and a final volume of 20 mL. The initial abundances of *Ostreopsis* cells and zoospores were 300 mL^{-1} and 3000 mL^{-1} , respectively. This low initial parasite abundance allowed to

study the development of the infection from its early stage, and thus host-parasite dynamics to be monitored over time. The incubations were maintained at 21°C and a 10 h:14 h light:dark cycle. The infections were followed for 20 days, with the flasks sampled on days 2, 5, 10, 16, and 20. The collected samples (2 mL) were fixed with formaldehyde (final concentration 3.6%) and then counted in a Sedgewick-Rafter chamber by light microscopy to determine the number of healthy and infected *Ostreopsis* cells as well as chytrid prevalence.

Growth of chytrids using an alternative nutrient source

The ability of the chytrids to grow on organic sources other than dinoflagellate cells was examined using *D. arenysensis* strains ICMB1101, ICMB1103, and ICMB1104. Strain ICMB1103 is a monoclonal culture of *D. arenysensis* isolated from Tossa Beach ($41^{\circ}42'55.9'' \text{ N}$, $2^{\circ}55'56.4'' \text{ E}$) in August 2020. Pine pollen (*Pinus pinea*) was added to the chytrid culture in a 10 mL 6-well plate. The presence of infections was checked by inverted light microscopy of the plates after 4 and 6 days of incubation. Thereafter, an inoculum of healthy *Ostreopsis* culture (strain ICMB293) was added to the wells to determine the viability of the sporangia and their ability to infect dinoflagellate cells.

RESULTS

Diversity and relative abundance of the eukaryotic community

The epiphytic protist community (8 to 100 µm fraction) on macroalgae was represented by 2153 ASVs, based on metabarcoding of the V4 18S rDNA region. The metabarcoding data could be categorized into three temporal phases according to community composition and the relative abundance of specific groups, including as Dinoflagellata, Ochrophyta (diatoms), Ciliophora, and Fungi (Fig. 1). The three phases of the community structure were observed from a Non-Metric Multidimensional Scaling (NMDS) analysis (Supplementary Fig. 1) and its statistical significance was confirmed through a Permutational multivariate analysis of variance using distance matrices (ADONIS. $R^2 = 0.48$, p -value < 0.001).

The differentiation between these phases was mainly attributed to the presence of distinct dominant taxonomic groups in each respective phase. The first phase, corresponding from June 14th to July 8th, was characterized by the dominance of Ochrophyta (diatoms), which accounted for $> 50\%$ of the relative abundance. This was followed by other groups of microalgae, including Dinoflagellata (dinoflagellates) and Ciliophora (Fig. 1a). In the second phase, observed from July 15th to July 22nd, the relative abundance of dinoflagellates ranged from $> 60\%$ to nearly 100%. During the third phase, from July 29th to September 1st, the relative abundance of dinoflagellates remained high ($\sim 50\%$) but that of other groups, including Fungi, Apicomplexa, Chlorophyta, Sagenista, Mesomycetozoa, and unassigned taxa, increased (Fig. 1a). However, microscopy of the samples showed slightly different dynamics of the epiphytic protist assemblage. The proportions of diatoms and dinoflagellates determined by microscopy were based on the biovolume (Fig. 1c). Thus, during the first phase, the biovolume of epiphytic diatoms and dinoflagellates was $< 0.1 \text{ mm}^3 \text{ gFW}^{-1}$. In the second phase, the fraction of epiphytic dinoflagellates increased to $21.8 \text{ mm}^3 \text{ gFW}^{-1}$ while that of epiphytic diatoms remained unchanged. In the third phase, the dinoflagellate fraction reached a maximum of $14.7 \text{ mm}^3 \text{ gFW}^{-1}$ (August 4th) followed by a progressive decrease to $0.2 \text{ mm}^3 \text{ gFW}^{-1}$. During the month of August, the dominant fraction of organisms was dinoflagellates, consistent with the metabarcoding results. However, the biovolume of diatoms gradually increased such that by September the epiphyte assemblage was dominated equally by dinoflagellates and diatoms (Fig. 1c), which was not observed in the metabarcoding data.

Diversity and relative abundance of the dinoflagellate assemblage

Given that dinoflagellates were the most abundant taxonomic group on macroalgae along the epiphytic succession, the



Fig. 1 Temporal dynamics of the epiphytic assemblage on macroalgae. **a** Bar plot showing the variation (%), in replicates, of the different epiphytic eukaryote groups at each sampling date. **b** Bar plot showing the variation in the epiphytic dinoflagellate assemblage on macroalgae. Relative abundances (%) are based on the total protist community. **c** Non-stacked area plot showing the variations in the biovolumes of epiphytic diatom and dinoflagellate groups. Vertical dashed lines represent the three temporal phases described in the text.

structure of the epiphytic dinoflagellate assemblage was evaluated in detail. The metabarcoding results showed structural differences between the three phases described above (Fig. 1b), with a high diversity of species, including those belonging to the genera *Amphidinium*, *Coolia*, *Symbiodinium* and unassigned taxa, during the first phase. *Ostreopsis* sp. appeared in early July, with a relative abundance of < 6%. During the second phase, the relative abundance of this species increased from 40% to almost 100% of the dinoflagellate and protist sequences. During the third phase, the relative abundance of *Ostreopsis* sp. decreased from 25% (July 29th) to < 6% in subsequent samples. The composition of the dinoflagellate sequences during the third phase was more diverse than during the first and second phases and was dominated by Suessiaceae, unidentified taxa, and, to a lesser extent, *Prorocentrum* after August 4th (Fig. 1b). By contrast, according to the microscopic analyses *Ostreopsis* sp. dominated among the dinoflagellate during both the second and the third phases, accounting for 89% to almost 100% of the epiphytic dinoflagellate biomass (Fig. 1c). Other dinoflagellate species, belonging to the genera *Coolia*, *Amphidinium* and *Prorocentrum*, were detected at low abundance beginning on July 15th, consistent with the metabarcoding results.

Diversity and relative abundance of fungal and chytrid assemblages

The fungal community based on metabarcoding of the V4 18S rDNA region was represented by 203 ASVs. During the first and second phases, fungi accounted for < 1% of the protist community, with the exception of the samples taken on June 30th and July 8th, in which the inter-replicate values ranged from 2% to 30%. However, on July 29th, corresponding to the third phase, fungi accounted for almost 30% of the protist community, followed by a gradual decrease to < 5% by September 1st. (Fig. 1a). The analysis of the fungal assemblage revealed that the elevated relative abundances during the first phase were due to the contributions of Basidiomycota (June 30th) and Ascomycota (July 8th). During the third phase, Chytridiomycota emerged as the dominant group in the fungal community, but its contribution gradually declined and its dominance eventually alternated with that of Ascomycota (Fig. 2a). Between mid-August and September, overall fungal diversity increased.

The diversity of the fungal community was further examined using the ITS region of fungal representatives (Fig. 2b). Seventy-seven percent of the ITS sequences were discarded as they were not identified as belonging to fungi. A total of 466 ASVs were obtained, revealing differences in community composition during the three phases. During the first and second phases, the community was dominated by fungal sequences with unknown taxonomic affiliations, albeit with Ascomycota and Basidiomycota present to a lesser extent. Between July 29th and August 4th, and thus during the third phase, the proportion of Chytridiomycota significantly increased and accounted for almost 100% of the fungal community. However, the relative abundance of Chytridiomycota gradually declined, accompanied by an increase in other groups, such as Ascomycota, Basidiomycota, and sequences of unclassified taxonomy.

Further analysis of the Chytridiomycota based on ITS region analysis revealed 19 ASVs (Fig. 2c). During the first and second phases, the composition of Chytridiomycota was diverse but the total relative abundance was < 5%. During the third phase, ASV2 emerged as the dominant Chytridiomycota sequence, accounting for almost 100% of the total Chytridiomycota and fungal sequences during the samplings of July 29th and August 4th, followed by ASV29 at much lower relative abundance. Although ASV2 remained the dominant Chytridiomycota sequence, its abundance relative to total fungal sequences gradually decreased. On August 25th and September 1st, the relative abundance of ASV2 was lower, and the relative abundance of ASV83 increased.

ASV2 and ASV29 were only differentiated by one single nucleotide, showing an identity of 99.7%. Both sequences were taxonomically assigned to the species *Dinomyces arenysensis*, likely representing intraspecific variants. ASV83 was assigned as an unknown species of Lobulomycetales.

Identification and prevalence of chytrids in field samples

Chytrid infections were detected by light and epifluorescence microscopy from the second phase (beginning July 15th) onwards (Fig. 3a, b and Table 1). During this period, two species of chytrids were identified by the molecular characterization of cultured strains established, or single cell isolates from natural samples (*Dinomyces arenysensis* and Lobulomycetales sp.). The last one was isolated, cultivated and identified as a new, yet undescribed species of Lobulomycetales (strain ICMB1108) (Fig. 3e, f and Table 1).

Only the prevalence of one species, *D. arenysensis*, was determined. The morphology of mature sporangia observed by epifluorescence was compared and confirmed with sporangia from cultures (Fig. 3a, b). The prevalence of *Dinomyces* infection during this second phase was < 0.5%. In the third phase, after August 4th, chytrid infections were recurrently detected in field samples by light microscopy, facilitating the establishment of chytrid cultures and thus their molecular characterization. Spherical epibiotic sporangia without discharge papillae were observed, and the subsequent molecular characterization of these strains revealed them to be *D. arenysensis* (ICMB1109–ICMB1112) (Fig. 3c, d and Table 1). Infection prevalence at this stage reached up to 2.7% (Table 1). Infections in the lab of the two chytrid species found in this study (*D. arenysensis* and Lobulomycetales sp.) were equally observed in the mucilage of *Ostreopsis* sp. (Fig. 3i). All infections, isolations and cultures were originated from the dinoflagellate *Ostreopsis* sp. No infections by chytrids were observed in any other microalgae during the study period.

Facultative parasitic strategy of *D. arenysensis*

The ability of *D. arenysensis* to utilize non-algal organic matter for its life cycle and propagation was investigated by incubating previously established chytrid strains (ICMB1101, ICMB1103 and ICMB1104) with pine pollen grains. After 4 days of incubation, ICMB1104 was the only strain that had formed sporangia on the pollen grains, with chytrids growing in the main body of the grain and forming single or multiple sporangia (Fig. 3g, h). After 6 days, more mature sporangia were found, although no living *Ostreopsis* sp. cells from the initial inoculum were observed. The viability of the newly produced sporangia on the pollen was tested by inoculating new, healthy *Ostreopsis* sp. cells into the same well, which resulted in re-infection of the dinoflagellate by the chytrid 2 days later. These infections originated from zoospores released from the sporangia present on the pollen grains. Once the dinoflagellate cells were added and infected, no new infections on the pollen grains were observed.

Temporal dynamics of *D. arenysensis* strains and *Ostreopsis* sp

The levels of infection development and virulence differed among *Dinomyces* strains (ICMB1101, ICMB1102, ICMB1104) in infections of *Ostreopsis* sp. (strain ICMB293) (Fig. 4). In infections with strain ICMB1101, < 2% of *Ostreopsis* sp. cells were infected during the first 10 days of the experiment and the abundance of healthy *Ostreopsis* sp. cells gradually increased from 300 cells mL⁻¹ to 900 cells mL⁻¹. Thereafter, the prevalence of infection increased exponentially such that by the end of the experiment 30% of the population was infected and the population of healthy *Ostreopsis* sp. cells decreased to > 300 cells mL⁻¹. When *Ostreopsis* sp. was inoculated with chytrid strain ICMB1104, the infection rate did not exceed 1% and the growth of *Ostreopsis* sp. cells was comparable to that observed in an uninfected control treatment. In infections with strain ICMB1102, the highest prevalence of infection occurred

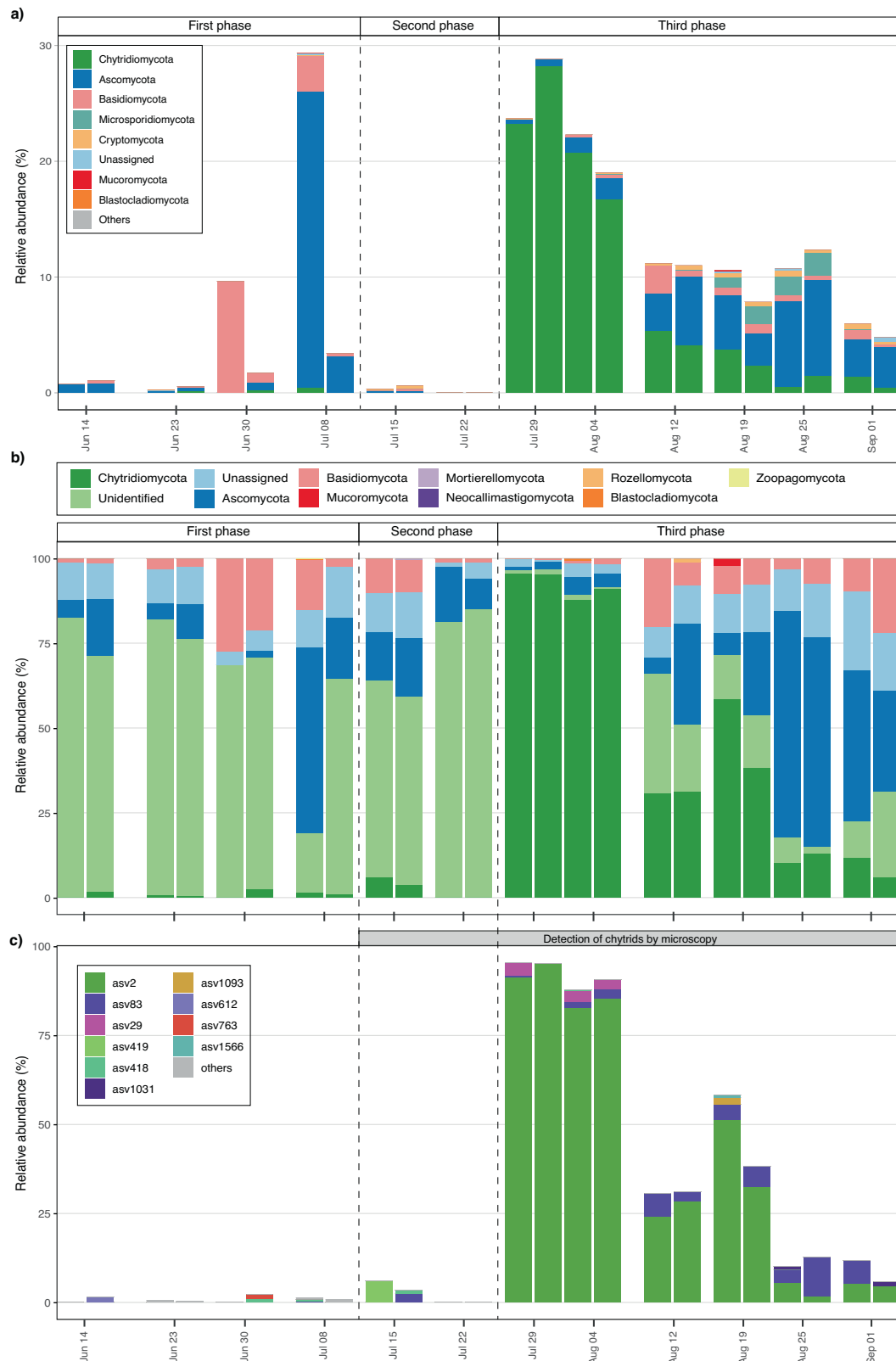


Fig. 2 Temporal dynamics of the epiphytic fungal assemblage as determined using the V4 18S rDNA and ITS regions. **a** Bar plot showing the variation, in replicates, of the epiphytic fungal assemblage as determined using the V4 18S rDNA region. Relative abundances (%) are based on the total protist community. **b** Bar plot showing the relative abundance of the fungal groups as determined using the ITS region. **c** Bar plot showing the relative abundance of Chytridiomycota with respect to the fungal community based on the ITS region. The ten most abundant Chytridiomycota ASVs are shown; the remaining nine ASVs are included in the “other” category. The shaded area between (b) and (c) indicates the period when the presence of chytrids was detected by light microscopy, epifluorescence microscopy or incubation of natural samples.

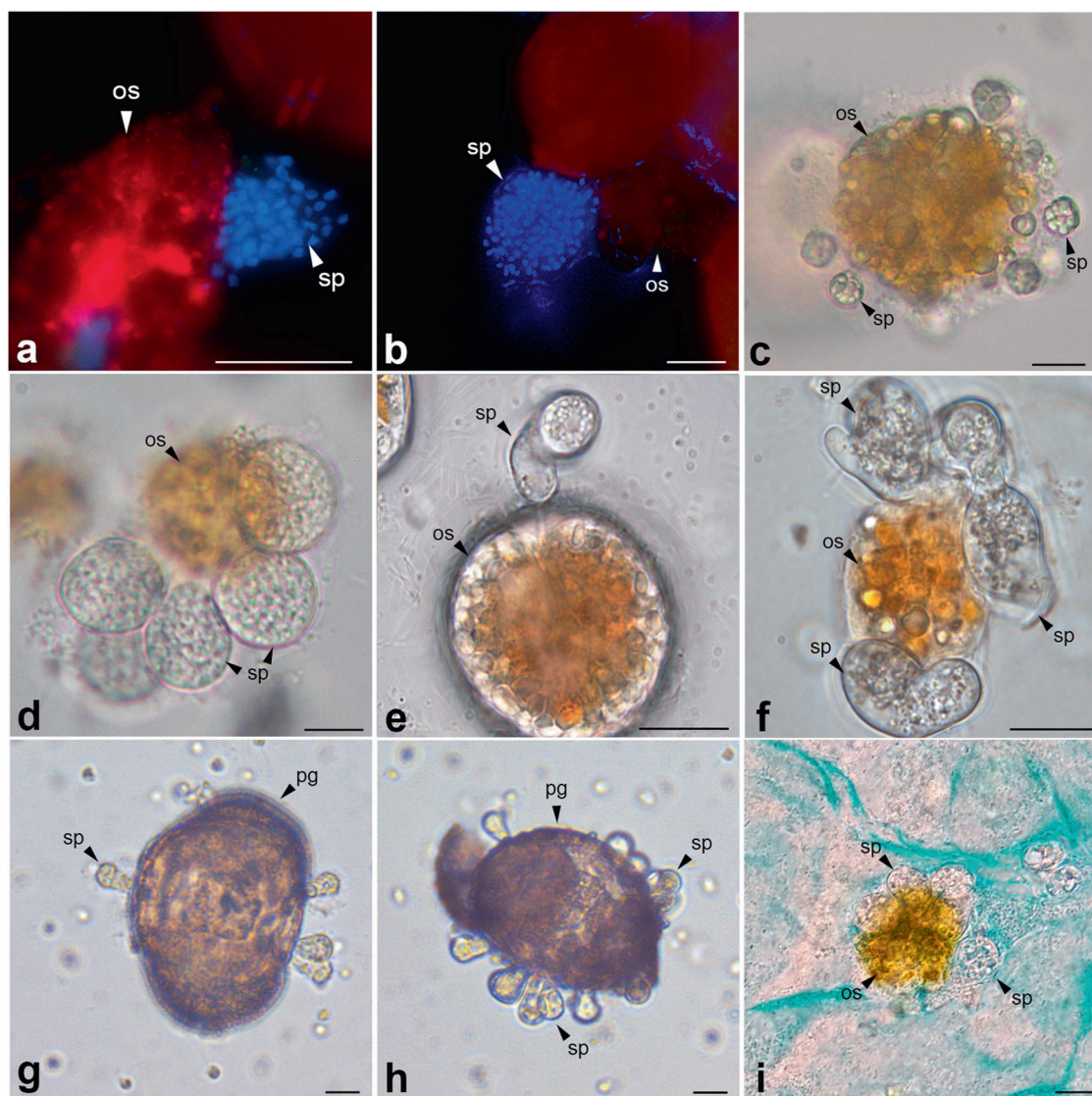


Fig. 3 Microscopy images of chytrids. Epifluorescence microscopy: **a** DAPI staining of mature sporangia from natural samples. **b** DAPI staining of mature sporangia of *Dinomyces arenysensis* from laboratory cultures. Light microscopy. **c** Early infection stage of *D. arenysensis* strain ICMB1109 infecting *Ostreopsis* sp. (ICMB293). **d** Immature sporangium stage of strain ICMB1109 infecting *Ostreopsis* sp. **e** Early infection stage of Lobulomycetales strain ICMB1108 infecting *Ostreopsis* sp. **f** Immature sporangium stage of strain ICMB1108 infecting *Ostreopsis* sp. **g, h** Pine pollen grains with multiple sporangia of *D. arenysensis* ICMB1103, observed 4 days after inoculation of the chytrid **i** Alcian blue staining of mucilage present in cultures of Lobulomycetales sp. sp: sporangium, os: *Ostreopsis* sp., pg: Pine pollen grain. Scale bars = 10 μ m.

after 5 days, at which time 5% of the population was infected. On subsequent days, the prevalence decreased gradually, to < 1% on day 20. During the first 10 days of exposure, the *Ostreopsis* population remained stable, with cell numbers similar to the starting value. From day 10 until the end of the experiment, the *Ostreopsis* population increased from 300 cells mL^{-1} to 900 cells mL^{-1} while the prevalence of infection remained low.

DISCUSSION

This study investigated the diversity and temporal dynamics of marine epiphytic protist assemblages on macroalgae, with a focus on fungi, Chytridiomycota and dinoflagellates. Our findings provide new insights into the complex interactions between marine fungi and their host communities. Specifically, the results showed that both the composition and the relative abundances of fungal communities are influenced by the succession of epiphytic microalgae. Over time, the community structure of the epiphytic

fungi changed, evidenced by changes in the dominance of Ascomycota, Chytridiomycota, and unidentified fungal organisms. The abundance of the Chytridiomycota was highly dependent on epiphytic dinoflagellate species assemblages, with *Dinomyces arenysensis* and a new species of Lobulomycetales as the primary representatives of this group. The peak in the abundance of the dinoflagellate *Ostreopsis* sp. coincided with the highest relative abundance of chytrids, particular *D. arenysensis*. Laboratory experiments showed that the latter species is characterized by intraspecific variability in its prevalence, with a capacity of growth on pollen demonstrated in one of the tested strains.

The identity and diversity of the eukaryotic protist community was explored using the V4 18S rDNA region, and that of the fungal community using the V4 18S rDNA and ITS regions. The two markers provide complementary information about the diversity of fungi present in the samples along with that of the other microbial components of the biofilm from which they were obtained. However, while V4 18S rDNA enables accurate

Table 1. Sampling dates, cell abundance of *Ostreopsis* sp. on macroalgae as determined by light microscopy counts, and chytrid prevalence on *Ostreopsis* sp. in field samplings. –: not determined. Chytrids were isolated for culture and sequenced by single-cell PCR. Strains grown under laboratory conditions are named after the culture collection (ICMB). The 18S rDNA molecular sequences and the resulting taxonomic identities of the strains are indicated. White rows mark the first and third sampling-based phases of the epiphytic assemblage; shaded rows mark the second phase.

	Date	<i>Ostreopsis</i> sp. abundance on macroalgae (cell*gFW ⁻¹)	Chytrid prevalence on <i>Ostreopsis</i> sp. (%)	Isolated chytrids and established cultures	GenBank Accession Number	Taxonomic identification of strain
First phase	2021-06-14	19	–			
	2021-06-23	13	–			
	2021-06-30	43	–			
	2021-07-08	1,502	–			
Second phase	2021-07-15	13,509	0.59	ICMB1108	OQ911493	Unknown Lobulomycetales sp.
				Single-cell from natural sample	OQ911487	<i>Dinomyces arenysensis</i>
	2021-07-22	548,326	0.23			
Third phase	2021-07-29	379,949	0.86	Single-cell from natural sample	OQ911488	<i>D. arenysensis</i>
	2021-08-04	666,983	1.99	ICMB1109	OQ911489	<i>D. arenysensis</i>
	2021-08-12	213,928	1.63	ICMB1110	OQ911490	<i>D. arenysensis</i>
	2021-08-19	8,423	2.74			
	2021-08-25	43,783	1.20	ICMB1111	OQ911491	<i>D. arenysensis</i>
	2021-09-01	64,711	0	ICMB1112	OQ911492	<i>D. arenysensis</i>

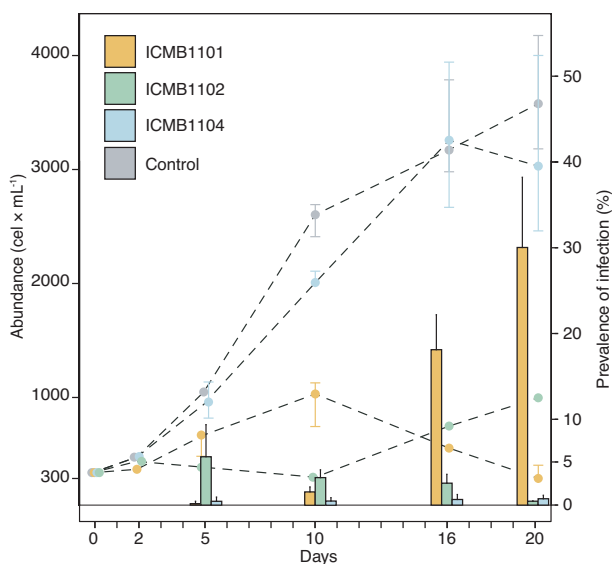


Fig. 4 Temporal dynamics of infection. Cell abundance (cells mL⁻¹) of host *Ostreopsis* sp. strain ICMB293 and the prevalence (%) of strains of the chytrid parasite *D. arenysensis* (ICMB1101, ICMB1102 and ICMB1104) over time (days). Dashed lines correspond to cell abundance, and bars to prevalence. Whiskers represent the standard deviation.

assignments at high taxonomic levels, such as phylum and order, in fungal organisms the region is too conserved to allow species-level discrimination [49, 50]. The latter can be achieved with the ITS region, which allows precise taxonomic assignments at the

genus and species levels. Nonetheless, the accuracy of the taxonomic assignments obtained with both markers heavily relies on the availability and quality of reference databases. The large number of unknown sequences in this study well demonstrates the need to expand our knowledge of fungal diversity. Identification of unknown fungal species will provide valuable insights into their ecological roles.

The main finding of the study is that the composition of the fungal community is influenced by the temporal dynamics of epiphytic protists on macroalgae. During the first phase, diatoms and dinoflagellates accounted for very few of the epiphytic protists on macroalgae. This phase can thus be considered as the initial period of colonization and development of the microalgae that will subsequently dominate the epiphytic community [22]. The fungal community on the macroalgae during the first phase was mainly composed of members of the Ascomycota and Basidiomycota, based on V4 18S rDNA. However, 70% of the fungal sequences obtained using the ITS region could not be attributed to any known fungal taxon, indicating a significant gap in our understanding of the diversity of marine fungal communities in these epiphytic assemblages, as has been reported in other studies [51–53]. As the protist biofilm on the macroalgae was not fully formed, this fungal assemblage may have been an epiphytic fungus of macroalgae rather than of the protist assemblage. In fact, Ascomycota and Basidiomycota are the main groups of marine fungi found in metabarcoding studies, and the most abundant and diverse fungal groups in benthic ecosystems [11, 54]. Studies conducted in the Mediterranean Sea have shown that these two groups, including Ascomycetes saprophytes of *Posidonia oceanica* and the macroalga *Dictyota dichotoma* [55], represent 97.2% of the fungal community present on vegetal substrates [56].

During the second phase, the abundance of epiphytic dinoflagellates increased rapidly, from <25% to almost 100%. This proliferation corresponded specifically to the dinoflagellate *Ostreopsis* sp., a well-known blooming species characterised in previous studies of the NW Mediterranean during the summer months [22]. *Ostreopsis* sp. blooms are likely driven by the same factors that promote macroalgal growth, i.e., increases in temperature, irradiance, and substrate availability [57]. Furthermore, the mucilage secretions characteristic of these blooms enable the establishment of the microorganisms that will ultimately form the epiphytic summer biofilm on macroalgae [58]. The proportion of Ascomycota, Basidiomycota, and the unidentified set of taxa in the fungal community during the second phase remained the same as during the first phase. However, a week after the increase in *Ostreopsis* abundance, the fungal composition changed such that by the beginning of the third phase the fungal fraction accounted for nearly 25% of the total protist community, with an increase in the number of Chytridiomycota members, specifically *D. arenysensis*. The time lag between the maximum cell abundance of the host and the increase in parasite abundance aligns with typical predator-prey dynamics, where predator (in this case, parasite) and prey (host) alternate in abundance over time. Starting from a growing host population, the increase in this food resource will promote the growth of the parasite population. Parasitic pressure, in turn, can adversely affect host population abundance, leading to a subsequent decline in parasite population. The specific observed time lag can be considered as the period necessary for the parasite to complete its infective life cycle, in a situation of high host abundance [59]. In the case of chytrids, their complete development, from the onset of infection to the formation of new infective zoospores released into the environment, occurs within 2–3 days [10, 60]. A similar time lag was observed in studies that likewise evaluated the temporal dynamics of chytrids and their microalgal hosts [8, 61].

The abrupt increase in the relative abundance of Chytridiomycota during the beginning of the third phase coincided with the period of maximum infection prevalence in the samples and the highest abundance of *Ostreopsis* sp. This observation was consistent with the blooming strategy of chytrid species during phytoplankton blooms, which has been described as a ruderal strategy based on chytrid-phytoplankton parasitism during bloom events [62]. The subsequent decrease in dinoflagellate abundance occurred in parallel with an increase in other groups, such as Fungi, Apicomplexa, Mesomycetozoa, Sagenista and unassigned taxa. The dominant fungal group in this third phase was Chytridiomycota, although towards the end of this phase its abundance decreased and alternated with a relatively high abundance of Ascomycota. These changes, during the third phase, suggest that the decline of the dominant dinoflagellate *Ostreopsis* sp. allowed the development of other protist groups, thereby changing the composition of the fungal community. This trend reflects changes in community assembly following the proliferation and decline of previously dominant *Ostreopsis* sp.

Dinomyces arenysensis was the dominant species in the fungal community examined in our study. This species is found in benthic marine-coastal habitats and has been described as a generalist, biotrophic parasite with a preference for the dinoflagellate *Ostreopsis* [9, 60]. However, the prevalence of *D. arenysensis* infections during the period of maximum *Ostreopsis* sp. abundance was relatively low, ranging from 1% to 2.7%, which is comparable to the values reported for other host-parasite systems within plankton. For example, the prevalence of two chytrid species of the genus *Rhizosiphon* on the cyanobacterium *Anabaena* increased from non-detection to 6% [7]. In a study examining chytrids associated with freshwater diatoms, the prevalence of infection varied widely among chytrid species, with those exhibiting specialized parasitic behaviour attaining an infection prevalence as high as 70%. By contrast, the infection

prevalence of species with facultative parasitic behaviour, and thus able to assimilate food resources other than diatoms, such as pollen, was low, around 2% [2]. The behaviour of *D. arenysensis* as observed in our study suggested that this species has a facultative parasitic life style. Previous research on the growth dynamics of *D. arenysensis* has reported its inability to grow on agar media or soil extracts, indicating an obligate parasitic lifestyle [60]. However, our laboratory experiments demonstrated that this chytrid can successfully use pine pollen, present in abundance in the atmospheric pollen above the Catalan Coast, to complete a viable life cycle. This ability to utilize diverse resources suggest that *D. arenysensis* is a facultative parasite. Moreover, it provides further evidence of intraspecific variability in virulence, as the ability to infect pine pollen was observed in only one of the studied strains. Likewise, sporangia were not observed on pollen when *Ostreopsis* sp. was present, indicating a preference for a parasitic life-style and that pollen serves only as a temporary resource. The ability of chytrids to infect both phytoplankton and pollen is common in freshwater environments [2, 63]. Our results suggest similar interactions in marine habitats, specifically in seasonal epiphytic assemblages, where other resources sustain the parasite until its preferred host again becomes available. However, survival of the parasite in winter, when the substrate of its host (macroalgae) is no longer available, suggests a reservoir within the plankton or benthonic compartment, allowing the recurrence of *Dinomyces* in *Ostreopsis* blooms [9].

Low infection prevalence of *Dinomyces* in *Ostreopsis* sp. could indicate benign parasitism (as described in ref. [64]). This suggests that chytrids and dinoflagellates can coexist without significant harm to the host population, allowing for a recovery from parasite pressure. Previous studies suggested that marine chytrid outbreaks prematurely terminate or suppress phytoplankton blooms [65, 66]. However, this was not supported by the prevalence of infection observed in the field in our study, as the infections observed in natural samples did not explain the decrease in *Ostreopsis* abundance. Rather, our findings are consistent with the coexistence of chytrids and dinoflagellates. In fact, laboratory studies have shown that parasites and dinoflagellate host populations can coexist if the equilibrium between them is sustained by a minimum host density threshold [67]. Coexistence at low infection prevalence was also observed in studies of other protist parasites, such as *Amoebophrya* sp., and can be interpreted as reducing the fitness of the dinoflagellate host without causing significant damage to the host population [68].

In contrast to the field studies suggesting coexistence, in the lab experiments the impact of parasitism ranged from harmless to lethal, depending on the host affinity of the different strains of *D. arenysensis*. Thus, the coexistence between chytrids and dinoflagellates observed in the field might reflect differences in the virulence of the different strains of chytrids. The presence in the field of multiple parasite strains with varying degrees of virulence would support parasite-host equilibrium and thus coexistence. Studies of the relationship between chytrids and cyanobacteria have shown that differences in the performance of multiple strains of parasitic chytrids can lead to changes in the infection strategy of these organisms [69]. Intraspecific diversity can also promote species coexistence and influence community dynamics, by promoting niche complementarity between genotypes [70, 71].

Mucilage secretion, a prominent feature of *Ostreopsis* and another microalgal species, provides structure to the epiphytic assemblage [72, 73], and serves as a trap for prey acquisition [74]. In a recent study, it has been proposed that the mucilage acts as a protective barrier against chytrid infection in diatoms [75]. However, the two chytrid species identified in our study (*D. arenysensis* and *Lobulomycetales* sp.) were capable of infecting the dinoflagellate *Ostreopsis* despite mucilage secretion. The ability to overcome the protective mucilage barrier suggests that these chytrid species have evolved specific mechanisms to infect

their host. Understanding these mechanisms could provide valuable insights into the interactions between fungi and dinoflagellates, and the competitive advantage that means using these resources.

In summary, this study characterized epiphytic protist succession on macroalgae with focus on chytrids and dinoflagellates. Our investigation of the host-parasite dynamics of *Dinomyces arenysensis* showed that the infection peak is influenced by the predominant resource, *Ostreopsis* sp., but also that the parasite is facultative with respect to its host resources. The coexistence of chytrids and dinoflagellates without significant harm to the host organism was supported by field studies. Concurrently, laboratory experiments indicated that this coexistence is partly attributed to the variability in the virulence levels exhibited by different strains of chytrids. Our findings contribute to a better understanding of the dynamics of epiphytic biofilm and host-parasite interactions in less-explored marine benthic habitats.

DATA AVAILABILITY

The datasets presented in this study are available in the NCBI Sequence Read Archive under Bioproject numbers PRJNA891539, PRJNA891506, and are included in the supplementary datasets of this published article.

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ACKNOWLEDGEMENTS

This study was funded by the Spanish Ministry of Science, Innovation, and Universities (MICINN) Project SMART PID2020-112978GB-I00, with institutional support from the “Severo Ochoa Centre of Excellence” accreditation (CEX2019-000928-S). High-performance computing analyses were run at the Marine Bioinformatics Service (MARBITS) of the Institut de Ciències del Mar (ICM-CSIC) in Barcelona. ADF-V is funded by the MICIU grant “Ayudas para contratos predoctorales para la formación de doctores 2018 (PRE2018-084893)”.

AUTHOR CONTRIBUTIONS

AR, EG, and ADF-V designed the study; all authors performed sampling, laboratory procedures, and data analysis; ADF-V, AR, and EG wrote the manuscript, with contributions from all co-authors.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s43705-023-00304-x>.

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9.4 Supplementary material of chapter 3

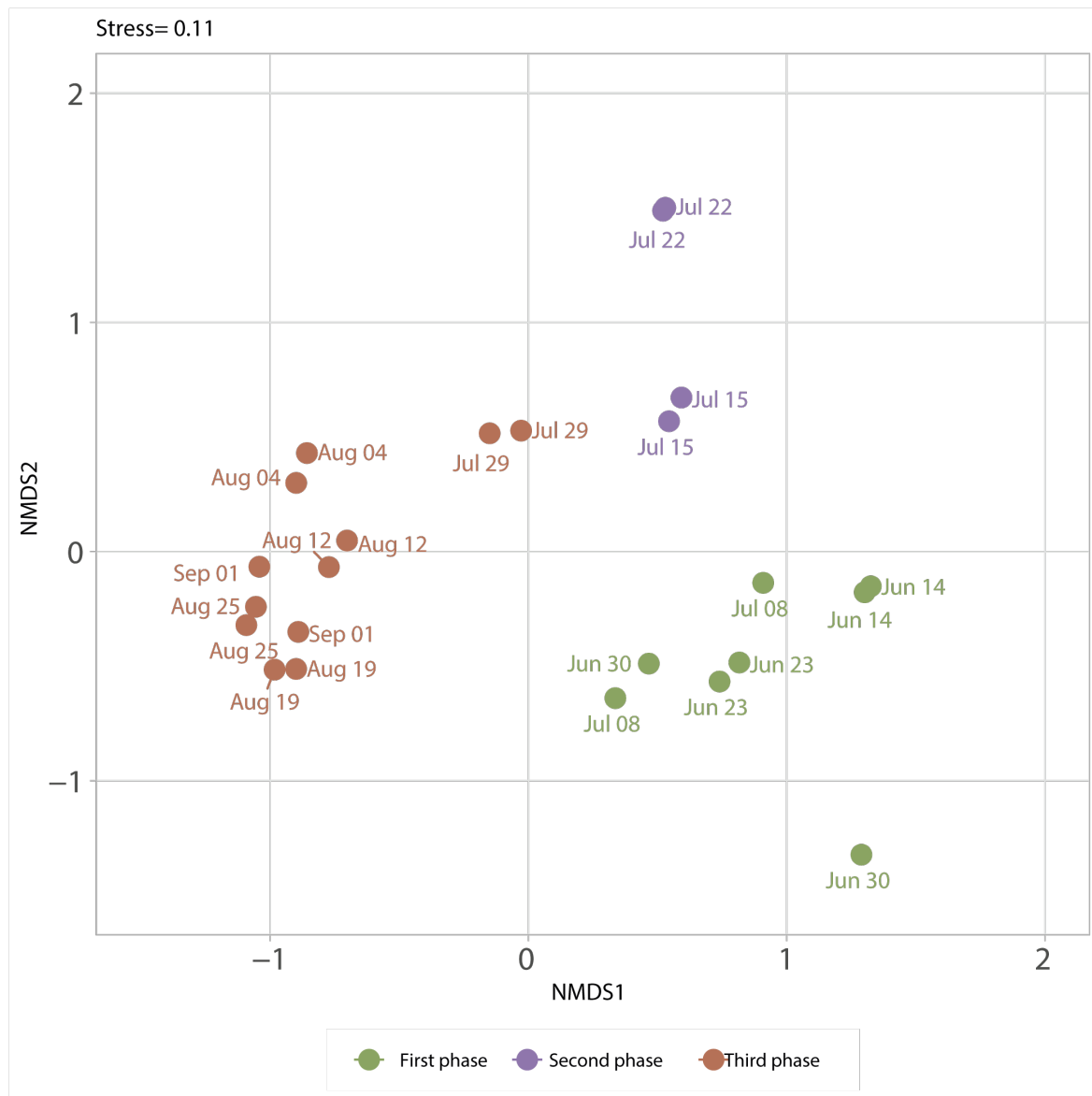


Figure 9.1: **Supplementary Figure 1.** Non-metric multidimensional scaling (NMDS) conducted using samples obtained during the three temporal sampling phases.

9.5 Original article of chapter 4

Newly identified diversity of Dinomycetaceae (Rhizophydiales, Chytridiomycota), a family of fungal parasites of marine dinoflagellates.

Fernández-Valero, A. D., Karpov, S. A., Sampedro, N., Gordi, J., Timoneda, N., Garcés, E., & Reñé, A. (2024). Newly identified diversity of Dinomycetaceae (Rhizophydiales, Chytridiomycota), a family of fungal parasites of marine dinoflagellates. *European Journal of Protistology*, 126053. Available from: <https://doi.org/10.1016/j.ejop.2024.126053>

Newly identified diversity of Dinomycetaceae (Rhizophydiales, Chytridiomycota), a family of fungal parasites of marine dinoflagellates

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ARTICLE INFO

Keywords:

Chytridiomycota
Coastal ecosystems
Dinoflagellate
Fungi
Molecular phylogeny
Ultrastructure
Fungal parasites

ABSTRACT

We identified two new parasite species of Chytridiomycota isolated during blooms of the dinoflagellate *Alexandrium minutum* in the coastal Mediterranean Sea. Light and electron microscopy together with molecular characterization of the nuclear 18S, ITS, and 28S rDNA regions led to their identification as two new species, *Dinomyces gilberthii* and *Paradinomyces evelyniae*, both belonging to the family Dinomycetaceae, order Rhizophydiales. *Dinomyces gilberthii* differs from the previously described *D. arenysensis* by the presence of discharge papillae and the development of a drop-shaped sporangium. *Paradinomyces evelyniae* differs from the previously described *P. triforaminorum* by the prominent lipid globule present in early sporangia and by the pointed end producing a rhizoid. The two chytrids differed in their geographical distribution. *Dinomyces gilberthii* was detected in several Mediterranean habitats, including harbours and beaches, and was particularly prevalent during summer dinoflagellate blooms. Its widespread occurrence in coastal ecosystems suggested a high level of adaptability to this environment. *Paradinomyces evelyniae* had a more restricted distribution in the coastal-marine environment, occurring in harbour sediments and only occasionally in the water column during winter and early spring. *Paradinomyces evelyniae* has previously been detected in the Baltic Sea, suggesting that its distribution encompasses contrasting coastal environments, although its presence is rare.

1. Introduction

Recent environmental sequencing studies of marine protist populations have led to a growing interest in fungi present in marine habitats and their ecological characteristics (Debeljak and Baltar, 2023; Hassett et al., 2019; Pernice et al., 2015; Richards et al., 2015; Tisthammer et al., 2016). The basal fungal clade of Chytridiomycota includes saprotrophs and parasites that infect diatoms (Duan et al., 2018), dinoflagellates (Fernández-Valero et al., 2022; Lepelletier et al., 2014; Reñé et al., 2022), and other marine microorganisms (Gerphagnon et al., 2013; Xue et al., 2018). Infections lead to the death of host cells and can cause the host population to decline (Van den Wyngaert et al., 2022), but parasite and host populations can also coexist (Fernández-Valero et al., 2023). Fungal-host interactions thus have the potential to shape the structure and diversity of marine microbial communities.

Parasitic Chytridiomycota, or chytrids, are a group of zoospore fungi

with a characteristic life cycle. Free-living zoospores attach to the host surface and encyst. After germination, they penetrate the host, often forming rhizoids to absorb nutrients. Cyst growth results in the formation of a sporangium, which contains several nuclei. During sporangium maturation, its multinuclear content differentiates into individual cells, which develop a posterior flagellum and become zoospores. The latter are released through the sporangial wall and infect new host cells (Medina and Buchler, 2020; Trinci et al., 1994). The dependence on a host to complete the life cycle of chytrids defines them as parasitoid fungi, i.e., parasites that consume and eventually kill the host in order to grow and reproduce (Eggleton and Gaston, 1990).

Chytrids have a greater presence in the marine environment than initially believed, which has motivated studies aimed at understanding their ecological role. However, while chytrids have been detected in a large number of environmental sequencing studies. The species identity of those sequences is largely unknown (Grossart et al., 2016), and also

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their ecological function. Identification of these organisms relies on cell isolation and culture efforts, together with microscopic observations. The integration of molecular techniques along with the morphological and ultrastructural characterization allows a correct taxonomic assignment of fungal zoosporic parasites and insights into their specific ecological functions. Indeed, characterizing the diversity of zoosporic fungi in marine environments has been the focus of several studies, covering localities ranging from the Baltic Sea, where *Ericiomyces syringoforeus* Karpov & Reñé (Karpov et al., 2021) and *Paradinomyces triforamini* Reñé & Karpov (Reñé et al., 2022) were identified, through the Hebrides Sea, where chytrid strains infecting diatoms have been described (Garvetto et al., 2019), to the Mediterranean Sea, where Fernández-Valero et al. (2022, 2023) examined a broad spectrum of habitats, including seawater, sediment, and epiphytic niches.

Nonetheless, Chytridiomycota are often overlooked in ecological studies, and their role in the marine ecosystem food web, and consequently in nutrient cycling remains poorly understood. In this study, we describe a new diversity in the family Dinomycetaceae Karpov & Guillou (Chytridiomycota), based on the identification of two new species of Chytridiomycota isolated from marine water samples of the coastal Mediterranean Sea. Both parasitize the dinoflagellate *Alexandrium minutum* Halim. These findings have implications for the dynamics of dinoflagellate populations, the main hosts of the new species, in addition to shedding light on chytrid interactions with other organisms in aquatic ecosystems.

2. Materials and methods

2.1. Sampling, isolation and cultivation of chytrids

On March 10th and April 1st 2021, samples were obtained from two harbours along the Catalan coast (NW Mediterranean Sea), Arenys de Mar (41°34'45.5" N, 2°33'19.8" E) and Vilanova i la Geltrú (41°12'54.9" N, 1°43'57.9" E) (Fernández-Valero et al., 2022), during blooms of the dinoflagellate *A. minutum* (Table 1). Sampling was performed when dinoflagellate abundance in the water column exceeded 10⁵ cells L⁻¹. At each location, integrated water column sampling using a tube connected to an electric pump allowed the collection of a 5-L composite sample from a depth range of 2.5–4.0 m, extending from the water surface to the bottom layer. An additional 5-L sample was collected exclusively from the bottom layer. The temperature and salinity of the water samples were measured in situ with a conductivity meter (WTW model Cond 3301), and all laboratory measurements were conducted with the same equipment. Surface sediments were obtained using a sediment corer attached to a pole, with ~ 30 g of sediment collected in a 50-mL Falcon tube using a scoop.

In the laboratory, the seawater samples were filtered through an 80-µm mesh to remove metazoans and large particles. Subsequently, 1 L of each seawater sample was filtered through a 10-µm mesh to retain dinoflagellate cells as well as potential dinoflagellate infections. The 10- to 80-µm fractions were concentrated to 10-mL and incubated in 6-well plates. For sediment samples, portions were placed in double-bottom tubes separated by an 8-µm mesh, with *A. minutum* added on top to promote the vertical swimming of zoospores through the mesh to separate the parasite from the sediment. Incubations were performed in culture chambers at a constant temperature of 20 ± 1 °C with a 12:12 h light:dark photoperiod, with illumination of ~ 40 µmol photons m⁻² s⁻¹ provided by a LED fluorescent tube. The presence of chytrid infections in the incubations was monitored for up to two weeks. Infected dinoflagellate cells were manually isolated to establish clonal chytrid cultures.

Chytrid cultures were maintained in 24-well plates by transferring a 1-mL aliquot of mature sporangia to a new well containing 1 mL of an exponentially growing, uninfected host stock culture every three or four days. Host cultures were maintained under non-axenic conditions in L1 medium with a salinity of 36. Chytrid and dinoflagellate host cultures were maintained under the conditions described above. The dinoflagellate host strains used in this study were obtained from the culture collection of the Institut de Ciències del Mar (ICM-CSIC). *Alexandrium minutum* strain ICMB292 served as a host in the maintenance of newly isolated strain ICMB1106, but the latter also survived with *Ostreopsis* sp. strain ICMB293 as a host. *Ostreopsis* sp. (ICMB293) mixed in equal proportions with *A. minutum* (ICMB292) was used to maintain the newly isolated strain ICMB1107. *Ostreopsis* sp. was chosen because the time required for its consumption by the parasite was longer than that of other dinoflagellates, due to its larger biovolume, thus prolonging the maintenance period of the cultures in the lab.

2.2. Light microscopy

The life cycle of strains ICMB1106 and ICMB1107 was investigated by light microscopy. Regular observations of newly produced infections were conducted to identify the different life-cycle stages and sporangium development. A drop of each chytrid culture was placed on a glass slide and observed with a Nikon Eclipse Ts2R-FL inverted microscope. Photographs were taken with a Nikon DS-10 digital camera, using a PLAN APO 100X magnification objective and differential interference contrast. The NIS Elements v5.3 software was used to capture the images. Time-lapse 40 × photographs from an aliquot of each chytrid strain placed on glass-bottom plates were taken every 10 min for four days using a Zeiss Axio Observer Z1 microscope, an Axiocam 503 mono camera, and the Zen pro 2012 software.

Table 1
Origin and characterization of the studied chytrid strains.

Taxonomic identification	<i>Dinomyces gilberthii</i> sp. nov.	<i>Paradinomyces evelyniae</i> sp. nov.
Culture code	ICMB1106	ICMB1107
Isolation date	01 April 2021	10 March 2021
Location	Vilanova i la Geltrú	Arenys de Mar
Sampling type	Bottom layer	Integrated water column
Host species in field	<i>Alexandrium minutum</i>	
Host abundance (cells L ⁻¹)	1.1 × 10 ⁵	9.5 × 10 ⁴
Salinity	35.4	24.4
Temperature (°C)	15.1	16.3
Host in culture	<i>A. minutum</i>	<i>A. minutum</i> , <i>Ostreopsis</i> sp.
Culture code of the host	ICMB292	ICMB292; ICMB293
18S rDNA gene	ON081639; ON081640	ON081641; ON081642
Length (base pairs; bp)	1325; 1337	964; 1117
ITS rDNA gene	OQ865234; OQ865235	OQ865236
Length (bp)	643; 607	526
28S rDNA gene	OQ865232; OQ865233	OQ865231
Length (bp)	900; 892	904

2.3. Scanning electron microscopy (SEM)

The structure of the sporangia was examined by SEM. Five mL of ICMB1106 and ICMB1107 cultures containing mature sporangia were fixed in 2% glutaraldehyde for 15 min at room temperature. The ICMB1107 sample was post-fixed with osmium tetroxide with a final concentration of 1% for 15 min at room temperature. The pH was not adjusted. The fixed sporangia were then filtered through a Nucleopore polycarbonate filter (Pleasanton, California, USA) with a diameter of 13 mm and a pore size of 3 µm and washed in distilled water. After dehydration in an ethanol series (25, 50, 75, 90, 96, and 3 × 100%, 10 min each), the filters were critical-point-dried using a Bal-Tec CPD030 apparatus (Leica Microsystems, Vienna, Austria), mounted in holders, and sputter-coated with gold on a Quorum Q150R S (Quorum Technologies, Ltd, Laughton, UK). They were then examined with a variable pressure scanning electron microscope working at 5 kV (Hitachi S-3500 N, Hitachi High Technologies Corp., Japan) at the Servei de Microscòpia Electrònica (ICM-CSIC).

2.4. Transmission electron microscopy (TEM)

The ultrastructure of zoospores was analyzed by TEM. Strains ICMB1106 and ICMB1107 were grown in 6-well plates until a large number of free-living zoospores were observed in the cultures. The supernatant from each well was collected in a 50-mL Falcon tube and centrifuged at 3893 rcf for 6 min at room temperature to concentrate the sample to 2 mL. A threshold concentration of 2.5×10^5 zoospores mL⁻¹ was established for initiating block preparation for TEM analysis. The 1.5-mL sample was fixed in 2% glutaraldehyde, postfixed with osmium tetroxide, dehydrated with acetone, and embedded in Spurr resin. Ultrathin serial sections (50–70 nm) obtained using a Leica ultramicrotome UC7 (Leica Microsystems) were stained with 2% uranyl acetate for 10 min and lead-staining solution for 5 min before they were observed with a JEOL JEM-1010 transmission electron microscope fitted with a Gatan Orius SC1000 (model 832) digital camera at the TEM-SEM Electron Microscopy Unit, Scientific and Technological Centers of the University of Barcelona (CCiTUB).

2.5. Molecular procedures and phylogenetic analyses

Individual mature sporangia were isolated from the cultures using glass micropipettes and the single-cell PCR protocol was implemented as described by Reñé and Hoppenrath (2019) and Reñé et al. (2021). The first PCR was performed using the primers EK82F and 28S-1611R (López-García et al., 2001; Moreira et al., 2007) to amplify the entire ribosomal operon followed by (semi-) nested PCR. The region corresponding to the 18S rDNA was amplified using the specific primers CRYPTO22F and EUK1520R (Lazarus and James, 2015; López-García et al., 2003) as reported by Fernández-Valero et al. (2022). The ITS rDNA region was amplified using ITS1 and ITS4 primers (White et al., 1990), and the 28S rDNA region using primers D1R and D3B (Nunn et al., 1996; Scholin and Anderson, 1994). Both ITS and 28S rDNA were amplified in 25-µL reactions containing 1 µL of the first PCR product as template, 2.5 µL of 10 × buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.4 µM of forward and reverse primers, and 2 U of Taq DNA polymerase. The PCR program was as follows: initial denaturation at 94 °C for 2 min, 35 cycles including denaturation at 94 °C for 15 s, polymerization at 55 °C for 30 s, and elongation at 72 °C for 60 s, followed by a final elongation at 72 °C for 60 s (Reñé et al., 2021). Sanger sequencing with the aforementioned forward and reverse primers was performed by an external service (Genoscreen, France). The molecular sequences obtained were deposited in GenBank with accession numbers OQ865231–OQ865233 (28S rDNA) and OQ865234–OQ865236 (ITS rDNA).

A concatenated 18S rDNA + ITS + partial 28S rDNA dataset covering the diversity of Rhizophydiales Letcher was constructed following that

used by Reñé et al. (2022). It was aligned using MAFFT v7 (Katoh and Standley, 2013) and the resulting alignment was manually trimmed to remove poorly aligned positions. The final alignment comprised 52 sequences and 4113 positions. The best-fit substitution model was determined using jModeltest v2.1 (Darriba et al., 2012), resulting in GTR + I + GAMMA under both the Akaike information criterion and the Bayesian information criterion. A maximum likelihood (ML) phylogenetic tree was constructed with RAxML v8.2.12 (Stamatakis, 2014), inferring the best tree from 1000 different starting trees. Bootstrap statistical support was evaluated using 1000 pseudoreplicates. The ML analyses were run at the Marine Bioinformatics Service (MARBITS) of the Institut de Ciències del Mar (ICM-CSIC). A Bayesian reconstruction was conducted using MrBayes v3.2.1 (Ronquist et al., 2012), based on the GTR + I + GAMMA model, four MCMC chains, and 1,000,000 generations. Convergence of the MCMC analyses was confirmed in that the average standard deviation of split frequencies was below 0.01, the potential scale reduction factor was close to 1, effective sample sizes were > 200, and no trends were observed in the plots of generations versus log probability. The first 10% of trees were then considered as burned-in and the Bayesian posterior probabilities (BPP) were determined for the majority rule consensus tree.

2.6. Exploration of metabarcoding datasets

The presence and global distribution of the two species were explored in public V4 18S rDNA metabarcoding datasets, specifically, metaPR2 v2.0.1 (Vaulot et al., 2022), a compilation of 59 published metabarcoding datasets from different surveys that employed environmental metabarcoding targeting the V4 region of the 18S rRNA gene. The search was performed using the 18S rDNA sequences obtained from cultures of ICMB1106 (ON081639) and ICMB1107 (ON081641). These two sequences were also queried for sequence similarity using NCBI-BLASTN against different metabarcoding datasets available from the Catalan coast. Spatial distributions were assessed using the COPAS BioProject PRJNA630546 (Fernández-Valero et al., 2022; Reñé et al., 2021), and temporal distributions using SMART Bioprojects PRJNA1023727 and PRJNA891539. A detection was considered positive when the query coverage was > 80% and the sequence identity > 99%.

3. Results

Chytrids infecting the dinoflagellate *Alexandrium minutum* were detected in samples from the harbours of Vilanova i la Geltrú and Arenys de Mar (Catalan Coast, NW Mediterranean Sea) obtained during blooms of that species (Table 1). In both cases, samples were collected during spring, when the water temperature was 15–16 °C and host abundance was $\sim 10^5$ cells L⁻¹. The salinity values differed significantly between the two sites, i.e., 35.4 and 24.4, respectively.

3.1. Phylogeny

Molecular analyses were conducted to ascertain the phylogenetic relationships of the two chytrid strains (Fig. 1). Both were determined to belong to the family Dinomycetaceae (order Rhizophydiales). This cluster (99% bootstrap /1.00 BPP) comprised two sister clades, one including *Dinomyces arenysensis* Karpov & Guillou, strain ICMB1106, and *Dinomyces* sp. P242 - RCC3408, and the other including *Paradinomyces triforaminorum* and strain ICMB1107, with maximum statistical support for both. The 28S rDNA (OQ865232, OQ865233) and ITS (OQ865234, OQ865235) sequences of ICMB1106 showed 100% identity with *Dinomyces* sp. P242 - RCC3408 (KJ027549 and KJ027550) sequences, respectively (Supplementary Table S1). The 18S rDNA sequence of *Dinomyces* sp. P242 - RCC3408 was not available. The sequence identity between ICMB1106 and *D. arenysensis* was 98.2% for 18S rDNA, 77.3% for ITS, and 91.6% for 28S rDNA. The sequence

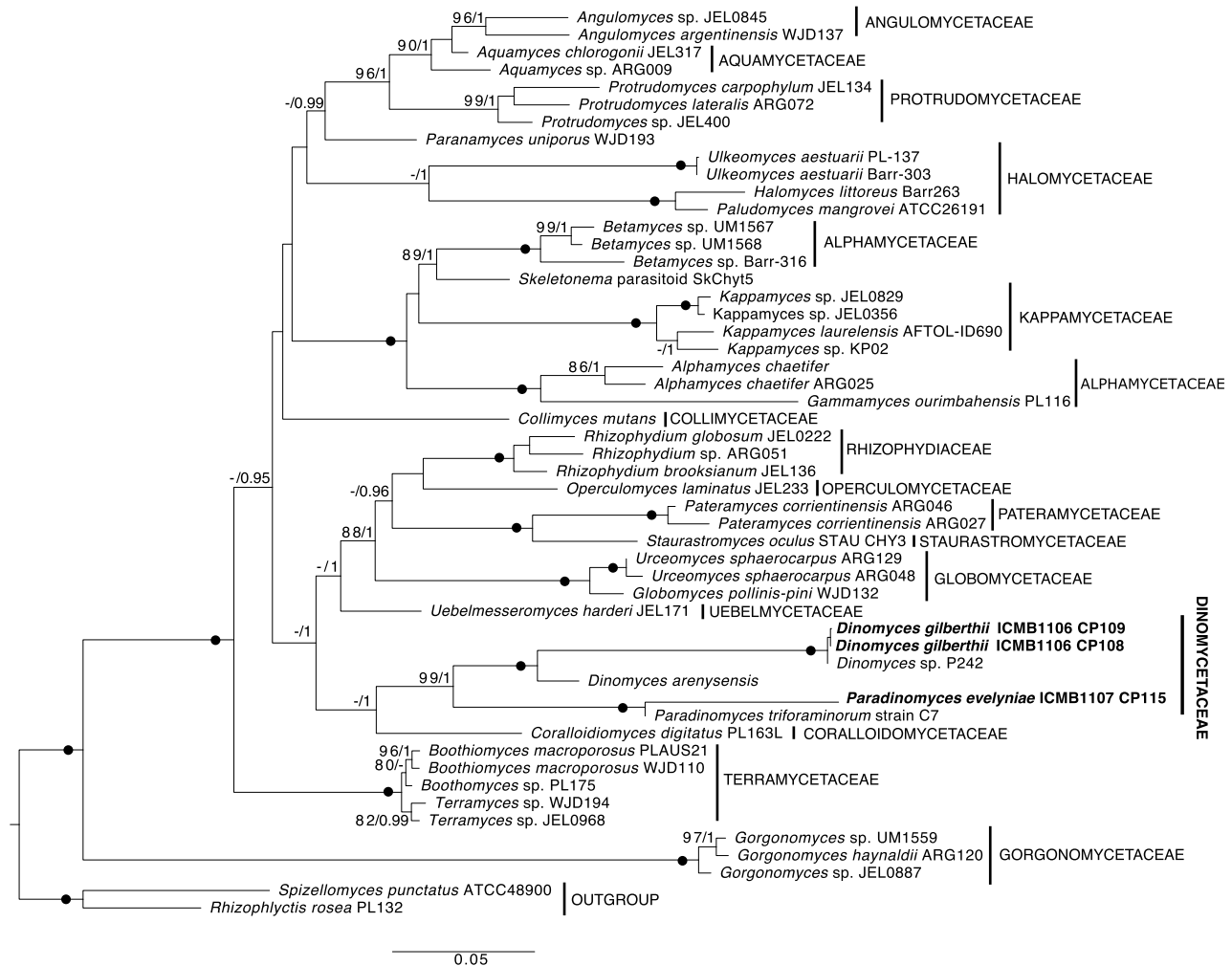


Fig. 1. Phylogenetic tree of Rhizophydiales based on concatenated 18S + ITS + 28S rDNA sequences. Sequences in bold were obtained in this study. *Spizellomyces punctatus* (Spizellomycetales) and *Rhizophlyctis rosea* (Rhizophlyctidales) sequences were used as an outgroup. The numbers above branches indicate the statistical support (ML bootstrap/Bayesian posterior probability); only values > 80% and 0.95, respectively, are shown. Black dots indicate maximum statistical support.

identity of ICMB1107 and *P. triforminorum* was 98.6%, 83.8%, and 94.5%, respectively. These results justified the designation of strains ICMB1106 and ICMB1107 as representing two new species belonging to the family Dinomycetaceae, namely, *Dinomyces gilberthii* sp. nov. and *Paradinomyces evelyniae* sp. nov., respectively.

3.2. Taxonomy

Dinomyces gilberthii sp. nov. Karpov, Fernández-Valero & René

Order Rhizophydiales Letcher, 2006
Family Dinomycetaceae Karpov & Guillou, 2014
Genus *Dinomyces* Karpov & Guillou, 2014
Index Fungorum: IF 900632.

GenBank accession numbers: ON081639–ON081640 (18S rDNA), OQ865234–OQ865235 (ITS), and OQ865232–OQ865233 (28S rDNA).

Diagnosis: Parasitoid of dinoflagellates with a likely host preference for *Alexandrium minutum*. Drop-shaped epibiotic sporangium 15–25 µm in length on the host *Alexandrium minutum*, with one or two papillae and connected to the host through a rhizoid with an apophysis. Spherical zoospores 2.0–2.6 µm in diameter, with a posterior lipid globule and a flagellum 8–11 µm long. Infects the dinoflagellates *Alexandrium minutum* and *Ostreopsis* sp.

Etymology: The species is named in honour of Gilberth Fernández, the father of the first author of this study.

Type: Fig. 2B.

Type strain: ICMB1106, isolated infecting the dinoflagellate *Alexandrium minutum* in Vilanova i la Geltrú harbour (41°12′54.9″ N, 1°43′57.9″ E), Catalonia (NW Mediterranean Sea), in April 2021.

Hapantotype: Fixed specimen of zoospores and sporangia derived from strain ICMB1106 embedded in a resin block and a stub for electron microscopy, deposited in the Marine Biological Reference Collections (CBMR) at the Institut de Ciències del Mar (ICM-CSIC, Barcelona, Catalonia, Spain) under the accession number ICMCBMR000651 (Guerrero et al., 2023).

Habitat: Marine coastal waters.

Morphological and ultrastructural description of *Dinomyces gilberthii* life-cycle stages

Zoospores of this chytrid species are spherical, with a diameter of 2.0–2.6 µm. They have a posterior flagellum 8–11 µm long and a posterior lipid globule (Fig. 2I). Once attached to the host, the zoospore encysts and a rhizoid with an apophysis develops inside the host cell (Fig. 2B, D, F). The rhizoid branches feed the chytrid and allow the growth of the sporangium (Fig. 2A, B, C, D), which develops one or two papillae (Fig. 2J). Multiple infections are common for this strain, as several zoospores were seen to settle on the host, encyst, and germinate within the dinoflagellate (Fig. 2A, B, G). Furthermore, multiple infections may not occur simultaneously (Fig. 2E, G). The number of infections in a single host cell determines the size of the sporangium and

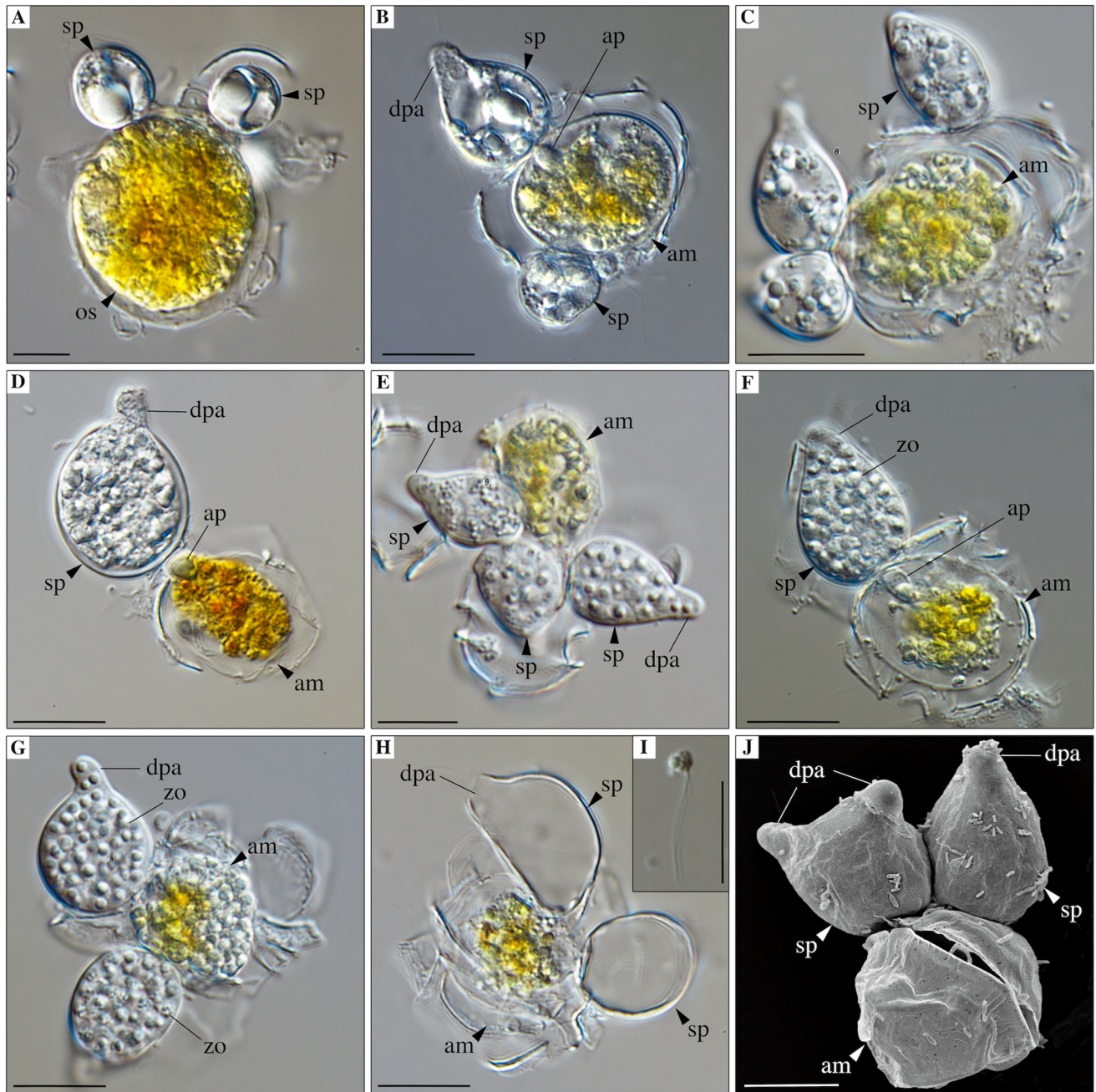


Fig. 2. Life-cycle stages of *Dinomyces gilberthii* sp. nov. in the light microscope (A–I) and in the SEM (J). (A) *A. minutum* infected by two young sporangia. (B, C) Young sporangium with a developed discharge papilla. (D) Sporangium before zoospore formation. (E) Sporangia showing the lipid globule compartmentalization during zoospore formation. (F, G) Mature sporangium with a discharge papilla. (H) Empty sporangium with a discharge papilla. (I) Zoospore. (J) Two mature sporangia with one and two discharge papillae. Abbreviations: am, *A. minutum*; ap, apophysis; dpa, discharge papilla; os, *Ostreopsis* sp.; sp, sporangium; zo, zoospores. Scale bars: 10 µm.

thus the number of zoospores produced per sporangium. If a host cell is infected by a single zoospore, the final biovolume of the sporangium will be similar to that of the host cell (Fig. 2D, F). However, if there are multiple infections of the same cell, the biovolume of each sporangium will be lower, and so will the number of zoospores produced per sporangium (Fig. 2 E, G). Mature sporangia are drop-shaped, with a length of 15–25 µm when a single infection of an *A. minutum* cell occurs, and typically develop one to two, rarely three, discharge papillae. After reaching its maximum size, sporangia show a gradual compartmentalization of the lipid globule (Fig. 2E, F, G), zoospores begin to individualize, and they are eventually released through the papillae once mature (Fig. 2H). The presence of sporangia with two discharge papillae is

common, observed in ~ 29% of sporangia (Fig. 2J), while the development of three discharge papillae is rare (<0.1%, not shown). The complete development of the parasitoid, from the cyst to the mature sporangium and zoospore release, takes ~ 25 h (Supplementary Video S1).

The ultrastructural characters of zoospores are key to the determination of their taxonomic affiliation. The general ultrastructural organization of *Dinomyces gilberthii* zoospores is shown in Fig. 3A–D. The nucleus lies near the center of the cell, slightly shifted towards one side (Fig. 3B–D). The ribosomal core occupies the central part of the zoospores, separating the nucleus from the mitochondrion and the microbody-lipid globule complex. A large lipid globule is present,

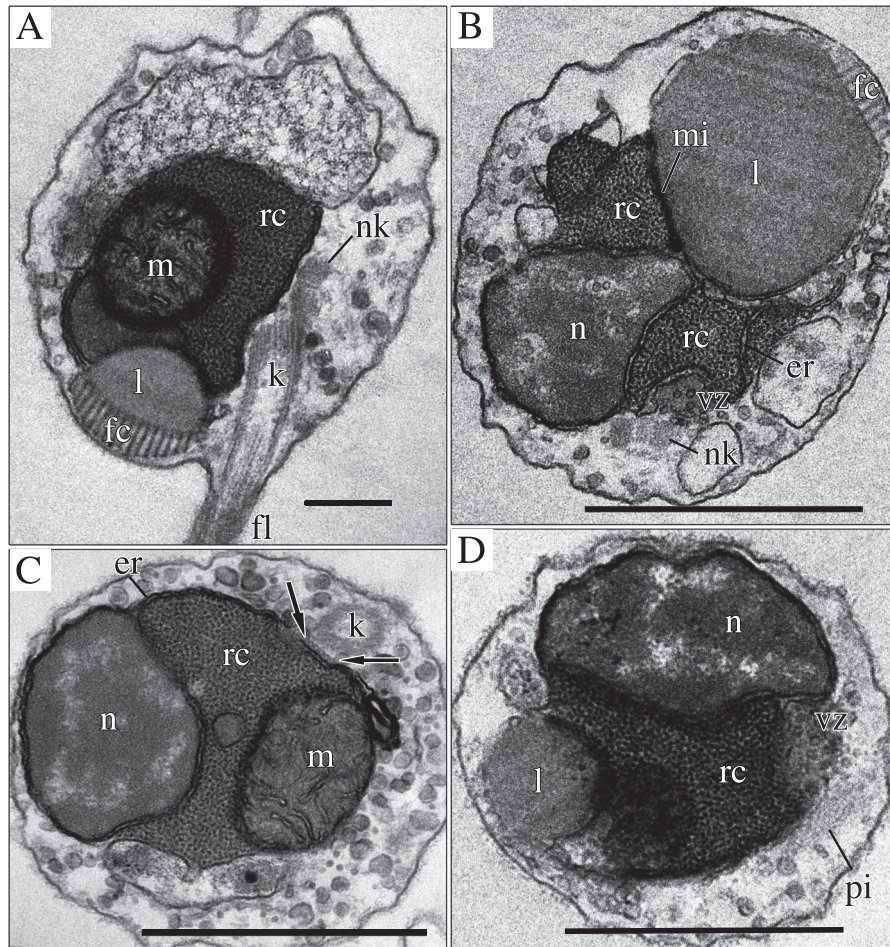


Fig. 3. Ultrastructure of *Dinomyces gilberthii* sp. nov. zoospores. (A) Longitudinal section showing the posterior flagellum. (B–D) General organelle disposition and cytoskeletal structures in different planes: cross-section of the flagellar kinetosome and five-microtubular root (C, arrows), longitudinal section of non-flagellar kinetosome (B), and paracrystalline inclusion (D). Abbreviations: er, endoplasmic reticulum; fc, fenestrated cisterna (botrosome); fl, flagellum; k, flagellar kinetosome; l, lipid globule; m, mitochondrion; mi, microbody; n, nucleus; nk, centriole (non flagellar kinetosome); pi, paracrystalline inclusion; rc, ribosomal core; vz, vesiculated zone. Scale bars: 400 nm (A) and 1 µm (B–D).

showing a prominent fenestrated cisterna (rumposome) facing the cell surface and covered with a flat microbody (Fig. 3A, B). One mitochondrial profile with flat cristae is attached to the ribosomal core outlined by the endoplasmic reticulum (Fig. 3A–C). Small dense bodies are scattered throughout the dense cytoplasm (Fig. 3A–C) and a vesiculated zone in the cytoplasm is present in the ribosomal core cavity near the kinetid (Fig. 3B, D). At the cell posterior, a paracrystalline inclusion was observed (Fig. 3D). The zoospores also contain a rather prominent vacuole with amorphous contents (Fig. 3A).

The kinetid is composed of a flagellar kinetosome and a short non-flagellar kinetosome (here called a centriole) together forming a 90° angle and connected by a short fibrillary bridge (Fig. 3A). The kinetosome has an anterior root of five microtubules, which is typical for Rhizophydiales (Fig. 3C).

Paradinomyces evelyniae sp. nov. Karpov, Fernández-Valero & Reñé

Order Rhizophydiales Letcher, 2006

Family Dinomycetaceae Karpov & Guillou, 2014

Genus *Paradinomyces* Reñé & Karpov, 2022

Index Fungorum: IF 900676.

GenBank accession numbers: ON081641–ON081642 (18S rDNA), OQ865236 (ITS), and OQ865231 (28S rDNA).

Diagnosis: Parasitoid of dinoflagellates, with a likely host

preference for *Alexandrium minutum* and *Ostreopsis* sp. Pear-shaped epibiotic mature sporangium 30–35 µm in length when infecting an *Alexandrium minutum* cell, with a pointed end producing a rhizoid, without papillae. Spherical zoospores 2.1–2.8 µm in diameter, with a single posterior lipid globule and a flagellum 11–13 µm long. Zoospores released from the sporangium through 1–3 discharge pores.

Etymology: The species is named in honour of Evelyn Valero, the mother of the first author of this study.

Hapantotype: Fixed specimen of zoospores and sporangia derived from strain ICMB1107 embedded in a resin block and a stub for electron microscopy, deposited in the Marine Biological Reference Collections (CBMR) at the Institut de Ciències del Mar (ICM-CSIC, Barcelona, Catalonia, Spain) under the accession number ICMCBMR000652 (Guerrero et al., 2023).

Type: Fig. 4D.

Type strain: ICMB1107 isolated infecting the dinoflagellate *Alexandrium minutum* in Arenys de Mar harbour (41°34'45.5" N, 2°33'19.8" E), Catalonia (NW Mediterranean Sea), in March 2021.

Habitat: Marine coastal waters.

Morphological and ultrastructural description of *Paradinomyces evelyniae* life-cycle stages

Free-living zoospores of this chytrid species are spherical, with a diameter of 2.1–2.8 µm. They have an 11 to 13 µm-long posterior flagellum and a posterior lipid globule (Fig. 4I). Once the zoospore attaches to a host cell, it encysts. The early trophic phase begins when the cyst

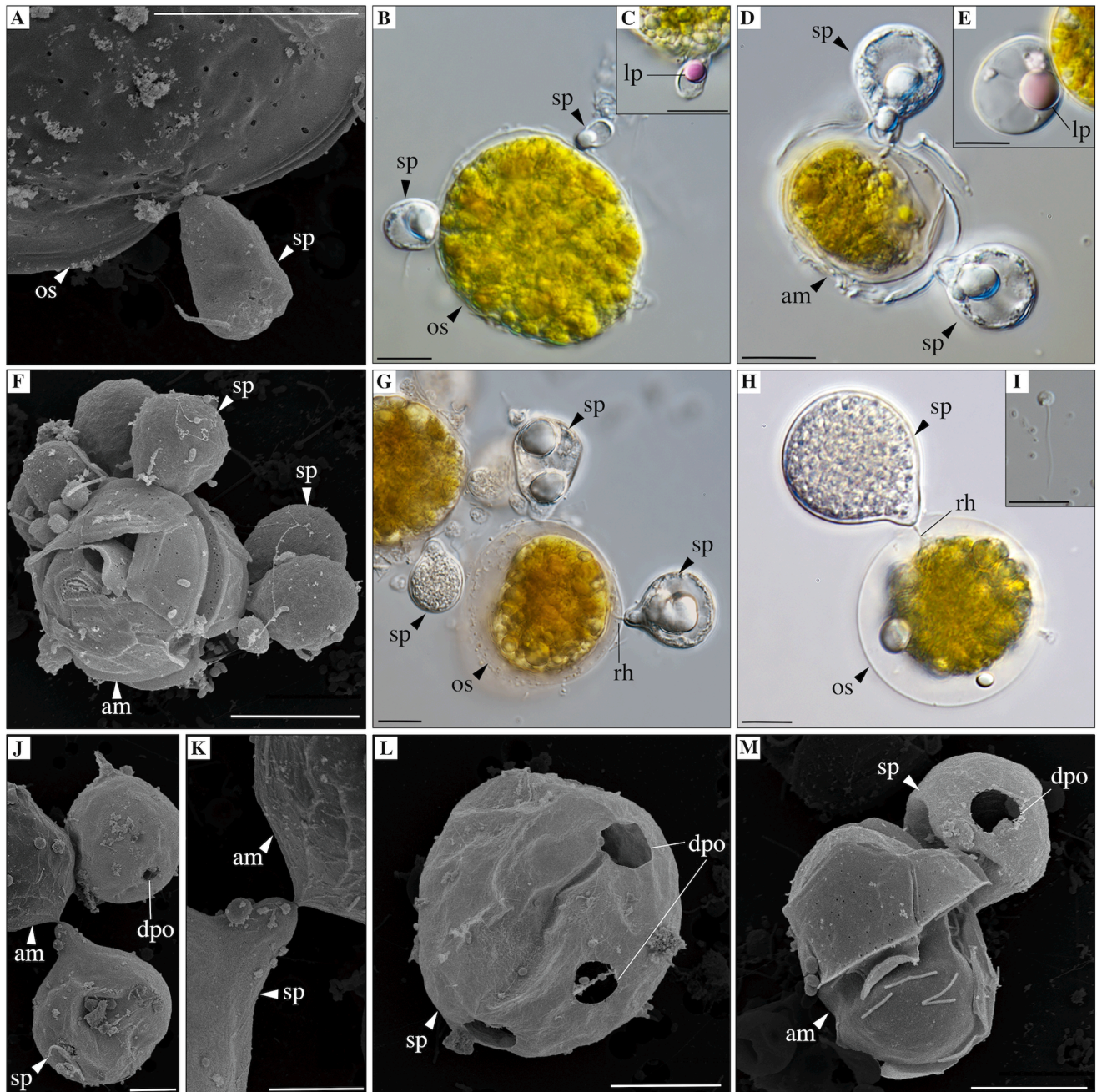


Fig. 4. Life-cycle stages of *Paradinomyces evelyniae* sp. nov. in the light microscope (B, C, D, E, G, H, I) and in the SEM (A, F, J, K, L, M). (A) Early sporangium. (B) Two young sporangia infecting an *Ostreopsis* sp. cell. (C) Nile Red staining of a lipid globule from a young sporangium. (D) Development of the infection of two sporangia in a cell of *A. minutum*. (E) Growth of the lipid droplet in a Nile Red-stained sporangium. (F) Multiple infections in a cell of *A. minutum*. (G) Division of the lipid droplet and growth of the sporangium in a cell of *Ostreopsis* sp. (H) Mature sporangium containing zoospores. (I) Zoospore. (J, K) Base of the sporangium connected to a host cell. (L) Three open discharge pores of an empty sporangium. (M) Empty sporangium with a discharge pore. Abbreviations: am, *A. minutum*; dpo, discharge pore; lp, lipid globule; rh, rhizoid; sp, sporangium; os, *Ostreopsis* sp. Scales bars: 10 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

produces a rhizoid, which penetrates the host cell. Young sporangia are spherical to pear-shaped, with a prominent central lipid globule (Fig. 4B–E). Mature sporangia are pear-shaped, 30–35 μ m in length when derived from a single infection of an *A. minutum* cell, and with a pointed end producing the rhizoid (Fig. 4H). Subsequent division of the plasmodium results in the formation of numerous small zoospores (Fig. 4H, I; [Supplementary Video S2](#)). The sporangia do not have visible structures related to zoospore discharge. The release occurs through discharge pores instead (Fig. 4J, L, M). The number of infections in the same host cell and the host species biovolume determine the final size of

the sporangium (Fig. 4F, H) and the number of zoospores. As infections do not occur synchronously, different stages of infection were observed among sporangia within the same host. The development of the parasitoid, from the cyst to the mature sporangium and release of the zoospores, takes approximately 30 h ([Supplementary Video S2](#)).

The general ultrastructural organization of *P. evelyniae* zoospores is shown in Fig. 5. The nucleus lies near the center of the cell, slightly shifted towards one side (Fig. 5A). The ribosomal core occupies the central part of the zoospore, separating the nucleus from the mitochondria and the microbody-lipid globule complex. A large lipid globule

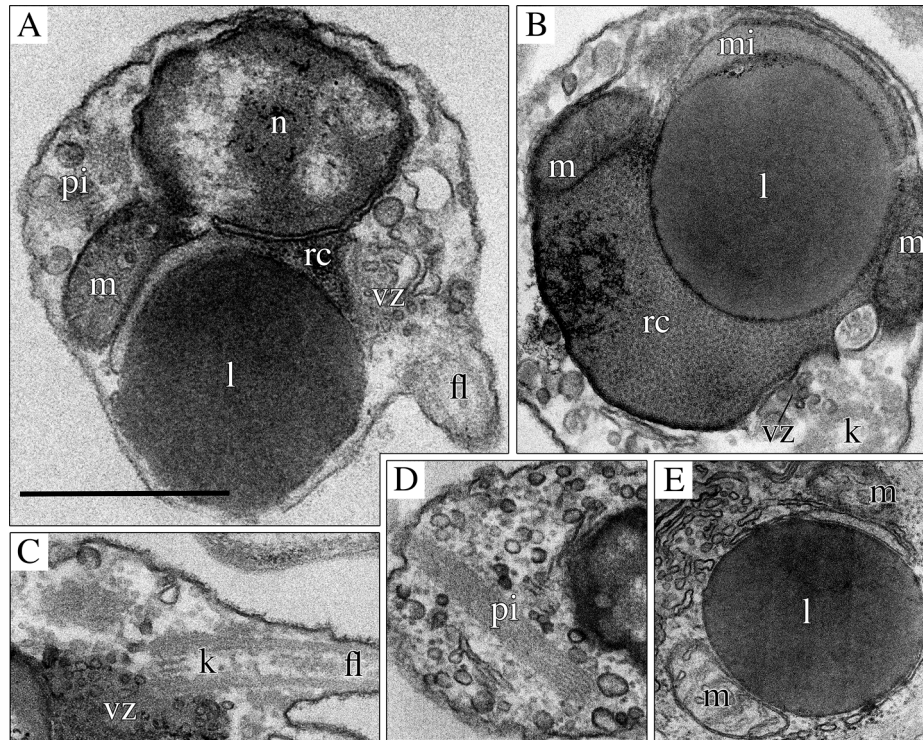


Fig. 5. Ultrastructure of *Paradinomyces evelyniae* sp. nov. zoospores. (A, B, E) Nucleus and general organelle disposition. (C) Longitudinal section of the kinetosome and flagellum. (D) Longitudinal section of paracrystalline inclusion. Abbreviations: fl, flagellum; k, flagellar kinetosome; l, lipid globule; m, mitochondrion; mi, microbody; n, nucleus; pi, paracrystalline inclusion; rc, ribosomal core; vz, vesiculated zone. Scale bar: 500 nm.

is covered with a flat microbody and has no fenestrated cisterna (Fig. 5B). Two mitochondrial profiles with flat cristae are attached to the ribosomal core outlined by the endoplasmic reticulum (Fig. 5A, B, E). Small dense bodies are scattered throughout the dense cytoplasm, with a vesiculated zone seen in the ribosomal core cavity near the kinetid (Fig. 5A, C). A paracrystalline inclusion lies in the cytoplasm near the nucleus (Fig. 5A, D). The kinetid is composed of the flagellar kinetosome (Fig. 5B, C) and a short non-flagellar kinetosome (not shown).

3.3. Geographical and temporal distribution of the novel fungal species

The Chytridiomycota species described in this study were detected in available metabarcoding datasets representing various locations along the Catalan coast (Table 2; Reñé et al., 2021). Examination of the spatial distribution of chytrids along the Catalan coast revealed the presence of *Dinomyces gilberthii* in other harbours (Cambrils, El Masnou and Barcelona) besides Vilanova i la Geltrú (from where it was isolated) during blooms of the dinoflagellate *A. minutum*. It was detected at the beach of Pals during a bloom of the dinoflagellate *Gymnodinium litoralis* Reñé and at the beach at the mouth of the La Muga river, concurrent with a bloom of mixed dinoflagellates dominated by *Prorocentrum micans* Ehrenberg (Reñé et al., 2021) as well. In a study examining the temporal patterns of parasites (unpublished) during coastal dinoflagellate blooms, *D. gilberthii* was detected at El Grau beach during the summer months (late June, July, August) and at L'Estartit beach (late June, July). The dinoflagellate communities in those samples were dominated by *G. litoralis*, but other dinoflagellate species, such as *A. taylori* Balech and *Levanderina fissa* (Levander) Moestrup, Hakanen, Gert Hansen, Daugbjerg & M.Ellegaard, were present as well. In a monthly sampling at Port Olímpic, *D. gilberthii* was detected in the water column during January and December 2022, when the dinoflagellate community was dominated by *A. minutum* and *Prorocentrum triestinum* J.Schiller, respectively. *Dinomyces gilberthii* was also found in sediment samples from January,

April, and July 2021. The dominant dinoflagellate in parallel water column samples was *Biecheleria* spp. Seawater temperature and salinity ranges during the detections were 11.8–27.1 °C and 28.6–38.2, respectively (Table 2).

Paradinomyces evelyniae, isolated during an *A. minutum* bloom at Arenys de Mar, was also detected in the water column from El Masnou harbour during *A. minutum* blooms and in sediments from Cambrils harbour. During monthly sampling at Port Olímpic, *P. evelyniae* was detected in seawater during December 2021 and January and December 2022, when the dominant dinoflagellate in the water column was *A. minutum*. The chytrid was also identified in sediment samples obtained in January, February, and May 2022, but was not detected again until December 2022, when *Biecheleria* sp. dominated the water column though other dinoflagellates were present as well. The seawater temperature and salinity ranges of the sampling sites were 11.8–21.9 °C and 36.4–38.0, respectively. *Paradinomyces evelyniae* was also occasionally found in samples from El Grau beach.

Consultation of the metaPR2 database for records of the two species revealed only the presence of *P. evelyniae* in the Baltic Sea. In that case, sampling had been conducted at the Tvärminne zoological station (entrance to the Gulf of Finland, Storfjärden) and in the coastal region of Krogarviken. The species was found in both the surface and the euphotic layer (down to ~ 15 m in depth) at those locations, as well as in the bottom water of Storfjärden, at depths ranging from 15 to 30 m. The temperature and salinity ranges at the sampling sites were 7.3–11.5 °C and 5.4–6.2, respectively (Table 2).

4. Discussion

4.1. Identity and characteristics of the newly described species

The present phylogenetic tree based on 18S, ITS, and 28S rDNA sequences showed two main clades within Dinomycetaceae. One included

Table 2Origin of amplicon sequence variants (ASVs) detected by metabarcoding and identified as *D. gilberthii* sp. nov. & *P. evelyniae* sp. nov., and characteristics of corresponding samples.

Species	Type	Location	Sampling date	Compartment	Dominant dinoflagellate	Temperature (°C)	Salinity	ASV match and reference	
<i>Dinomyces gilberthii</i>	Harbour	Cambrils ¹	15 MAY 2019	seawater, sediment	<i>Alexandrium minutum</i>	18.7	36.9	ASV84; René et al. (2021)	
		Barcelona ¹	28 MAR 2019	seawater, sediment		15.3	38.2		
		El Masnou ¹	2 MAR 2018	seawater		11.8	37.7		
			7 MAR 2018	seawater, sediment		13.2	38.0		
	Beach	La Muga ¹	5 JUN 2019	seawater	mixed dinoflagellates dominated by <i>Prorocentrum micans</i>	26.4	37.4		
		Pals ¹	4 JUL 2019	seawater, sediment	<i>Gymnodinium litoralis</i>	21.5	35.7		
	Harbour	Port Olímpic ¹	11 JAN 2022	seawater	<i>A. minutum</i>	12.9	36.6	ASV4101; Unpublished results	
			11 JAN 2022	sediment	<i>Biecheleira</i> spp.	12.9	36.6		
			26 JAN 2022	sediment		12.7	37.0		
			5 APR 2022	sediment	<i>Biecheleria</i> sp., <i>Alexandrium ostenfeldii</i>	13.2	36.4		
			29 JUL 2022	sediment	<i>Pyrophacus steinii</i> , <i>Biecheleria</i> sp.	27.1	37.6		
		L'Estartit ¹	15 DEC 2022	seawater	<i>Prorocentrum triestinum</i>	14.7	36.4		
			21 JUN 2022	seawater	<i>G. litoralis</i>	21.7	37.0		
			29 JUN 2022	seawater	<i>G. litoralis</i> , <i>Alexandrium taylori</i> , <i>Levanderina fissa</i>	24.4	37.2		
			08 JUL 2022	seawater	<i>Heterocapsa</i> spp.	22.8	37.3		
			El Grau ¹	21 JUN 2022	seawater	<i>G. litoralis</i>	22.9		32.9
	08 JUL 2022	seawater			23.0	35.9			
	18 JUL 2022	sediment		<i>A. taylori</i>	26.9	30.3			
	27 JUL 2022	seawater		<i>G. litoralis</i>	26.3	28.6			
	19 AUG 2022	seawater		<i>Heterocapsa</i> sp.	24.6	37.5			
	Harbour	Cambrils ¹	15 MAY 2019	sediment	<i>A. minutum</i>	18.7	36.9	ASV613; René et al. (2021)	
			2 MAR 2018	seawater		11.8	37.7		
			7 MAR 2018	seawater, sediment		13.2	38.0		
		Harbour	Port Olímpic ¹	14 DEC 2021	seawater	<i>A. minutum</i>	14.3	36.5	ASV5858; Unpublished results
				11 JAN 2022	seawater		12.9	36.6	
				11 JAN 2022	sediment	<i>Biecheleira</i> sp.	12.9	36.6	
				26 JAN 2022	sediment	<i>Biecheleria</i> sp., <i>Pyrophacus steinii</i>	12.7	37.0	
23 FEB 2022				sediment	<i>Biecheleria</i> sp.	14.0	36.9		
Grau ¹			19 MAY 2022	sediment	<i>Heterocapsa</i> sp., <i>Suessiaceae</i>	21.9	36.8		
			15 DEC 2022	seawater	<i>Prorocentrum triestinum</i>	14.7	36.4		
	19 AUG 2022		sediment	<i>Heterocapsa</i> sp.	24.6	37.5			
	Krogarviken ²		8 OCT 2012	water	<i>Heterocapsa triquetra</i> , <i>Biecheleria baltica</i> , <i>Dinophyceae</i> sp.	11.6	6.1		
			Beach	Storfjärden ²	8 OCT 2012	surface water	<i>Heterocapsa triquetra</i> , <i>Biecheleria baltica</i>	11.9	
8 OCT 2012	bottom water	<i>Heterocapsa triquetra</i> , <i>Biecheleria baltica</i>			11.9	6.1			
19 OCT 2012	bottom water	<i>Biecheleria baltica</i>			7.3	6.3			
29 OCT 2012	surface water	<i>Heterocapsa triquetra</i> , <i>Biecheleria baltica</i>			9.0	5.4			
29 OCT 2012	bottom water	<i>Gymnodinium corollarium</i>			10.5	5.6			

¹ NW Mediterranean Sea, ² Baltic Sea.

Dinomyces arenysensis and the new species *D. gilberthii*, while the other comprised *Paradinomyces triforaminorum* and the new species *P. evelyniae*. The identity between sequences belonging to the same clade was > 98% for 18S rDNA, >70% for ITS, and > 90% for 28S rDNA, while the identity between sequences belonging to different clades was much lower. As mentioned above, *D. gilberthii* corresponds to the species previously recorded as *Dinomyces* sp. P242 - RCC3408 (Lepelletier et al., 2014). Morphological or ultrastructural information was not provided in that report, and the species was tentatively classified as a *Dinomyces* representative based on the low divergence between its ITS and 28S rDNA sequences and those of *D. arenysensis*. Our results are in agreement with that interpretation.

The morphological, ultrastructural, and molecular results obtained in this study justify the description of the species included in each clade as belonging to the genera *Dinomyces* and *Paradinomyces*. A morphological distinction between them is in that the sporangia of *D. gilberthii* and *D. arenysensis* have an apophysis, whereas those of *P. evelyniae* and *P. triforaminorum* have branched rhizoids. Ultrastructural differences were also observed between the respective zoospores. Zoospores of *D. gilberthii* have fenestrated cisternae and kinetid characteristics similar to those of *D. arenysensis*, whereas the zoospores of *Paradinomyces triforaminorum* and *P. evelyniae* lack fenestrated cisternae. Although the kinetid was not studied in detail, that of *D. gilberthii* has the same anterior five-microtubular root seen in kinetids of other Dinomycetaceae, and an inclined kinetosome orientation was found in both *D. gilberthii* and *P. evelyniae*. Zoospores of the two strains contain a paracrystalline inclusion, as also found in *P. triforaminorum* (René et al., 2022). A paracrystalline inclusion was not detected in zoospores of *D. arenysensis* (Lepelletier et al., 2014), but the reinvestigation of its original TEM images revealed a bundle of microtubules at the zoospore's anterior side, as visible in Fig. 4D of Lepelletier et al. (2014). The paracrystalline inclusion consists of a pool of tubulins that may also assume a microtubular appearance, as observed in zoospores of *P. evelyniae* (Fig. 5). Given that zoospores of all described members of Dinomycetaceae contain a paracrystalline inclusion, it can be regarded as a character of the whole family.

Regarding interspecies differences, the two strains differ genetically and morphologically from the previously described species of *Dinomyces* and *Paradinomyces*. In the case of *Dinomyces*, the sporangia of *D. gilberthii* exhibit a drop-shaped growth derived from the development of discharge papillae for zoospore release. This structure is lacking in *D. arenysensis*, whose sporangia are more spherical. Furthermore, zoospores of these two species differ in their dimensions. In the case of *Paradinomyces*, early sporangium development in *P. evelyniae* is marked by a prominent lipid globule. In addition, the sporangia of *P. triforaminorum* have three openings for zoospore release, whereas *P. evelyniae* has a variable number (1–3) of discharge pores. However, zoospores of the two *Paradinomyces* species are of the same dimensions and have the same general ultrastructure.

4.2. Factors influencing the distribution and behaviour of *Dinomyces* and *Paradinomyces* species in marine environments

Both the establishment of chytrid-dinoflagellate interactions and the characterization of host preferences can be challenging when based only on metabarcoding information. However, it is clear that chytrid occurrence is influenced by the presence of preferred protist hosts, as demonstrated in the present study and that of Fernández-Valero et al. (2022). *Dinomyces gilberthii* was detected in various locations along the Catalan coast, both on beaches during summer and in harbour sediments throughout the year. This widespread coastal distribution of *D. gilberthii* highlights its adaptability. The role of this species in the dynamics of coastal dinoflagellate blooms could be similar to that studied in *D. arenysensis*, which varies from host-parasite coexistence (observed in the field and laboratory experiments) to near extinction of the host population (observed in laboratory experiments) (Fernández-Valero

et al., 2023).

The persistence of *D. gilberthii* in the water column during spring and summer coincided with high biomass blooms of the dinoflagellates *A. minutum* and *G. litoralis* or mixed communities dominated by these dinoflagellates. However, despite the co-occurrence of *G. litoralis* blooms and *D. gilberthii* indicated by metabarcoding datasets, attempts to induce infections of *G. litoralis* strain ICMB235 in the laboratory were unsuccessful (data not shown). In previous research, the inability of *Dinomyces* sp. P242 - RCC3408 (= *D. gilberthii*) to infect *G. litoralis* (strain ICMB226) was observed in cross-infection experiments (Lepelletier et al., 2014). Co-occurrence in the field but failure infections in the laboratory suggest strain-strain incompatibilities or *D. gilberthii*'s preference for alternative species during *G. litoralis* blooms. The need for further exploration of the chytrid's host range, with early indications of specificity for members of the genera *Alexandrium* Halim, *Scrippsiella* Balech, and *Ostreopsis* J. Schmidt (Lepelletier et al., 2014), is a reminder of the caution required when interpreting the simultaneous presence of two species in a metabarcoding dataset, as it may not represent a definitive relationship between them.

In contrast to *D. gilberthii*, *P. evelyniae*, with a more restricted occurrence in the water column, was mainly detected in harbour sediments, during winter and early spring, despite an exceptional detection in beach sediments in summer. The presence of *A. minutum*, one of the *P. evelyniae*'s dinoflagellate hosts, in the water column may have influenced the occurrence of the chytrid. Its more limited distribution along the Catalan coast, i.e., only during *A. minutum* blooms, compared to that of *D. gilberthii*, suggests a higher host specificity.

The frequent detection of chytrid-dinoflagellate pairs in coastal ecosystems implies greater importance of biotic factors, especially the host presence, than abiotic factors in the parasite development. On the other hand, the detection of chytrids in sediments points to this compartment as a species reservoir, when primary hosts are absent from the water column. Nonetheless, abiotic factors may also influence species distribution. The presence of *D. gilberthii* and *P. evelyniae* in Mediterranean samples and the occurrence of *Paradinomyces* spp. in the Baltic Sea question previous conclusions regarding the abiotic preferences of chytrids with respect to salinity. Notably, *P. triforaminorum* was previously described in low-salinity waters from the Baltic Sea during a bloom of the dinoflagellate *Kryptoperidinium foliaceum* (F.Stein) Lindemann (René et al., 2022), and *P. evelyniae* was detected in Northern Baltic Sea waters (Enberg et al., 2018). Further studies on how salinity modulates the geographical distribution of chytrids could provide deeper insights into their ecological dynamics within diverse aquatic ecosystems, especially for species able to adapt and survive in marine ecosystems, which thus far are assumed to include only a few (Rojas-Jimenez et al., 2019).

Indeed, zoospore fungi have been described as being less resistant to marine environments than other fungal groups, such as Basidiomycota and Ascomycota (Jones et al., 2022) and they show optimal growth under low salinity conditions (Guo et al., 2023). This may explain why locations, such as the Baltic Sea or sea ice, host a relatively high proportion of chytrids (Hassett et al., 2019). However, the same study also mentions locations of the Mediterranean Sea and Red Sea, both of which are characterized by high salinity and the presence of a high richness of Chytridiomycota. Therefore, whether salinity is a key factor in the chytrid biogeography is still an open debate.

Our study offers perspectives on the spatial and temporal distribution of two newly identified marine chytrids, including their recurrent presence in specific localities. Repetitive occurrences may be mainly influenced by the presence of the species in sediments along the year and host availability in the water column. The description of these species expands our understanding of the biological components that interact directly with dinoflagellate species, revealing added complexity in the dynamics of phytoplankton blooms. In the absence of primary hosts, parasites able to infect secondary hosts or enter dormant states (in the sediments) would have strong survival advantages. Both survival

mechanisms and the persistence of *D. gilberthii* and *P. evelyniae* in the sediment reservoirs remain to be examined in order to elucidate the direct role of these chytrid species in the coastal ecosystems.

Assessing the global presence of Chytridiomycota species in metabarcoding datasets from marine regions is challenging due to limited detections in global molecular datasets. The scarcity of these detections can be attributed to inadequate sampling efforts, particularly in regions where these fungi occur prominently, such as in the benthic and epiphytic compartments (Fernández-Valero et al., 2022). Additionally, limitations may arise from the lack of sampling during conditions favorable for chytrid growth, such as microalgal blooms (Fernández-Valero et al., 2023).

Undetected taxa are not proof of absence because a rare biosphere might not be revealed by metabarcoding due to insufficient sequencing depth. This study underscores the need to employ diverse sampling methods in diverse habitats and to integrate molecular and morphological techniques to gain a thorough understanding of the lesser-studied fungal diversity within marine ecosystems.

CRedit authorship contribution statement

Alan Denis Fernández-Valero: Investigation, Methodology, Writing – original draft, Writing – review & editing. **Sergey A. Karpov:** Writing – original draft, Visualization, Validation, Supervision, Formal analysis. **Nagore Sampedro:** Writing – review & editing, Resources, Methodology. **Jordina Gordi:** Resources, Methodology. **Natàlia Timoneda:** Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Esther Garcés:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Data curation, Conceptualization. **Albert Reñé:** Writing – review & editing, Writing – original draft, Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This study was funded by the Spanish MICINN Project SMART PID2020-112978GB-I00, with institutional support from the “Severo Ochoa Centre of Excellence” (CEX2019-000928-S). A. D. Fernández-Valero was funded by the MICIU grant “Ayudas para contratos predoctorales para la formación de doctores 2018” (PRE2018-084893). This work was partly supported by the Russian Science Foundation, grant no. 21–74–20089 (light and electron microscopy analysis, manuscript writing). SAK thanks Saint-Petersburg State University for a research grant (project ID Pure 115624290) and ZIN RAS program 122031100260-0. We thank the TEM-SEM Electron Microscopy Unit from Scientific and Technological Centers (CCiTUB), Universitat de Barcelona, and its staff for their support and advice during the TEM studies. We thank Dr. J.-M. Rintala (University of Helsinki, Finland) and Dr. M. Majaneva (Norwegian Institute for Nature Research, Norway) for providing metadata from their metabarcoding dataset. We also thank Dr. M. Antó and Dr. I. Ruiz (Institut de Biologia Evolutiva IBE-CSIC) for their time and for providing access to their laboratory equipment.

Data availability statement

Molecular sequences generated in this study are publicly available in GenBank. Phylogenetic alignments analysed in the present study are

available from the corresponding author upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejop.2024.126053>.

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9.6 Supplementary material of chapter 4

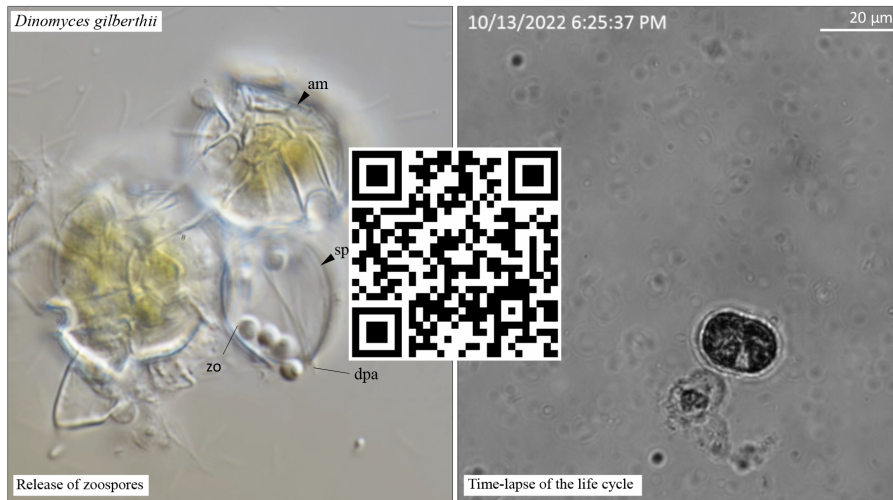


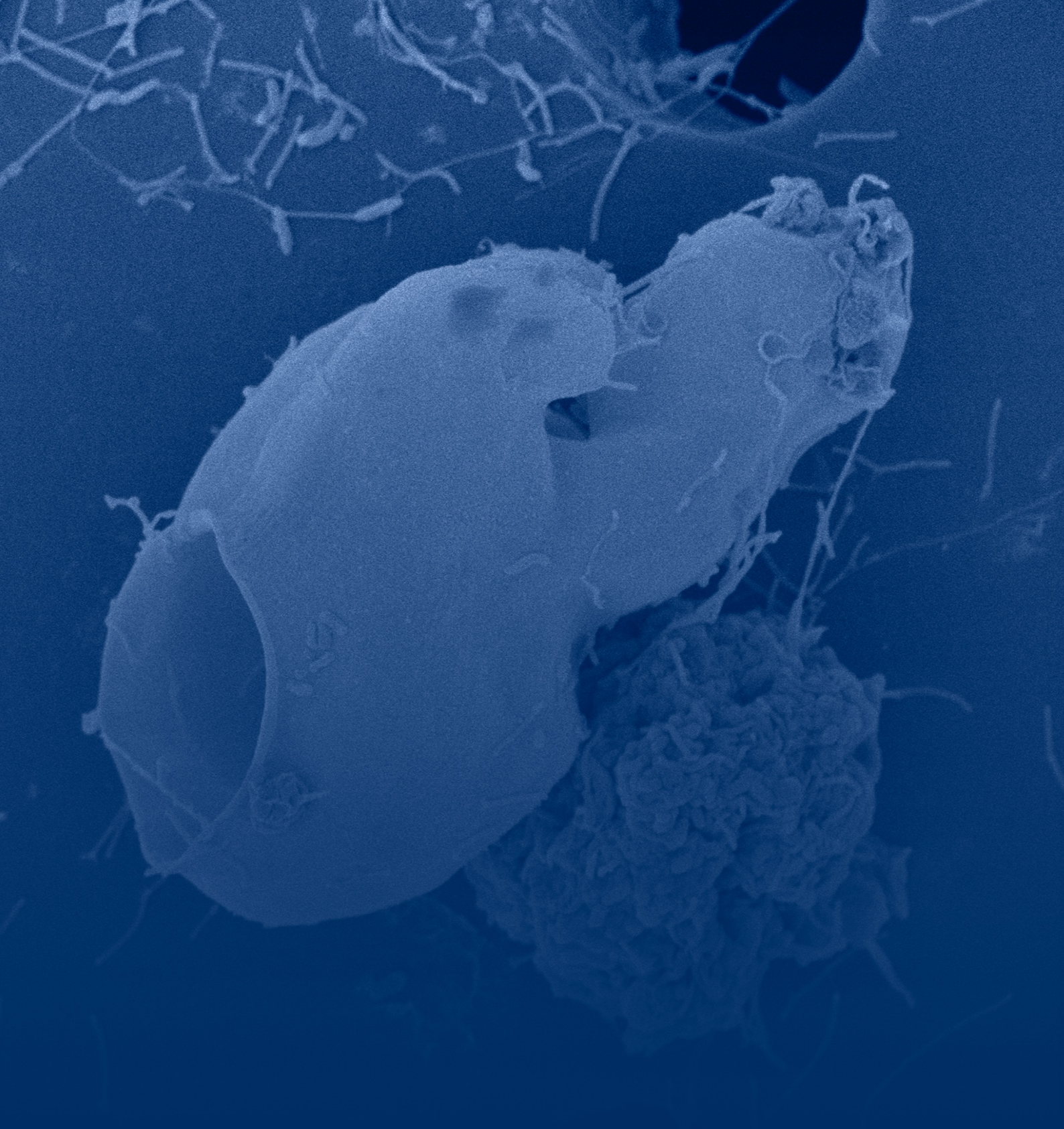
Figure 9.2: **Supplementary Video 1.** Time lapse of *Dinomyces gilberthii* infection stages. Abbreviations: am-A. minutum, dpa-discharge papilla, sp-sporangium, zo-zoospores.



Figure 9.3: **Supplementary Video 2.** Time lapse of *Paradinomyces evelyniae* infection stages. Abbreviations: os-Ostreopsis sp., sp-sporangium, zo-zoospores.

Table 9.2: **Supplementary table 1.** Matrix of pairwise identity values (%) between Dinomycetaceae representatives, based on their 18S rDNA, ITS, and 28S rDNA sequences. The 18S rDNA sequence of *Dinomyces* sp. P242 is not available.

18S rDNA	<i>Paradinomyces triforaminorum</i>	<i>Paradinomyces evelyniae</i>	<i>Dinomyces arenysensis</i>	<i>Dinomyces gilberthii</i>	
<i>Paradinomyces triforaminorum</i>		98.6%	97.5%	96.4%	
<i>Paradinomyces evelyniae</i>			96.6%	95.4%	
<i>Dinomyces arenysensis</i>				98.2%	
<i>Dinomyces gilberthii</i>					
ITS	<i>Paradinomyces triforaminorum</i>	<i>Paradinomyces evelyniae</i>	<i>Dinomyces arenysensis</i>	<i>Dinomyces gilberthii</i>	<i>Dinomyces</i> sp. P242
<i>Paradinomyces triforaminorum</i>		83.3%	66.6%	60.9%	55.5%
<i>Paradinomyces evelyniae</i>			61.9%	59.5%	62.0%
<i>Dinomyces arenysensis</i>				77.3%	75.0%
<i>Dinomyces gilberthii</i>					100%
<i>Dinomyces</i> sp. P242					
28S rDNA	<i>Paradinomyces triforaminorum</i>	<i>Paradinomyces evelyniae</i>	<i>Dinomyces arenysensis</i>	<i>Dinomyces gilberthii</i>	<i>Dinomyces</i> sp. P242
<i>Paradinomyces triforaminorum</i>		94.5%	88.0%	87.0%	87.0%
<i>Paradinomyces evelyniae</i>			87.3%	86.5%	86.5%
<i>Dinomyces arenysensis</i>				91.6%	91.9%
<i>Dinomyces gilberthii</i>					100 %
<i>Dinomyces</i> sp. P242					



10. Notes

Lined area for notes, consisting of multiple horizontal dashed lines.

