Host-parasite interactions: The *Parvilucifera sinerae* model in marine microalgae

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RESUM DE LA TESI

El parasitisme és una interacció generalitzada, que ha evolucionat pràcticament en totes les branques de l'arbre de la vida. Històricament no s'ha tingut en compte en l'estudi dels sistemes microbians marins, limitant el coneixement de les xarxes tròfiques marines i dels cicles biogeoquímics. Recentment, les eines moleculars han revelat moltes associacions hoste-paràsit fins ara desconegudes, situant els paràsits com a components clau de les comunitats planctòniques i bentòniques marines. El fitoplàncton sosté la major part de la producció primària, i de vegades causa proliferacions massives que poden tenir conseqüències negatives per als éssers humans i l'ecosistema. Les proliferacions de dinoflagel·lades sovint tenen lloc en la zona costanera, i co-ocorren amb paràsits de tipus zoosporic. En ocasions concretes, les infeccions causades pels paràsits poden ser la causa principal de mortalitat de les dinoflagel·lades, regulant la fi de la proliferació, de manera que s'ha suggerit el seu ús com a agents de control biològic. En la actualitat, hi ha descrits tres grups de paràsits eucariotes de dinoflagel·lades: els *Amoebophrya* (Sindinial), els *Parvilucifera* (Perkinsozoa) i els *Dinomyces* (Chytridiomycota). Ja que aquests paràsits poden controlar l'abundància dels seus hostes, poden afectar la dinàmica del fitoplàncton, l'estructura de la comunitat i la seva diversitat. Tanmateix, se sap molt poc sobre la seva ecologia i diversitat.

El gènere *Parvilucifera* és un dels grups que s'han descrit recentment dins dels Perkinsozoa. Aquest gènere comprèn 5 espècies, la majoria descrites recentment. La major part del seu coneixement inclou les seqüències del 18S rDNA, que permeten la seva classificació filogenètica, i els caràcters morfològics rellevants taxonòmicament. Per a això, la present tesi té com a objectiu entendre millor les interaccions hoste-paràsit de les comunitats planctòniques marines estudiant el sistema *P. sinerae*-dinoflagel·lades com a model. La seva interacció s'ha estudiat a diferents escales, des de cèl·lula a cèl·lula, a la població i a escala de comunitat, combinant l'experimentació al laboratori i l'estudi de camp.

La combinació de tècniques de microscòpia i eines moleculars, ha permès la descripció del cicle de vida del *P. sinerae* i la seva cinètica d'infecció. El *P. sinerae* té un cicle de vida directe que causa la mort de l'hoste, amb un temps de generació curt i una alta taxa de reproducció, produint una gran descendència a partir d'una sola infecció (Capítol 1). També hem identificat el sulfur de dimetil com el senyal químic que activa les zoòspores dins de l'esporangi, provocant el seu alliberament (Capítol 2). La nostra capacitat de cultivar el *P. sinerae* i les dinoflagel·lades, ha permès fer experiments d'infecció creuada, resultant en la classificació del *P. sinerae* com un paràsit generalista capaç d'infectar 15 gèneres de dinoflagel·lades (Capítol 3). A més, es va determinar que *P. sinerae* té preferència per certes espècies d'hoste, on assoleix una alta taxa de reproducció i de transmissió (Capítol 4). L'estudi de la detecció a la natura dels *Parvilucifera* i l'estimació del flux d'infecció usant trampes de sediments, ens ha permès caracteritzar i quantificar l'ocurrència, la dinàmica i l'impacte de la infecció per *Parvilucifera* durant les proliferacions de l'*Alexandrium minutum* (Capítol 5). Hem demostrat que aquestes proliferacions sempre van acompanyades per infeccions dels *Parvilucifera*, presentant una dinàmica temporal similar a la de la interacció entre depredador i presa, i contribuint a la fi de la proliferació amb una magnitud similar a d'altres factors biològics. L'anàlisi d'aquesta relació a diferents escales ha permès concloure que la dinàmica dels *Parvilucifera* està ben adaptada a la dels seus hostes, que formen aquestes proliferacions estacionals, les quals faciliten la transmissió dels *Parvilucifera*, mantenint-se la població de paràsits en ambients costaners marins.

SUMMARY OF THE THESIS

Parasitism is a widespread interaction that has evolved practically in all branches of the tree of life. It has historically been neglected in studies of marine microbial systems, limiting our understanding of marine food webs and biogeochemical cycles. Molecular tools have recently revealed many new host-parasite associations, placing parasites as key components of coastal marine planktonic and benthic communities. Phytoplankton sustains most of the marine primary production, sometimes causing massive proliferations or blooms, which may have negative consequences for humans and the ecosystem. Dinoflagellate blooms often occur in coastal areas, sometimes in co-occurrence with zoosporic parasite species. Occasionally, parasitic infections may be the main cause of dinoflagellate mortality, which can modulate bloom termination and consequently, their use has been suggested to biologically control natural blooms. Up to date, three groups of eukaryotic parasites of dinoflagellates have been described: *Amoebophrya* (Syndiniales), *Parvilucifera* (Perkinsozoa) and *Dinomyces* (Chytridiomycota). Such parasites can control the abundance of their hosts populations, and hence they can also affect phytoplankton dynamics, community structure and diversity. However, very little is still known about the ecology and diversity of these parasites, especially *Parvilucifera* and *Dinomyces*.

Parvilucifera genus is one of the recently described groups of Perkinsozoa. To date, the genus comprises only 5 species, some of them described very recently. Most of the knowledge about this genus is related to the 18S rDNA sequences that allow its phylogenetic classification, and also with the morphological characters valuable for taxonomy studies. For this reason, this PhD thesis aims to better understand the microbial host-parasite interactions of marine planktonic communities by studying *P. sinerae*-dinoflagellates as a model system. Here we studied these host-parasite interactions at different scales, from cell-cell, to population and at community level, combining laboratory experiments and field studies.

The use of several microscope techniques and molecular tools (TSA-FISH) have allowed the characterization of the life-cycle of *P. sinerae* and the kinetics of the infection stages. *P. sinerae* has a direct life cycle that causes the host death, with a short generation time and a high asexual reproduction rate, producing a huge offspring from a single infection (Chapter 1). Moreover, we unequivocally identified dymethilsulfide as the chemical cue that triggers zoospore activation and release from the dormant sporangium (Chapter 2). Our capacity to culture both partners of the association in the lab, *P. sinerae* and dinoflagellates, allowed for a series of cross-infection experiments, which resulted in the designation of *P. sinerae* as a generalist parasitoid, being able to infect up to 15 genera of dinoflagellates (Chapter 3). Furthermore, we determined that *P. sinerae* exhibits preferences for certain host species, which enhance parasitoid reproduction rate and transmission (Chapter 4). The study of in situ *Parvilucifera* detection and estimates of the flux of infected host cells using sediment traps has allowed us to unveil the occurrence, dynamics, and impact of *Parvilucifera* infection during *Alexandrium minutum* natural blooms (Chapter 5). We showed that outbreaks of the dinoflagellate *A. minutum* were always accompanied by *Parvilucifera* infections, presenting a host-parasitoid temporal dynamic similar to predator-prey interactions, and contributing to bloom decrease with a similar magnitude than other biological loss factors. The analysis of this relationship at different scales has provided the necessary information to conclude that the ecology of *Parvilucifera* is well adapted to that of its blooming hosts, whose seasonal proliferations enhance *Parvilucifera* transmission, sustaining the parasitic populations in marine coastal environments.

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General Introduction

General Introduction

Types of association between organisms – Interspecies interactions

The biotic environment of an organism consists of all the other organisms that interact with and affect it. In nature, no organism exists in isolation, being all organisms, without exception, involved in a complex network of interactions between them. In an ecosystem, these relations between different species are called interspecific interactions. Most of them are critical for their survival, and play a major role in regulating population growth and abundance. There are many different approaches to categorize and define them. In 1878, de Bary defined the term 'symbiosis' as an assemblage of dissimilar organisms living together. It is a broad definition of symbiosis and there is no implication regarding to the length or the outcome of the association, nor does it imply physiological dependence or benefit or harm to the symbionts involved in the partnership. Thus, symbiosis include a continuum with a high variety of intimate partnerships in nature defined in Box 1. Later on, in order to categorize this continuum, parasitologists established artificial thresholds to divide interspecies interactions based on the dependency of the association, its durability, and the impact on each of the associated organisms (Box 1. Fig. B1.1). Within the durable relationships, there is a gradient from the modern concept of symbiosis, which include mutualism, where both organisms benefit, commensalism, one benefits and the other is unaffected, and parasitism, where benefit of one cause harm on the other. Moreover, predator-prey interactions are considered short-term interactions with the same partner's impact than parasitism (Combes, 2001). Recently, Goater et al. (2013) defined several categories of symbiosis related to trophic relationships and how energy is transferred between partners (Box 1. Fig. B1.2). This can be seen as a continuum with vague boundaries with a broad trend in evolution (Goater et al., 2013) .

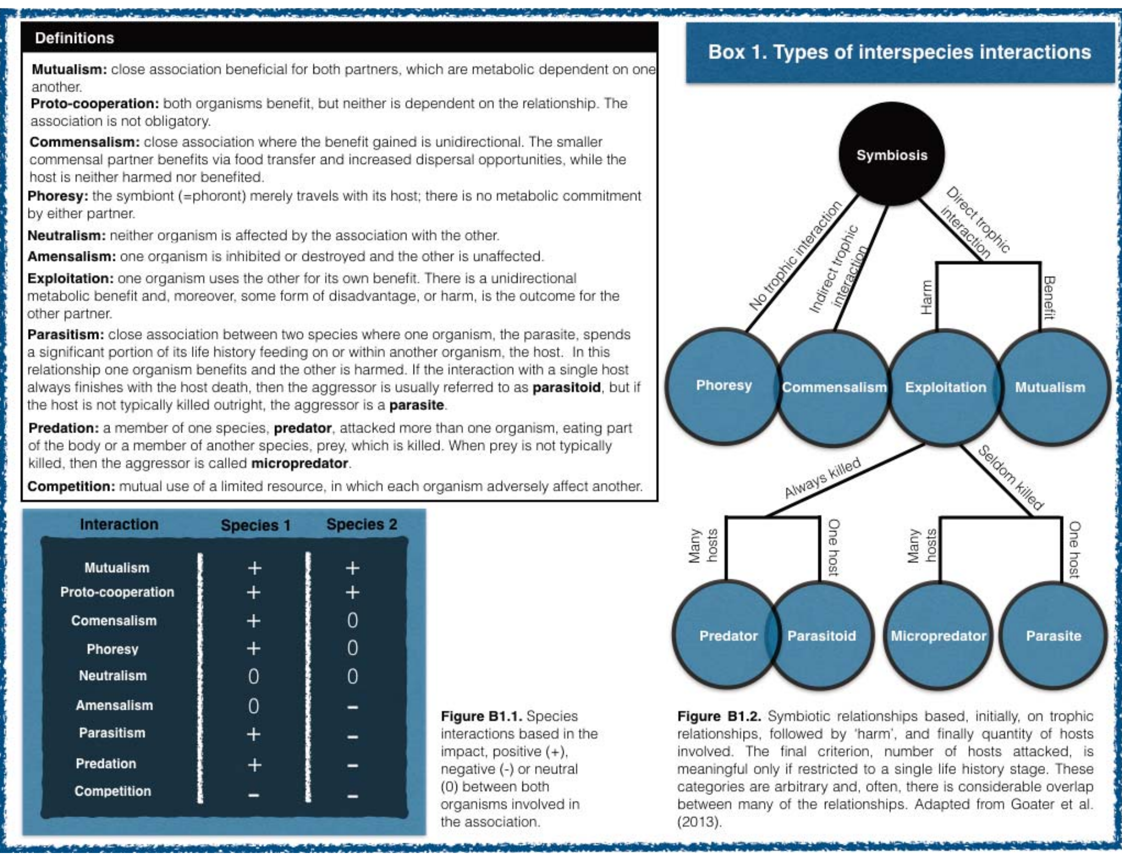
Identify the nature and strength of such interactions and associations is of prime importance to understand the structure and function of natural communities and the energy transfer within food webs. Of all the intimate and durable interspecies associations, parasitism has received a great attention, since it is considered to promote the major factors that have influenced the organization and evolution of life (Thomas et al. 1996). In the present thesis, we will focus on parasitic interactions in marine phytoplankton, one of the most important compartments of the marine planktonic food web.

Parasitism

Parasitism is considered as one of the most common life strategies on earth due to the large number of parasite species and their ubiquities in nature (Windsor, 1998). The term 'parasitism' has as many definitions as the number of scientists working on parasites. The classical definition hold that it is an intimate interspecific interaction where one organism (the parasite) spends the whole or part of its life feeding in or a single individual of another species (the host) (Price, 1980). When defining parasitism, the idea of expense or harm was introduced as a functional characteristic of the host-parasite relationship, implying a negative impact on the host. The parasite benefits give rise to the host harm, termed virulence, where important fitness traits of the host are often negatively affected by the parasite. A difference from predators, parasites cause a degree of damage but not use to cause death to its host, and host mortality is related to the reduction in body condition or physiological fitness. Other definitions of parasitism rely on the idea of genetic comple-

mentation (Combes, 2001), or metabolic dependence (Crofton, 1971) or both, which implies a long-term process of adaptation between the two partners or 'arms race' leading coevolution (Van Valen, 1973). These definitions attempt to understand the nature of the host-parasite relationship, that is the manner in which partners are tied to each other, both evolutionarily and ecologically. However, parasitologists concluded there is no distinct ecology, function, evolution, or physiology that discriminates and define all parasites from all non-parasite species, because parasites do not represent a monophyletic group, but a variety of organisms that converge in a mode of life.

Parasitism had appeared independently in many different lineages (de Meeûs and Renaud, 2002), including a very diverse group of organisms that have managed to spread across a large diverse taxonomic host groups converging in some morphological, ecological, and epidemiological traits. Poulin and Morand (2014) consider that there have been many independent evolutionary transitions from free-living to obligate parasites. Given the independent origins of parasitism across numerous Phyla, they have evolved a huge diversity of life cycles and lifestyles in order to exploit all the diversity of hosts, which can be translate in a high diversity of host-parasite interactions. Such diversity of interactions can be categorized within a set of more-or-less distinct parasitic strategies. Earlier categorizations based on shared traits rather than phylogeny, such as the parasite localization, the degree of dependency, their life cycle patterns, their host range, their mode of transmission, fail in describing parasite strategies because they consider a single trait, rather than taking a general approach of all parasite features and their associations. In 1979, Anderson



Box 1. Types of interspecies interactions

Definitions

Mutualism: close association beneficial for both partners, which are metabolic dependent on one another.

Proto-cooperation: both organisms benefit, but neither is dependent on the relationship. The association is not obligatory.

Commensalism: close association where the benefit gained is unidirectional. The smaller commensal partner benefits via food transfer and increased dispersal opportunities, while the host is neither harmed nor benefited.

Phoresy: the symbiont (=phoront) merely travels with its host; there is no metabolic commitment by either partner.

Neutralism: neither organism is affected by the association with the other.

Amensalism: one organism is inhibited or destroyed and the other is unaffected.

Exploitation: one organism uses the other for its own benefit. There is a unidirectional metabolic benefit and, moreover, some form of disadvantage, or harm, is the outcome for the other partner.

Parasitism: close association between two species where one organism, the parasite, spends a significant portion of its life history feeding on or within another organism, the host. In this relationship one organism benefits and the other is harmed. If the interaction with a single host always finishes with the host death, then the aggressor is usually referred to as **parasitoid**, but if the host is not typically killed outright, the aggressor is a **parasite**.

Predation: a member of one species, **predator**, attacked more than one organism, eating part of the body or a member of another species, prey, which is killed. When prey is not typically killed, then the aggressor is called **micropredator**.

Competition: mutual use of a limited resource, in which each organism adversely affect another.

Interaction	Species 1	Species 2
Mutualism	+	+
Proto-cooperation	+	+
Comensalism	+	0
Phoresy	+	0
Neutralism	0	0
Amensalism	0	–
Parasitism	+	–
Predation	+	–
Competition	–	–

Figure B1.1. Species interactions based in the impact, positive (+), negative (-) or neutral (0) between both organisms involved in the association.

Figure B1.2. Symbiotic relationships based, initially, on trophic relationships, followed by 'harm', and finally quantity of hosts involved. The final criterion, number of hosts attacked, is meaningful only if restricted to a single life history stage. These categories are arbitrary and, often, there is considerable overlap between many of the relationships. Adapted from Goater et al. (2013).

and May made a more general approach, which was one of the most important categorizations of parasites for epidemiology, distinguishing between micro- and macroparasites. This dichotomy was based in several biological traits of the parasite-host interaction to describe two different parasite dynamics in host populations (Anderson and May, 1979; May and Anderson, 1979). The latest attempts to classify the existing parasite strategies derived, in using several life history traits of parasites in a factorial design to identify and describe general evolutionary end points towards which most parasite lineages tend to converge (Poulin, 2011). The terminology used to describe the type of parasites, hosts, life cycles and parasitic strategies that exists is summarized in Box 2.

The number of parasite species on earth is still under debate, and the estimates on parasitic diversity differ depending on how broad we define the term parasite. Several studies calculate that between the 30-50% of the described species are parasitic at some stage during their life cycle (Price, 1980; Windsor, 1998; de Meeûs and Renaud, 2002; Poulin and Morand, 2014). Given that all living species are, at least, infected by one species of parasite, knowledge of parasite diversity links to knowledge of the scientifically known branches of the Tree of Life. In that sense, our view of life has focused in multicellular organisms, which are more conspicuous and familiar to us. The 96% of the eukaryotic described species belongs to animals, fungi and plants although they represent the 23% of all operational taxonomic units (OTUs) in environmental surveys (del Campo et al., 2014). So, the current knowledge of parasite diversity is mainly based on those ones that infect these three eukaryotic kingdoms. Taking this bias into account, the proportion of parasitic species is probably huge underestimated due to these differential efforts in studying the taxonomy of parasitic organisms as compared with the free-living ones, and the bias in knowledge on biodiversity of economically and pathologically relevant species at the expenses of the ecologically relevant ones (de Meeûs and Renaud, 2002). Thus, most of the studies of parasitism have placed a great effort in terrestrial ecosystems due to the impact of parasites in human health and agriculture, rather than in freshwater and marine ecosystems. Moreover, in the marine environment, research on parasitism focuses in parasites of economically relevant species, such as fish and shellfish (Rohde, 2005) although marine ecosystems are mainly composed by a huge diversity of unicellular life forms, such as archaea, bacteria, and eukaryotes. So that, very little is known about the ecology and diversity of parasites of microbial communities. For instance, the apicomplexans within the Alveolata, which is a parasitic group with representatives in all environments, are well studied in terrestrial ecosystems because they comprise important human and animal parasites, while very little is known about their representatives in the marine environment (Skovgaard, 2014). Moreover, ecology and diversity of other groups of parasitic Alveolates, such as perkinsozoans are much less known, although they can cause high mortalities in aquaculture producing important economic losses (Lafferty et al., 2015). In the present thesis, we will focus on *Parvilucifera* genus within the Perkinsozoa, a marine parasitic group of phytoplankton whose ecology and diversity remains largely unexplored.

Relevance of parasitism in marine planktonic communities

Primary productivity in the ocean is mostly carried out by protistan (unicellular eukaryotic) phytoplankton, accounting for approximately 50% of the total photosynthesis on Earth, which is close to the calculated for the terrestrial plants (Field et al., 1998). These microorganisms perform oxygenic photosynthesis, which remove carbon dioxide from the atmosphere to produce organic compounds, sustaining the whole marine trophic web and playing a key role in the biogeochem-

Box 2. Definitions related to parasitism

Types of parasites

Endoparasite: a parasite which lives within the body of the host.

Ectoparasite: a parasite which lives on the outside of the host.

Obligate parasite: a parasite which is completely dependent upon the host.

Facultative parasite: a parasite which is capable of living both freely and as a parasite.

Microparasite: usually small parasites (up to few hundreds of μm in size), typically referring to the viruses, bacteria, fungi, and protozoa. They present short generation times, and can multiply to large numbers within their host.

Macroparasite: large parasites, typically referring to helminths, parasitic insects, etc. They have generation time comparable to their hosts, sometimes just a single individual.

Parasitoid: parasites that have a free-living stage, while the juvenile lives in or on the host. Typically consists of parasitic insects. Often, but not always, parasitoids kill their hosts before progressing to their next stage in the life-cycle.

Hyperparasite: a parasite that parasitises another parasite.

Types of hosts

Definitive host: is the host in which a parasite reaches sexual maturity and undergoes sexual reproduction.

Intermediate host: the host in which asexual development of the parasite occurs.

Paratenic (transport) host: host in which parasite does not undergo any development but remains alive till it gains entry in the definitive or intermediate host. It bridges the ecological gap between the definitive and intermediate host.

Vector host: host in which a part of the life cycle of a parasite takes place and is also instrumental in the transmission of the parasite from main host to other.

Reservoir host: temporary host. Is the host where the parasite survives in the absence of regular hosts. This host become the source of infection to regular hosts.

Types of life cycle

Direct or simple life cycle: involve one single host.

Indirect or complex life cycle: involve two or more host (intermediate and definitive hosts).

Types of transmission

Direct transmission: the parasite transfers to a new host without any intervening other host or vector.

Paratenic transmission: the parasite transfers to a new host via another opportunistic host where the parasite does not develop, grow or multiply.

Vector transmission: the parasite transfers to a new host via an specialised (vector) host, which involves physiological interactions between the parasite and its vector. Inside the vector parasite can develop.

Horizontal transmission: parasite is transferred from one host to other hosts in the population.

Vertical transmission: parasite is transferred to offspring of the current host.

ical processes (Worden et al., 2015). Such marine protists have evolved different life forms that exploit many different conditions (Glibert, 2016) and their temporal dynamics and succession depends not only on abiotic factors (chemical and physical) but also on the biological ones, it is interactions with the other organisms, such as parasitism or grazing (Fig. 1). The combination of these factors influence on phytoplankton growth that sometimes results in a massive increase in their populations, producing transient proliferations referred as blooms. In pelagic systems, phytoplankton blooms are the principal resources by which flows of energy and matter feed higher trophic levels and export organic matter to deeper waters escaping to the coupled microbial loop (Kiørboe, 1993). Therefore, these blooms are integral to planktonic system dynamics and biogeochemical cycles, being essentials for ecosystem functioning.

Since carbon cycling and sequestration depends on photosynthesis and on carbon oxidation rates carried out by heterotrophic organisms, biological interactions within photosynthetic protists,

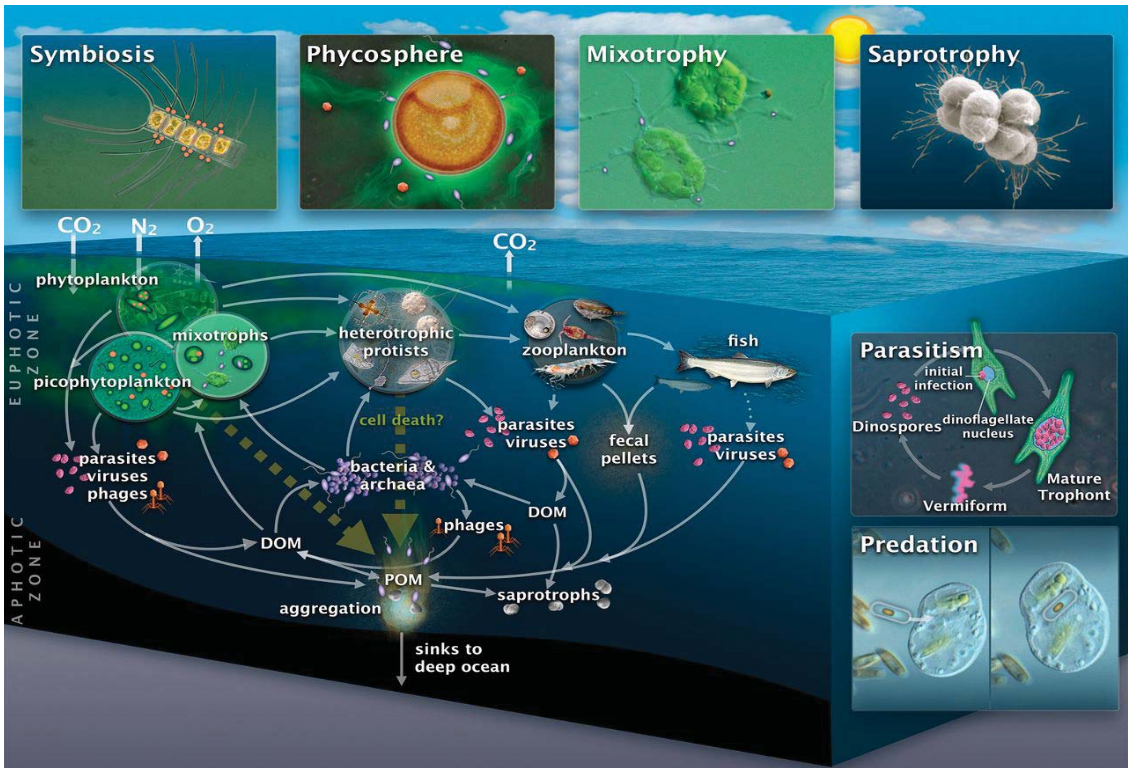


Figure 1. Microbes interact within them structuring ocean ecosystems and biogeochemical cycles. Photosynthetic protists and cyanobacteria fix and transform CO_2 into organic matter, that have diverse food web links. Eukaryotes are involved in a complex web of interactions, as represented in inset boxes. Eukaryotic parasites are one force of mortality that affects many types of eukaryotes (with host specificity), as do viral infections. Parasitic and viral infections produce a release of a variety of organic matter (i.e., POM and DOM). All microorganisms contribute to CO_2 respiration, which results in CO_2 release to the atmosphere and decreases organic carbon export to the deep ocean. Inorganic nutrients and direct release of CO_2 are not represented for simplification purposes. From Worden et al. (2015).

such as predation, grazing, and parasitism among others, will affect fluxes of matter, altering dissolved and particulate organic matter pools (DOM and POM). The recent view of complex marine food web support a multifarious network of phototrophic and heterotrophic strategies that decompose the diversity of organic compounds to make them available for other trophic levels, which in turn influence the budgets of energy transferred in the whole marine food web (Figure 1 inset). Although efforts have been made to include the main organism interactions in food web studies, the majority of protistan roles and linkages are not represented in ecosystem and carbon cycle models, and most of them have not yet been rigorously quantified.

Historically, predation and/or grazing were thought to be the interactions dominating marine planktonic food web linkages whereas parasitism had been largely overlooked. This was mainly because technical and sampling limitations related to the difficulty of studying parasites and the host-parasite interactions since: i) many microscopic organisms cannot be cultured in the laboratory; ii) most parasites have distinct or cryptic morphologies and present complex life cycles; iii) parasites are difficult to find as they are inside their (often unknown) hosts (Chambouvet et al., 2015b). Although parasitism have been reported in the different components of the marine plankton (Park et al., 2004; Skovgaard, 2014) there is only a few studies that have addressed its ecological importance, in contrast to large research efforts dedicated to the role of parasites in freshwater planktonic communities (Kagami et al., 2007; Lefèvre et al., 2008; Rasconi et al., 2012; Sime-Ngando, 2012). However, with the recent advances in molecular tools, environmental surveys have highlighted that parasitism is a widespread interaction in marine planktonic systems, unveiling a high diversity of unclassified parasites, and placing such parasites as key components of the microbial communities (de Vargas et al., 2015; Cleary and Durbin, 2016). Hypothetical parasitic links have been identified by co-occurrence studies (Lima-Mendez et al., 2015), and nowadays, the challenge is to validate them by characterizing the underlying mechanisms and constrains these interactions by empirical measurements. That means answering questions like, what taxa do they infect and by what mechanism? What is the outcome of the infection? Do they have direct or complex life cycles? Do they have free-living stages and how abundant they are? Answering these questions would help to quantify and parameterize these interactions into global biogeochemical models and assess their impact in the marine ecosystem.

A wide variety of prokaryotic and eukaryotic organisms had been identified as parasites of phytoplankton. Prokaryotic pathogens, including viruses and bacteria are usually reported infecting smaller phytoplankton species, such as chrysophytes, prymnesiophytes, prasinophytes, raphydophytes and cyanobacteria, whereas they appear less frequently infecting dinoflagellates and diatoms (Brussaard, 2004). By contrast, these larger phytoplankton cells are usually infected by a wide variety of eukaryotic parasites, including fungi, perkinsozoa, amoebae, syndiniales, euglenoids and kinetoplastids (Park et al., 2004; Skovgaard, 2014).

The effect that these parasites can cause in their protist hosts implies different ecological and evolutionary scales that are summarized in Figure 2. Since they produce direct changes in phytoplankton abundances, they may play a top-down control role in their host populations affecting population dynamics and succession (Toth et al., 2004; Chambouvet et al., 2008). The extent to which a parasite may impact different hosts populations strongly depends on their host range and the degree of host specificity (Keesing et al., 2010), which determines their ecological niche and the strength of their interactions. So that, parasites will exert asymmetrical pressures on different phytoplanktonic hosts, indirectly affecting other non-host species (Hatcher et al., 2012) and

in consequence, the evolution of phytoplankton dynamics. For instance, the specificity of a fungal parasitic infection on certain diatoms during the spring-summer bloom in an upwelling ecosystem, results in changes in diatom composition leading species succession (Gutiérrez et al., 2016). Such fluctuations in phytoplankton community composition can affect higher trophic levels that interact with them producing changes in the planktonic community structure and diversity (Hudson et al., 2006; Lafferty et al., 2006). In a larger scale, these asymmetric pressures on phytoplankton hosts and populations, may significantly influence the stability of the ecosystem and the coevolutionary dynamics of parasites and their hosts (Jephcott et al., 2016b).

The relevance of eukaryotic parasitism and their interactions with phytoplanktonic protists are of great relevance given that: i) all organisms are affected by parasites at some point in their life, ii)

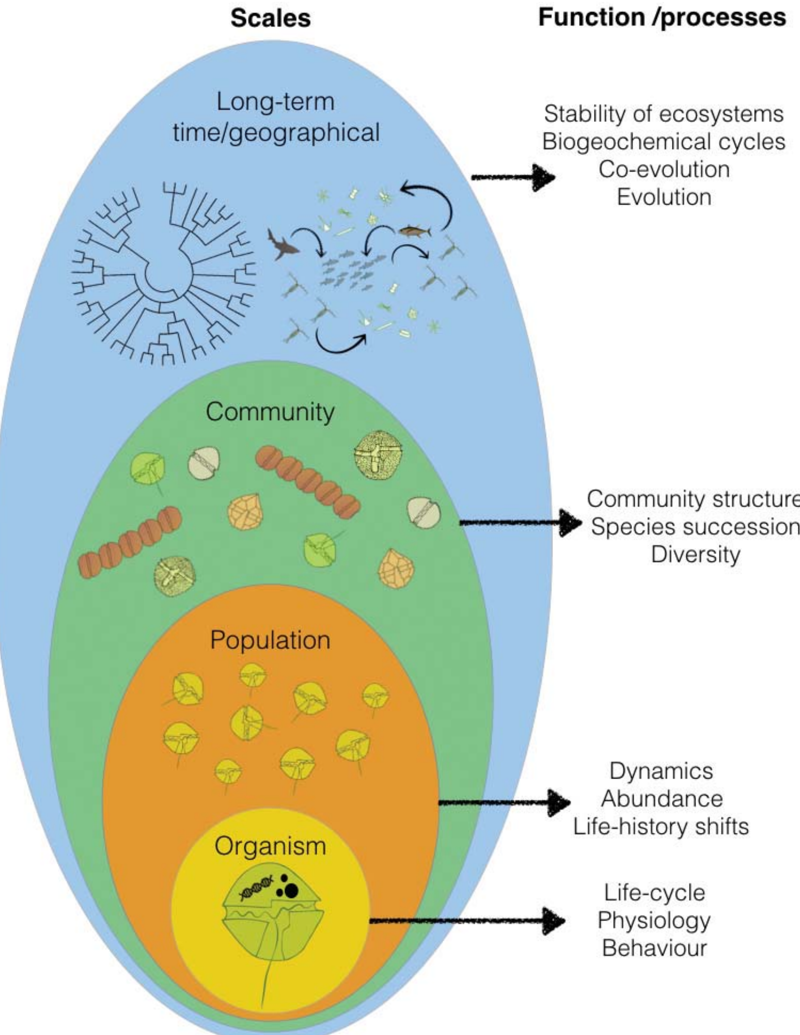


Figure 2. Effect of parasites on their hosts at different ecological and evolutionary scales.

they play a top-down control role in their hosts populations and iii) they may exert strong selective pressure within their host populations leading the evolution of their hosts.

Phytoplankton in coastal ecosystems: dinoflagellate blooms

Dinoflagellates are one of the most abundant and divers groups of microplanktonic protists in the ocean, comprising more than 2000 species (Taylor et al., 2008). They belong to the group Alveolata Cavalier-Smith 1991 clustering together with ciliates and apicomplexans (Van de Peer and De Wachter, 1997). They present a huge physiological diversity, sometimes flexible, including autotrophic, heterotrophic, symbiotic, parasitic and mixotrophic lifestyles (Taylor and Pollingher, 1987). Dinoflagellates occupy planktonic and benthic habitats in the marine environment (Hoppenrath et al. 2014) and have a wide range of ecological roles in the ecosystem (Fig. 3). They produce chemical compounds with wide impacts for the ecosystem as a result of their metabolism. Dinoflagellates can shape the marine sulphur cycle as a consequence of the biosynthesis of dimethylsulfoniopropionate (DMSP) (Caruana et al., 2012). Moreover, DMSP is the precursor of dimethyl sulfide (DMS), a volatile sulfur compound that play important role as a chemoattractant for a variety of marine organisms, including phytoplankton, bacteria, zooplankton, fish, and sea birds (Seymour et al., 2010; Garren et al., 2014; Savoca and Nevitt, 2014). Dinoflagellates also produce complex organic compounds as a result of their metabolism, comprising allelochemicals and toxins molecules. These compounds may play different roles for the producing organism. For instance, it can serve to immobilize a potential prey in mixotrophic species (Sheng et al., 2010) or can play a role in preventing predators (Selander et al., 2006). Dinoflagellate species also act as a host, harbouring viruses, bacteria and other protists. Viruses and virus-like particles have been reported infecting a wide range of dinoflagellates, including photosynthetic species (Tarutani et al., 2001), parasites (Soyer, 1978) and coral symbionts (Lohr et al., 2007). Most bacteria that inhabit dinoflagellates are thought to be commensals or mutualistics (Doucette, 1995), although some of them are still under debate if they are parasitic, due to they have a negative effect on the dinoflagellate host (Mayali and Doucette, 2002). Nonetheless, it seems than viruses and bacteria rarely act as pathogens of dinoflagellates compared to eukaryotic protists, which have been long described as parasites infecting many dinoflagellate species (Elbrächter and Schnepf, 1998). These eukaryotic parasites infecting dinoflagellates comprises other protists, such as Perkinsids, Syndiniales (Park et al., 2004) and chytrid fungi (Lepelletier et al., 2014a).

Under specific conditions dinoflagellates proliferate reaching high cell densities which are known as proliferations or blooms. Such blooms are natural phenomena in the marine environment, however, in coastal areas where human activities take place, proliferation of certain species are considered harmful from a human point of view. So that, they are referred as harmful algal blooms (HABs). Due to the interaction of anthropogenic, atmospheric, terrestrial and oceanic forces in coastal areas, those phenomena are reported to be more intense and occur more commonly than in open waters. HABs cause illness and death in humans and marine life through the production of toxins, or cause economic losses affecting aquaculture and recreational activities (Van Dolah, 2000; Zingone and Enevoldsen, 2000). Dinoflagellates account for the 75% of the HAB-forming species. Moreover, dinoflagellate blooms seem to be stimulated by eutrophication, coast modification (increase of enclosed areas), climate shifts and species dispersal (Anderson, 2009). Since coastlines worldwide have long suffered the effect of human activities, the number of this nutrient-rich sites with restricted hydrodynamics have increase vastly, becoming the ideal envi-

ronment for their proliferation (Heisler et al., 2008; Garcés and Camp, 2012).

Even though dinoflagellate blooms have been recognized as a major environmental challenge, studies have focused in the bottom-up factors that make them proliferate and little is known about their interactions with other species and what makes them decline. Recent studies have demonstrated that the top-down control exerted by biotic factors, such as parasitism and grazing may play an important role in bloom regulation (Calbet et al., 2003; Chambouvet et al., 2008; Montagnes et al., 2008; Jordi et al., 2015). Since such dinoflagellate blooms are temporary states of the phytoplankton community characterized by having a very high density and a low diversity, they are assumed to favour parasitic infection and transmission. Several studies reported high dinoflagellate mortalities during bloom events caused by eukaryotic parasitic infections (Coats et al., 1996; Chambouvet et al., 2008), to the extent they have been proposed as biological control agents for HABs mitigation (Taylor, 1968a; Mazzillo et al., 2011), in the same way that it is done in agricultural applications on land. The suggestion has faced opposition on the basis of the poor knowledge on their specificity, the mechanisms of infection, and the possible negative consequences (Anderson, 2009). The debate highlights the need for a better understanding of these interactions in this parasite-host system.

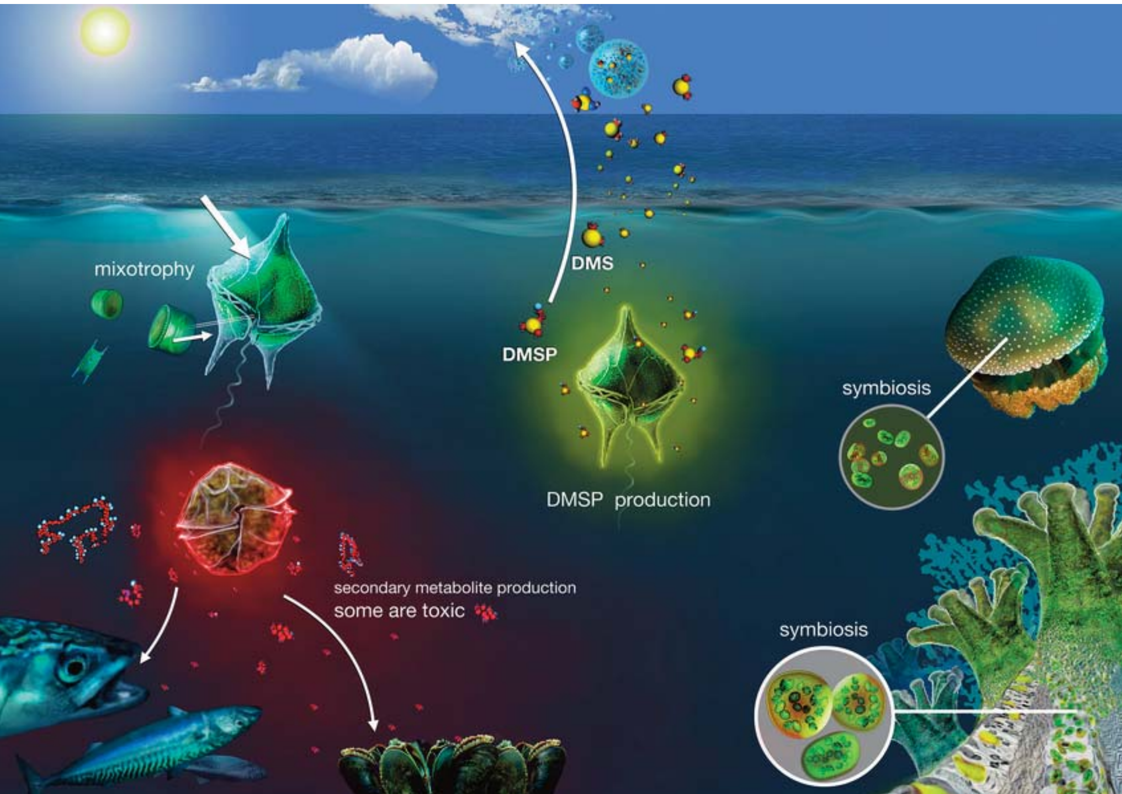


Figure 3. Ecological qualities of dinoflagellates: free living cells in the plankton, symbiotic relations with other organisms, DMSP production, mixotrophy, production of toxic and non-toxic metabolites that influence on other marine organisms. From Murray et al. (2016).

Zoosporic parasites of dinoflagellates

Zoosporic parasites are a heterogeneous group of eukaryotic aquatic organisms with similar morphological features and small size, that exhibit a parasitic lifestyle and produce many asexual flagellated (motile) (zoo)spores that are released into water. Zoospores are unicellular eukaryotic cells with only one nucleus, possessing one to several mitochondria, which are produced by sporogenesis.

Zoosporic parasites are divided into two major groups: (i) unikonts or opisthokont (zoospores with one flagellum), which comprises chytrids and aphelids and (ii) heterokonts (zoospores with two flagella) that includes the SAR supergroup, i.e. Stramenopiles, Alveolates and the Rhizaria (Baldauf, 2008; Adl et al., 2012). The number and type of flagella, and the shape of the zoospore have a great taxonomic value and their ultrastructure is a key feature in the taxonomy of these parasites.

Historically, these parasites have not been taken into account when studying ecology of microbial marine systems, limiting the understanding of microbial food webs and biogeochemical cycles. This is because they lack of distinctive morphological characters, and all zoospores are very similar under traditional microscope techniques, being not distinguishable in environmental samples. For this reason, they have been rarely reported in ecological studies, hidden within the group of heterotrophic pico- or nanoflagellates. Hence, these parasites are thought to be more numerous in marine environments than previously reported (Gleason et al., 2012).

Marine zoosporic parasites of dinoflagellates belong to Chytridiomycota, Syndiniales (or Marine Alveolate group II, MALVII), and Perkinsozoa taxa. Very little is known about the ecology and diversity of marine parasitic chytrids. Before starting the present thesis, there was no chytrid parasite species of marine dinoflagellates described, and to date there is only *Dinomyces arenysensis*, a unique representative. This chytrid was isolated from an *Alexandrium minutum* bloom in the NW Mediterranean Sea (Lepelletier et al., 2014a). *Amoebophrya* (Syndiniales) is an abundant group of parasites represented by a high diversity of environmental sequences, distributed worldwide (Guillou et al. 2008), and comprising one genus with seven described species (Cachon, 1964). The species complex *Amoebophrya ceratii* was the only one thought to parasitize dinoflagellates, which were reported from about 40 different free-living dinoflagellates, among 20 genera (Park et al., 2004). Later on, dinoflagellate infections due to *Amoebphrya* have been extended to other clades different to *A. ceratii* (Kim and Park, 2014; Chambouvet et al., 2008). Parasites belonging to Perkinsozoa are among the lately identified groups of marine protist parasites of phytoplankton, and environmental molecular studies have revealed a hidden diversity of unknown organisms which remain unclassified (Chambouvet et al., 2014). Of those that have been described, the genera *Parvilucifera* is the only confirmed parasites of dinoflagellates, comprising 5 species to date, which also have a world-wide distribution (Park et al., 2004).

All three groups of parasites infecting dinoflagellate hosts, typically have characters of parasitoids, i.e. the host cell is killed by the parasite for completion of its life cycle. In general terms, they present similar life cycles (Fig. 4) which include three main stages: host infection by zoospores, host body consumption via the trophocyte or feeding stage, and the production of zoospores for reproduction and transmission (Jephcott et al., 2016a). In the all three groups, the parasitic infection begins when a free-living zoospore penetrates into a host cell. Once infected, it becomes a

trophocyte (=trophont) that enlarges while feeding on the host by digesting all cell content. Then, trophocyte nucleus starts division and transforms into a sporocyte, and sporogenesis takes place. Finally, the zoospores are released into the marine environment to find a new host to infect. Although these similarities, they present some differences. The trophocyte of *Amoebophrya* and *Parvilucifera* grow inside the host cell (they are endoparasitoids), whereas the trophocyte of *Dinomyces* grows on the outside (it is an ectoparasitoid). Also, *Dinomyces* and *Parvilucifera* form the sporangium that is non-existent in *Amoebophrya*, which forms a vermiform stage before spreading the zoospores.

Host specificity of these parasites is hard to evaluate because it has been partially tested in laboratory studies and there is an absence of data from the field. Some of them are host-generalists, being able to infect a wide range of dinoflagellate species (Park et al., 2004; Lepelletier et al., 2014a) and others are specialists as in the case of *Amoebophrya* (Chambouvet et al., 2008). Inter-strain variability also exists, which also play a role in determining the outcome of infection (Turon et al., 2015). Thus, data is necessary to better understand the host range, their specificity in the field, and whether they present host preferences.

These parasites cause divers effects on their dinoflagellate hosts at different levels, and most of the knowledge comes from *Amoebophrya* parasitoids. Only one study exists addressing host changes at molecular level produced by these parasites. Upon infection, *Amoebophrya* produces significant changes in the expression levels of *Alexandrium* genes associated with specific metabolic pathways, suggesting that parasite infection increases the energy demand of the host (Lu et al., 2016). Moreover, the same study pointed that *Alexandrium* is able to respond to parasite attacks, as indicated by the increased expression of genes associated with defence and stress. At organismal scale, these parasites can alter the physiology of their hosts, for instance, *Amoebophrya ceratii* alter hosts photophysiology during the evolution of the infection (Park et al., 2002b). The same parasite can also cause important changes in host behaviour. Infected dinoflagellates reduce their swimming velocity in advanced infection stages, or loss the ability to perform diel vertical migrations, which is important for their survival and ecology (Park et al., 2002a). In host populations, parasites can produce shifts in their life-history stages, causing the formation

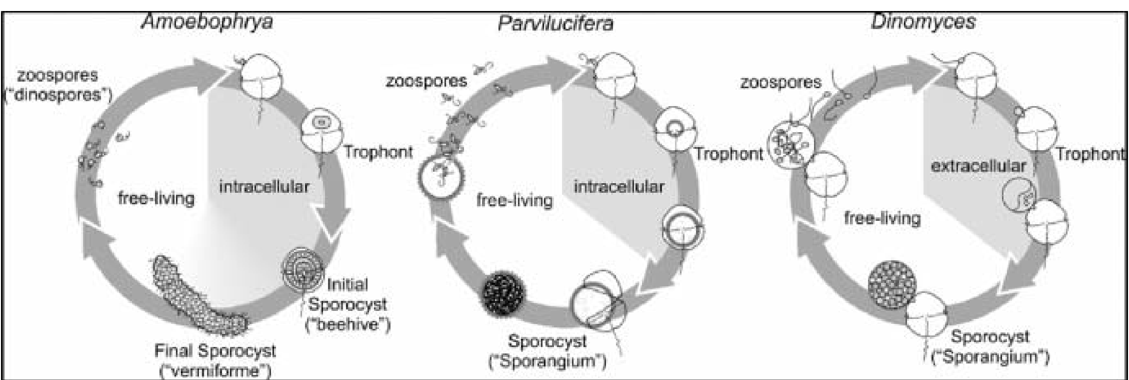


Figure 4. Similarities and differences between the life cycles of *Amoebophrya* (Syndiniales), *Parvilucifera* (Perkinsozoa), and *Dinomyces* (Chytridiomycota). From Jephcott et al. (2016a).

of temporary cysts (Toth et al., 2004) or enhancing sexual reproduction (Figueroa et al., 2010). This shifts, together with the high mortalities reached sometimes, which have been related to bloom decline (Taylor, 1968b; Mazzillo et al., 2011), produce changes in the dinoflagellate community and lead to species succession (Chambouvet et al., 2008).

Parvilucifera parasitoids (Perkinsozoa, Alveolata)

Perkinsozoa phylum is a diverse group of parasites infecting a wide range of species including fish (Freeman et al., 2017), shellfish (Bower et al., 1994), amphibians (Chambouvet et al., 2015a) and dinoflagellates (Park et al., 2004). It is a widespread group that have representatives in divers aquatic environments, including marine waters (de Vargas et al., 2015) and sediments (Chambouvet et al., 2014), salt marshes (Reñé et al., 2016), and diverse freshwater ecosystems, such as small rivers (Brugerolle, 2002b), lake epilimnion (Lepère et al., 2008) and sediment (Bråte et al., 2010), water reservoirs and ponds (Chambouvet et al., 2015a).

The systematic classification of Perkinsozoans has been for long debated, although its recent position as a sister group of dinoflagellates seems to be strongly supported (Moore et al., 2008). The classification of the first representative, *Perkinsus marinus*, was as a fungi, which was based only on ultrastructural morphological features (Mackin et al., 1950), being later transferred to the protozoan phylum Labyrinthomorpha (Mackin and Ray, 1966). Then, based on the ultrastructural analysis of the zoospore, the presence of organelles similar to an apical complex, placed *Perkinsus* within the Apicomplexa (Levine, 1978). Later on, studies based on the morphology as well as phylogenetic analyses of DNA sequences (Siddall et al., 1997), confirmed they do not belong to the phylum Apicomplexa, and concluded that *Perkinsus* was related to dinoflagellates. Actually, morphological studies of *Perkinsus* zoospores determined that they present a mastigoneme-bearing anterior flagellum, and a pseudo-conoid on the apical complex, which are features that differ from those of all other apicomplexans. More recently, Norén et al. (1999) erected the new phylum Perkinsozoa. Such classification was based on the similarity of both, zoospore ultrastructure and phylogenetic similarity of the 18S rRNA sequence, between *Perkinsus* genus and *Parvilucifera infectans*, bridging dinoflagellates and apicomplexans.

To date, most of the diversity of Perkinsozoa is represented by environmental sequences, and only comprises three described genera: *Perkinsus* (Mackin, Owen, Collier) Levine, *Parvilucifera* Norén et Moestrup, and *Rastrimonas* (= *Cryptophagus*) Brugerolle. However, there are no Rastrimonas molecular sequences available and its taxonomic classification within Perkinsozoa remains to be confirmed (Brugerolle, 2002b; a; 2003). So that, most of the species existing in culture and therefore most of the morphological information that are available belong to the genera *Perkinsus* and *Parvilucifera*.

Perkinsus spp. are known to be parasites of shellfish, being causative agents of the disease named Perkinsosis or 'Dermo', which have a broad distribution in marine waters worldwide and cause important economic losses as they are commercially important; therefore much of the research effort is placed in *Perkinsus* spp. (Villalba et al., 2004; Cho and Park, 2010; Smolowitz, 2013). While the pathogenicity of Perkinsus spp. has been relatively thoroughly investigated and quantified, *Parvilucifera* spp., which constitute a recently described group of parasites, there is no quantitative prevalence data available.

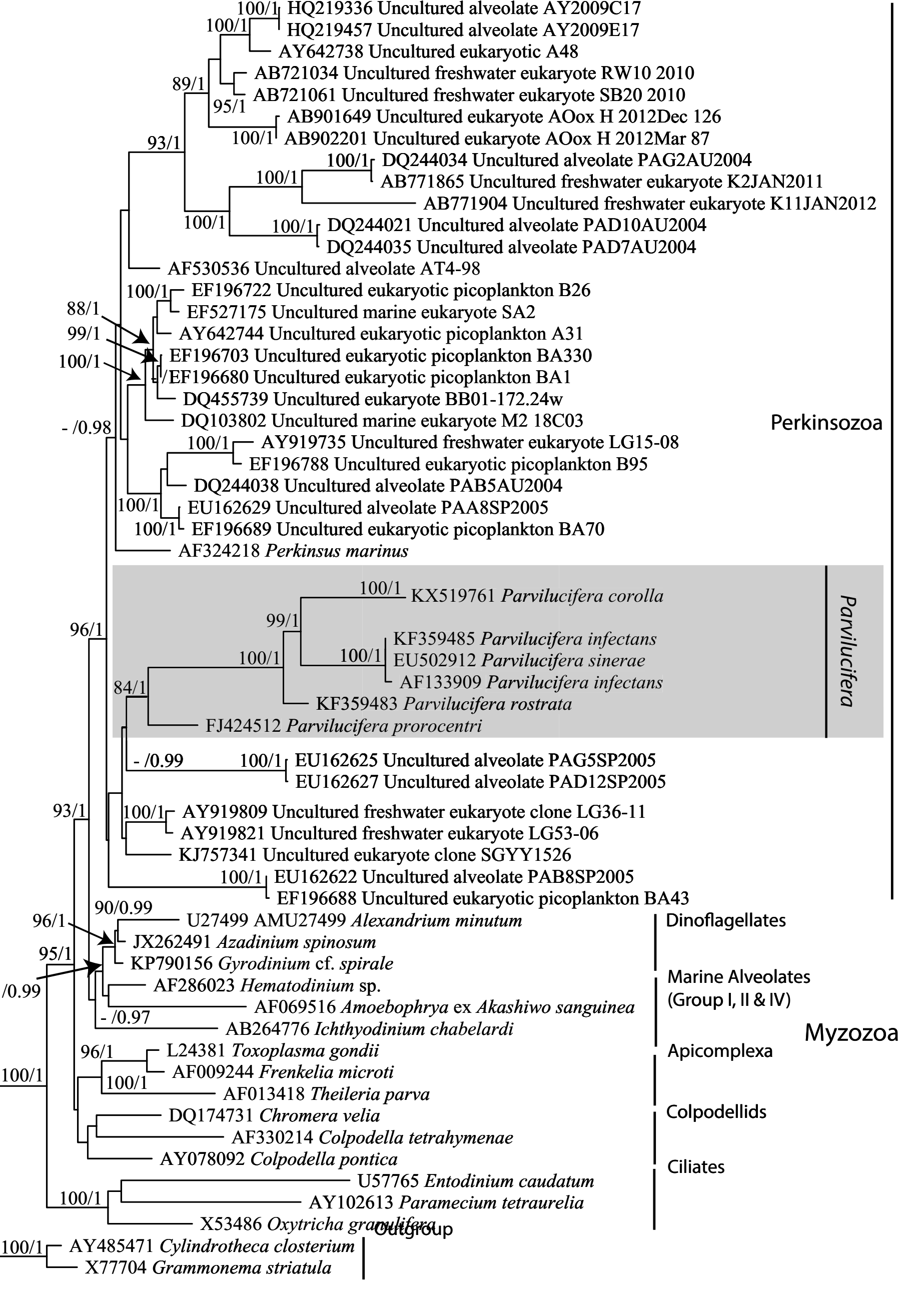


Figure 5. Maximum likelihood tree inferred from the SSU rDNA sequences of representatives of the phylum of Alveolata. Sequences of *Parvilucifera* species are highlighted in grey. Sequences of the diatoms served as the outgroup. The bootstrap (BS) and Bayesian posterior probabilities (BPP) are provided at each node (BS/BPP). Only BS and BPP values >80% and >0.95, respectively, are shown. Modified from Reñé et al. (2016).

Parvilucifera genus was established for the first time by Norén et al. (1999) based on the morphological and phylogenetic description of *P. infectans*. The distribution of this species covers from Australian to Norwegian coasts, where they commonly infect many different dinoflagellates, including, photosynthetic and heterotrophic, thecate and athecate, and toxic and non-toxic species (Norén et al., 2001; Park et al., 2004; Johansson et al., 2006). Then, the presence of *Parvilucifera-like* parasitoids were also reported in the Mediterranean Sea and on the French Atlantic coast, although its identification could not be confirmed (Delgado, 1999; Erard-Le Denn et al., 2000). Later on, two new species were described, *P. sinerae* from the Mediterranean Sea (Figueroa et al., 2008), and *P. prorocentri* from the Pacific North (Leander and Hoppenrath, 2008). At the beginning of the present thesis, these three species were the only ones described within the genus *Parvilucifera*, and to date, two more species were additionally described: *P. rostrata* (Lepelletier et al., 2014b) isolated from the Penzé Estuary in France (English Channel) and *P. corolla* (Reñé et al., 2016) from coastal salt marshes of the NW Mediterranean Sea. Figure 5 shows the phylogenetic position of the 5 *Parvilucifera* species within the Perkinsozoans and their relation to other Alveolata groups. Some authors have suggested that the ultrastructural similitude between *P. infectans* and *P. sinerae*, together with the fact that they are hardly separated phylogenetically, requires clarification (Lepelletier et al., 2014b). Although all species share some morphological and ultrastructural features, several differences between them exists, and together with the phylogenetic analyses of SSU rDNA (Fig. 5), suggest that *P. infectans*, *P. sinerae*, *P. rostrata* and *P. corolla* have more in common than *P. prorocentri*, which reclassification has been suggested by several authors (Hoppenrath and Leander, 2009; Reñé et al., 2016).

Parvilucifera species are intracellular parasitoids of dinoflagellates with part of the life cycle confined to the host (trophocyte) and with a part as a free-living, including a non-motile stage, the sporocyte or sporangium, and a swimming zoospore, which is the infective stage. As for the completion of their life cycle necessarily ends with the host death, they are termed parasitoids. Although the absence of data on dinoflagellates mortalities in the field caused by *Parvilucifera*, several authors suggested that these parasites may play a significant control top-down on their dinoflagellate host populations, preventing and controlling blooms (Delgado, 1999; Norén et al., 1999; Erard-Le Denn et al., 2000; Park et al., 2004). In fact, Norén patent *P. infectans* for a pesticidal use of a parasitic flagellate for eliminating or suppressing harmful algae blooms (Norén, 2003). Such idea comes up from the capability of *Parvilucifera* to kill their hosts, the wide range of dinoflagellates that are able to infect and the high virulence observed under laboratory conditions. However, despite their potential capacity, its effective use in natural environments for this purpose will depend on prior knowledge on ecology and molecular biology of the host-parasite interaction, i.e. the molecular mechanisms underlying specificity, the identification of the genes involved during infection, a complete understanding of parasite life-cycle, and field mortalities caused by *Parvilucifera*, among others.

In the present thesis, we focused in the study of the interactions of *Parvilucifera* with their dinoflagellate hosts in the marine coastal environment. Specifically, we study *P. sinerae*, which was isolated and cultured by Figueroa and Garcés in 2008 during a dinoflagellate bloom in Arenys de Mar harbour (NW Mediterranean Sea).

Aims of the thesis

Aims of the thesis

This thesis aims to advance in the knowledge of host-parasite interactions in the marine environment by studying the ecology of marine protist parasites of planktonic microalgae. Specifically it focuses on the model system comprising the parasitoid *Parvilucifera sinerae* (Perkinsozoa, Alveolata) and their dinoflagellate hosts.

The present thesis characterizes the *Parvilucifera*-dinoflagellates relationship at different scales, from organismal scale to population and at community level by combining laboratory experiments and fieldwork.

The specific objectives are listed below:

❶ To describe *Parvilucifera sinerae* life cycle and the kinetics of the infection.

❷ To assess host specificity of *P. sinerae* at inter- and intra-species level defining their potential host range.

❸ To characterize *P. sinerae* infection strategy in artificial host communities.

❹ To determine *Parvilucifera* occurrence and dynamics in the field.

❺ To quantify *in situ* the impact of *Parvilucifera* infection during dinoflagellate blooms.

The work conducted are reported in five chapters, each of which addresses some of the above-defined specific objectives.

The five chapters are structured as scientific articles, 4 of them already published and one submitted in peer-review SCI journals. This thesis concludes with a general discussion and general conclusions.

Chapter 1: NEW INSIGHTS INTO THE PARASITOID *PARVILUCIFERA SINERAE* LIFE CYCLE: THE DEVELOPMENT AND KINETICS OF INFECTION OF A BLOOM-FORMING DINOFLAGELLATE HOST

Alacid, E., Reñé, A., and Garcés, E. (2015). *Protist* 166(6), 677-699.

The knowledge of a parasite life cycle and all the different stages, where each of the stages develop, and their timing, give relevant information in parasite ecology and disease concerning: the individual infected, how each particular stage affects the host, the most plausible time for the parasite to be in a certain environment, the type of disease or pathology caused by the parasite, and the more important in parasitology, the vulnerable points in the life cycle for the best treatment and prevention.

In this chapter, we provide a detailed description of the life cycle of *P. sinerae*, based on optical, confocal (combined with TSA-FISH), and transmission electron microscopy observations, and its infection kinetics and dynamics in the toxic dinoflagellate host *Alexandrium minutum*.

Chapter 2: HOST-RELEASED DIMETHYLSULPHIDE ACTIVATES THE DINOFLAGELLATE PARASITOID *PARVILUCIFERA SINERAE*

Garcés, E., **Alacid, E**., Reñé, A., Petrou, K., and Simó, R. (2013). *ISME J* 7(5), 1065-1068. doi: 10.1038/ismej.2012.173.

The ability of organisms to perceive and respond to their environment is the basis of their survival and reproduction. Chemical signalling play a key role in marine ecology, mediating a variety of processes within organism interactions, such as mechanisms of defence or trophic relationships. For instance, chemical cues allow motile organisms to exploit nutrient patches, locate partners or hosts, and select or avoid prey.

This chapter focuses on the identification of the chemical signal that activates the parasite *Parvilucifera sinerae* from the dormant to the actively swimming infective stage of its life cycle.

Chapter 3: *PARVILUCIFERA SINERAE* (ALVEOLATA, MYZOZOA) IS A GENERALIST PARASITOID OF DINOFLAGELLATES

Garcés, E., **Alacid, E.,** Bravo, I., Fraga, S., and Figueroa, R.I. (2013). *Protist* 164(2), 245-260. doi: 10.1016/j.protis.2012.11.004.

Host-specificity is one of the most intriguing features in parasitism, being a key characteristic of a parasite because it relates to the concept of its ecological niche and evolutionary history. In nature, host-specificity is a continuum from extreme specialists, to generalist parasites. The degree of host-specificity has relevant implications, since it sets the potential effect in structuring ecological communities, the likelihood that a parasite is able to jump to another species, the possibility to eradicate a disease or the use of parasites as biological control agents.

In this chapter, we test the host specificity of the parasitoid *P. sinerae* under laboratory conditions. We also focus on the biological characteristics of the infection process within non-type hosts, such as sporangium size, the number of zoospores produced, and the time needed for their development. Finally, we characterize parasitoid infection of microalgal species in field samples.

Chapter 4: A GAME OF A RUSSIAN ROULETTE FOR A GENERALIST PARASITOID OF DINOFLAGELLATES

Alacid, E., Park, M.G., Turon, M., Petrou, K., and Garcés, E. (2016). *Frontiers in Microbiology* 7, 1-13. doi: 10.3389/fmicb.2016.00769.

Successful parasites have developed strategies that allow their survival and maximize their offspring and transmission in host populations. Theory predicts that parasites that can only survive for short periods outside the host develop strategies to minimize the search of a suitable host that maximizes parasite fitness, i.e. active host selection.

In this chapter, we explore the strategy by which *P. sinerae*, a generalist dinoflagellate parasitoid with a short-living motile infective stage, seeks out their hosts and whether it exhibits preferences for certain host species, highlighting key factors in determining a successful infection.

<u>Chapter 5:</u> ***IN SITU* OCCURRENCE, PREVALENCE AND DYNAMICS OF *PARVILUCIFERA* PARASITOIDS DURING RECURRENT BLOOMS OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM MINUTUM***

Alacid, E., Reñé, A., Camp, J., and Garcés, E. *Frontiers in Microbiology*, *submitted*.

Determine when parasites occur in their natural environment and their dynamics is essential to understand the role they play in their host populations and for hence in the food web. The impact these parasites cause in their hosts is usually expressed as prevalence in field studies, which give an idea of the relevance of parasitism on natural host populations. Parasites cause direct changes in host abundances, and in phytoplankton communities they have been mentioned to play a role in the control and prevention of harmful algal blooms.

In this chapter we assess the occurrence, prevalence and dynamics of *Parvilucifera* parasitoids during noxious dinoflagellate recurrent blooms. We also estimate and discuss the contribution of *Parvilucifera* parasitism to bloom termination.

Chapter 1

"New insights into the parasitoid *Parvilucifera sinerae* life cycle: The development and kinetics of infection of a bloom-forming dinoflagellate host"

Protist, 2015

New insights into the parasitoid *Parvilucifera sinerae* life cycle: The development and kinetics of infection of a bloom-forming dinoflagellate host

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ABSTRACT

Parvilucifera sinerae **is a parasitoid of dinoflagellates, the major phytoplankton group responsible for harmful algal bloom events. Here we provide a detailed description of both the life cycle of** ***P. sinerae*****, based on optical, confocal, and transmission electron microscopy observations, and its infection kinetics and dynamics.** ***P. sinerae*** **completes its life cycle in 3–4 days. The zoospore encounters and penetrates the host cell within 24 h after its addition to the host culture. Inside the host, the parasitoid develops a trophocyte, which constitutes the longest stage of its life cycle. The trophocyte replicates and divides by schizogony to form hundreds of new zoospores contained within a sporangium. Under laboratory conditions,** ***P. sinerae*** **has a short generation time, a high rate of asexual reproduction, and is highly prevalent (up to 80%) in the** ***Alexandrium minutum*** **population. Prevalence was shown to depend on both the parasitoid inoculum size and host density, which increase the encounter probability rate. The parasitoid infection parameters described in this study are the first reported for the genus** ***Parvilucifera*****. They show that** ***P. sinerae*** **is well-adapted to its dinoflagellate hosts and may be an important factor in the termination of** ***A. minutum*** **blooms in the natural environment.**

INTRODUCTION

Marine dinoflagellates are an abundant group of microplankton, some of which produce potent toxins and cause harmful algal blooms (HABs). HAB events have a negative impact on the exploitation of seafood resources, pose a threat to human health, and alter marine trophic structure (Van Dolah 2000; Zingone and Enevoldsen 2000). However, the microalgae that cause HABs serve as hosts for several eukaryotic parasites (Park et al. 2004 and references therein). Recent interest in parasites derives from the impact that they may exert in the control of dinoflagellate populations, especially HAB species. This has led to an increase in both experimental studies (Kim et al. 2004; Maranda 2001; Park et al. 2002a, b; Park et al. 2004) and modelling-based analyses (Llaveria et al. 2010; Montagnes et al. 2008; Salomon and Stolte 2010). Under specific conditions, some parasites cause high mortality of their hosts and thus facilitate the decline of their blooms (Coats et al. 1996; Mazzillo et al. 2011). Accordingly, the use of these parasites as control agents in bloom mitigation has been proposed (Anderson 1997; Chambouvet et al. 2008; Erard-Le Denn et al. 2000; Norén et al. 1999). However, the infection prevalence in natural populations of dinoflagellates is typically low to intermediate (Coats and Bockstahler 1994; Chambouvet et al. 2008; Velo-Suárez et al. 2013). In addition to causing mortality, eukaryotic parasites can induce a shift in the physiology and life-history stages of their hosts (Chambouvet et al. 2011; Toth et al.

2004) and they may also stimulate their sexual reproduction and therefore increase the rate of genetic recombination (Figueroa et al. 2010).

The greatest diversity of eukaryotic parasites belongs to Alveolates (including Dinoflagellates, Apicomplexa, and Perkinsozoa among others), which are characterized by the presence of cortical vesicles (alveoli) that subtend the plasma membrane (Cavalier-Smith 1993; Leander and Keeling 2003; Zhang et al. 2011). This group is well represented in aquatic and terrestrial ecosystems (Diéz et al. 2001; Guillou et al. 2008; Massana et al. 2004; Richards et al. 2005) and includes pathogenic species that cause economic losses (Mackin 1951) and important human diseases, such as malaria parasites of the genus *Plasmodium* (Kaplan et al. 2000; Nahlen et al. 2005). In the marine ecosystem, the genera *Amoebophrya* (Syndiniales, Dinophyceae) and *Parvilucifera* (Perkinsozoa) are widespread and composed exclusively of parasites that infect dinoflagellates, including HAB species (Garcés et al. 2013a; Guillou et al. 2008; Park et al. 2004). Studies of *Amoebophrya* account for most of the knowledge of parasitism in dinoflagellates whereas little is known about the diversity, ecology, and host effects of *Parvilucifera* species.

The genus *Parvilucifera* belongs to the Perkinsozoa together with the two parasitic genera *Perkinsus* and *Rastrimonas* (originally described as *Cryptophagus*) (Brugerolle 2003; Norén et al. 1999). As an early branch in alveolate evolution, *Parvilucifera* species possess many of the features of dinoflagellates and apicomplexans, making their study interesting from an evolutionary point of view (Leander and Keeling 2003). Thus far, four *Parvilucifera* species have been described, with *P. infectans* and *P. sinerae* as the most closely related species with respect to their morphology, host range, and molecular phylogenetics (Figueroa et al. 2008; Garcés et al. 2013a; Garcés and Hoppenrath 2010; Norén et al. 1999). The morphologies of the sporozoite and the sporangium distinguish *P. rostrata* from *P. infectans* and *P. sinerae*, but the host range of all three species is very similar (Lepelletier et al. 2014). By contrast, the morphological features of *P. prorocentri* are a combination of those of the perkinsids and syndineans (Leander and Hoppenrath 2008). These morphological differences and the phylogenetic distance with the other *Parvilucifera* species suggest the need for its reclassification (Hoppenrath and Leander 2009; Leander and Hoppenrath 2008; Lepelletier et al. 2014).

All four *Parvilucifera* species complete their life cycle within a single host organism that is then consumed and killed. Therefore they are referred to as parasitoids (Lafferty and Kuris 2002). Their life-cycle can be summarized as follows: A small biflagellate zoospore penetrates the host cell and then develops into the trophocyte. This trophont gradually becomes a sporocyte (referred to as the sporangium), which is the replicative stage resulting in many zoospores that are released into the marine environment to infect new hosts (Garcés et al. 2013a; Lepelletier et al. 2014; Norén et al. 1999). Several stages of the *P. sinerae* life cycle have been observed and both the time needed by sporangia to germinate and the infection rates in different host species have been established (Figueroa et al. 2008; Garcés et al. 2013a). The ultrastructure of the sporangium and of the zoospores of *P. sinerae* was described by Garcés and Hoppenrath (2010) and it supported the classification of these organisms in the genus *Parvilucifera*. However, the wide range of morphological and structural changes induced in infected host cells by *Parvilucifera* are poorly characterized with respect to parasitoid development and survival. Specifically, the life-cycle stages of the parasitoid during infection and the kinetics of infection, including parasitoid generation time, prevalence in susceptible hosts, and host mortality rate, have yet to be determined. Here we provide the first detailed characterization of the *P. sinerae* life cycle during its infection

of the bloom-forming toxic dinoflagellate *A. minutum*. By using several different microscopy techniques we were able to follow the kinetics of infection by this parasitoid, including determination of the different stages of infection, parasitoid development time, the duration of each stage of infection, parasitoid mortality and prevalence, and the host mortality rate, and to quantify the effect of the inoculum size on parasitoid prevalence. These data will contribute to an understanding of the potential effect of this parasitoid on its host population.

MATERIAL AND METHODS

Parasitoid culture and infection

A *Parvilucifera sinerae* culture (strain ICMB 852) was established from a bloom of *Alexandrium minutum* in Vilanova Harbor (Mediterranean Sea, Spain) in March 2009, as detailed in Garcés and Hoppenrath (2010). The parasitoid culture was propagated by transferring aliquots of mature sporangia (20–25) every 6–7 days into an uninfected host stock culture of exponentially growing *A. minutum* strain P4 in sterile polystyrene wells 32 mm in diameter and 18 mm deep (BD Biosciences). The *A. minutum* host culture belong to the culture collection of Instituto Español de Oceanografía (Vigo, Spain) , cloned for the last time in 2009 and maintained in exponential phase in 45-mL polystyrene tissue culture flasks (BD Biosciences) filled with L1 medium without silica (Guillard 1995). All cultures were incubated at 20±1°C in a 12:12 light:dark cycle of white fluorescent light at 90 µmol photons m^2 s^1. An *A. minutum* (P4) host culture at an initial density of approximately 3×10^4cells·mL^{-1} was inoculated with recently formed *P. sinerae* (ICMB 852) zoospores at a zoospore:host ratio of 1:1, in a total volume of 20 mL, and under the culture conditions described above. The infection process was monitored from sampled acquired twice daily (at hours 2 and 8 of the light period) to identify and quantify host abundances and the different parasitoid infection stages using the microscopy techniques described below. The infection was followed until there was no further increase in the number of infected *A. minutum* cells and mature sporangia had formed. Sample processing for each microscopy technique is explained below. The use of a large inoculum mimicked the concentration characteristic of a bloom event and ensured that the parasitoid achieved the maximum level of infection in the first generation. This facilitated synchronization of the infection, resulting in improved estimates of the time required for each stage of infection and of parasitoid losses between life-stage transitions.

Optical microscopy and lipid staining

To identify the different stages of infection (from early stages to the mature sporangium), 1-mL samples from the infected culture were fixed in formaldehyde (1% final concentration) and photographed using a Leica–Leitz DMIRB inverted microscope (Leica Microsystems, Wetzlar, Germany) and ProgRes CapturePro image analysis software (JENOPTIK Laser, Optik, Systeme).

The intracellular distribution of neutral lipids in the host during parasitoid infection was examined by staining a 3-mL suspension of the infected culture with 10 µL (7.8×10^4 M) of Nile Red fluorescent dye (Sigma-Aldrich, St. Louis, MO) dissolved in acetone (final concentration 0.26 µM). After a 15-min incubation at room temperature in the dark, the samples were examined by epifluorescence microscopy (Leica-Leitz DM-II; Leica Microsystems) with an excitation wavelength of 486 nm. Photographs were taken with ProgRes CapturePro image analysis software (JENOPTIK Laser, Optik, Systeme).

Spectral confocal microscopy

Two-mL samples of the infected culture were fixed by adding one volume of formaldehyde 37% to nine volumes of sample. After incubation in the dark for 2 h at 4ºC, the fixed samples were filtered onto 8-µm pore size polycarbonate filters (25 mm diameter) with a gentle vacuum of 150 mbar at room temperature. Cellulose acetate support filters were used because they promote the homogeneous distribution of the cells. The samples were subsequently stored at –80°C until further processing. On the day of the analysis, the filters were cut into pieces with a razor blade. To avoid cell loss, filter sections were dipped in low-gelling-point agarose (0.1%) and dried face down on Parafilm. FISH-TSA was then carried out using the temperatures and conditions described in Not et al. (2002). Briefly, filters dipped in agarose were incubated with 0.01 M HCl (to inactivate endogenous peroxidases) for 10 min at room temperature and then washed with Milli-Q water. For hybridization, they were treated with 18 µL of hybridization buffer (40% formamide, 0.9M NaCl, 20mM Tris-HCl pH 7.5, 0.01% sodium dodecylsulfate (SDS), 10% blocking agent (Boehringer Mannheim) and 2 µL of Parvi-2R probe (Johansson et al. 2006). Hybridization was performed at 35ºC overnight in a humid chamber. The filters were then washed twice for 10 min at 37ºC in freshly prepared washing buffer in which formamide was replaced by NaCl of equivalent stringency (37 mM NaCl for 40% formamide). After equilibration of the filters for 15 min in PBS buffer at room temperature in the dark, the TSA reaction was initiated by the addition of tyramide-labeled Alexa 488, as described in Pernthaler et al. (2004). Each filter piece was transferred to amplification buffer in an Eppendorf tube, incubated for 30 min in the dark at room temperature, and then transferred to PBS buffer baths for 10 min. The filter sections were then mounted on a microscope slide placed in a mixture consisting of four parts Citifluor and one part Vecta Shield containing 4’-6’-diamidino-2-phelylindole (DAPI) (final concentration 1 µg mL^{-1}) and stored at 4ºC in the dark until observed using confocal microscopy. The cells were visualized with a Leica TCS-SP confocal laser scanning microscope mounted on a Leica DCM IRB epifluorescence microscope equipped with a 50-W mercury lamp, a PL APO 60×/1.4 na oil objective, and the appropriate filter sets for Alexa 488 and DAPI stains (Leica Microsystems). Images were processed using Imaris x64 7.2.1 software (Bitplane).

Transmission electron microscopy

Cells from the infected culture were concentrated in Eppendorf tubes by aspirating the culture medium. The sample was pre-fixed with glutaraldehyde (2% final concentration), prepared by mixing one volume of 8% glutaraldehyde with three volumes of filtered seawater medium, without the addition of buffer. The pre-fixed samples were left to stand on ice (4°C) for 2 h and then washed with filtered seawater while still on ice for 5–10 min. They were then post-fixed at room temperature in an osmium tetroxide solution consisting of one volume of 4% osmium and three volumes of cacodylate buffer. The pellet was washed twice (10 min each), embedded in agar (2.5%), and dehydrated through a graded series of ethanol (30, 50, 70, 85, 2×95, 2×100%; 10 min each) followed by two 10-min rounds of dehydration with 100% acetone. Finally, the samples were infiltrated with 2:1, 1:1, and 1:2 mixtures of acetone: Spurr’s resin (approx. 2 h for each dilution) and embedded overnight in 100% Spurr’s resin. The material was sectioned on an Ultracut E (Reichert-Jung) microtome using a diamond knife (Diatome). Sections were collected on a 200-mesh grid coated with Formvar film, stained in 2% uranyl acetate and lead citrate following the method of Reynolds (1963), and examined in a JEOL JEM-1010 electron microscope operated at 80 kV. Micrographs were taken using a Gatan BioScan model 792 digital camera.

Time-course of infection and parasitoid intracellular development

FISH-TSA filters were used to estimate host abundances as well as the abundances of the different stages of parasitoid infection in the infected culture. A minimum of 400 cells on a filter section were counted on an Olympus BX61 epifluorescence microscope equipped with a 100-W mercury lamp and fluorescence filter sets appropriate for observing Alexa 488 and DAPI dyes. Host cells were defined as infected if *Parvilucifera* was detected either on the surface or inside the cell, otherwise they were considered to be healthy. Infected host cells were categorized with respect to *Parvilucifera* development as: early trophont (stage 1), in which a zoospore was present on or inside the host, or if the parasitoid occupied a small fraction of the host (Fig. 1B, C); late trophont (stage 2) if the parasitoid occupied a large portion or the entire host cytoplasm (Fig. 1D); early sporocyte (stage 3) if the parasitoid underwent schizogony (nuclei division) (Fig. 1E); and late sporocyte (sporangium) if the sporangium was filled with fully developed zoospores (after cytokinesis) (Fig. 1F).

Temporal differences in early- and late-stage infections were used to estimate the parasitoid intracellular development time. Sporangium dormant stage was not taken into account when carrying out all calculations. The duration of each consecutive intracellular stage (T_p and T_{p+1}) of the parasitoid, defined as the interval necessary for a cohort of cells to transition from one stage to the next, was estimated following the method of Carpenter and Chang (1988). The two consecutive stages were calculated as shown in Eqs. (1) and (2):

$$T_p = 2\alpha\beta/(\beta+1) \quad (1)$$

$$T_{p+1} = 2\alpha/(\beta+1) \quad (2)$$

where α is the time interval between the maximum fraction of cells transitioning to the next stage and the maximum fraction of cells that are in this stage, as shown in Eq. (3):

$$\alpha = t_2 - t_1 \quad (3)$$

t_1 and t_2 (the time at which the number of cells at stage P and P_{+1}, respectively, reach a maximum) were calculated after fitting a four-degree polynomial function to the frequency data. The ratio between T_p and T_{p+1} is **β**, defined according to Eq. (4):

$$\beta = \frac{\sum ln[1 + f_p(t_j) + (t_j)]}{\sum ln[1 + f_p(t_j)]} - 1 \quad (4)$$

where .and are the fractions of P and P_{+1} cells of the population as a function of time and are defined by Eqs. (5) and (6):

$$f_{p(t)} = \frac{n_p(t)}{N(t)} \quad ; \quad f_{p+1(t)} = \frac{n_{p+1}(t)}{N(t)} \quad (5)$$

$$N(t) = \sum_{i=0}^{k} n_j(t) \quad (6)$$

where n_p and n_{p+1} are the number of cells in the P and P_{+1} phases, respectively; is the number

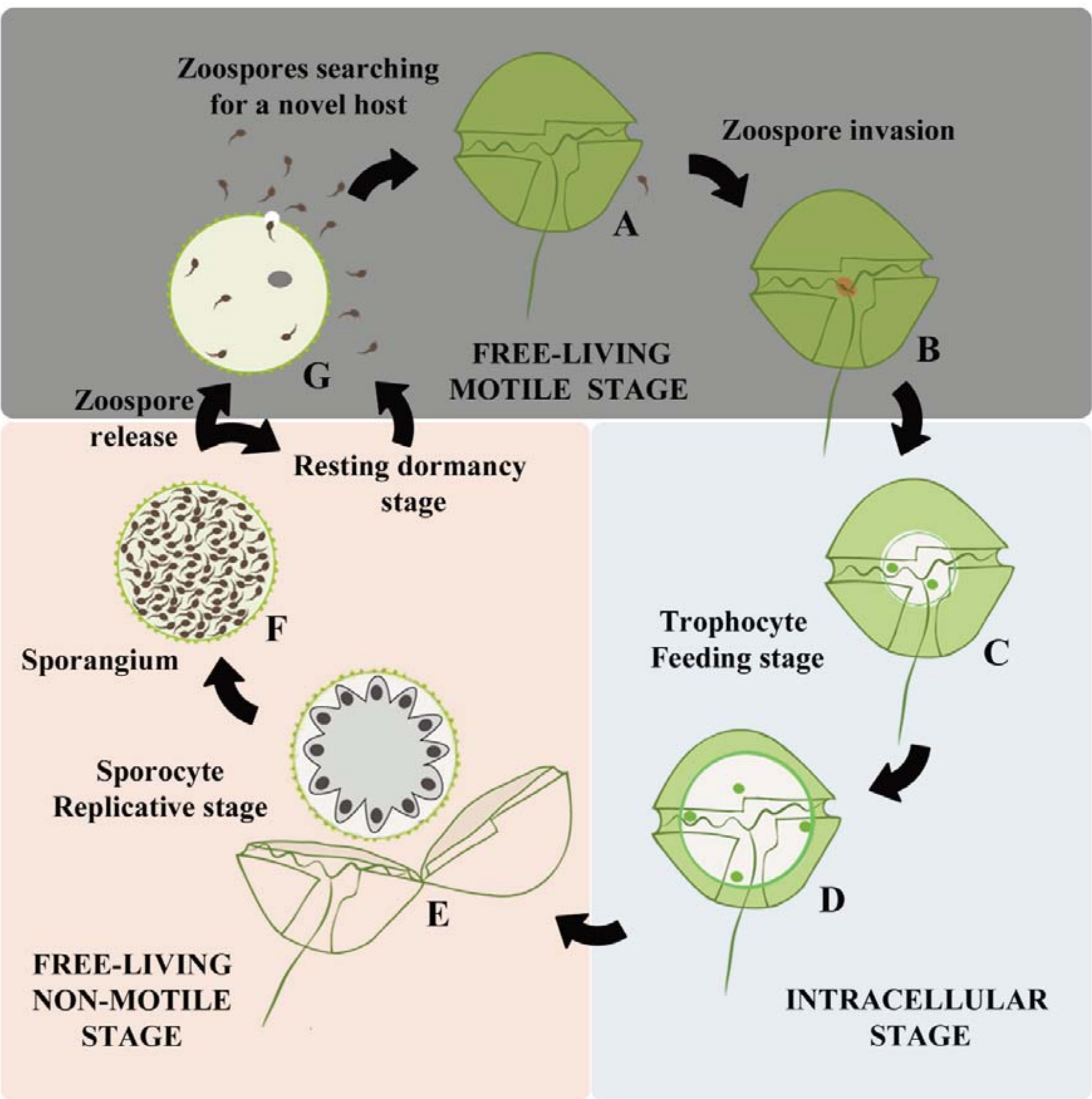


Figure 1. Schematic view of the life-cycle stages of the parasitoid *Parvilucifera sinerae* infecting the dinoflagellate host *Alexandrium minutum*. The parasitoid life cycle is divided in three main stages: The free-living motile stage begins with zoospore release and lasts until host-cell invasion (G, A, B). The intracellular stage is the period of parasitoid development and growth while feeding on its host (C–D). The free-living non-motile stage is a replicative stage, where the zoospores are produced inside the sporangium detached from its host. **A**. Healthy cell of *A. minutum* and a free-living zoospore. **B.** A zoospore invading a host cell. **C.** Early trophocyte. The parasitoid grows inside the parasitophorous vacuole (light gray). **D.** Late trophocyte. **E**. Early sporocyte. Zoospores divide by schizogony, starting from the peripheral areas of the early sporocyte first and then occupying the interior. **F**. A late sporocyte (sporangium) filled with fully developed zoospores. **G.** Infective zoospores abandoning the sporangium. Following their release, free-living zoospores seek out a suitable host cell and thereby initiate a new round of infection.

of cells in stage j; N is the total number of cells in the population; t is the time; t_j is the time needed to obtain the jth sample; and k is the last stage in the parasitoid life cycle.

Parasitoid mortality, parasitoid prevalence, and host mortality rate

Parasitoid mortality between successive life-stages was expressed using *k* values (killing power), as described in Vaughan (2007) and originally developed to study insect ecology. Individual *k* values were calculated as the difference between the densities of two consecutive life stages, expressed as logarithms, and represent the intensity of parasitoid losses during the transition from one stage to another. The total parasitoid mortality during the transition from zoospores to the mature sporocyte (K) was calculated by summing the individual *k* values. The percentage of loss was calculated as: 100–100 (1/antilog *k*) (Gouagna et al. 1998).

Parasitoid prevalence (percentage of infected cells) during the infection process (comprising all stages of infection) was estimated by counting at least 400 cells. Host mortality was calculated as the decrease in host cell density due to parasitic infection, following the method of Guillard (1973) and shown in Eq. (7):

$$\mu = \frac{1}{(t_2 - t_1)} \ln \frac{N_2}{N_1} \qquad (7)$$

where μ is the mortality rate in d^{-1} and N_2 and N_1 are the cell concentrations at t_2 and t_1, respectively.

Parasitoid prevalence as a function of inoculum size

Parasitoid prevalence was determined as a function of inoculum size in an experiment carried out in sterile vials containing 10 mL of host cells at an initial density of 10^3 mL^{-1}. The initial host density used to quantify the differences in parasitoid prevalence due to the effect of the inoculum size was lower in this experiment than in the previous one. The parasitoid inocula were adjusted to obtain zoospore:host ratios of 1:1, 2:1, 5:1, 10:1, 20:1, 30:1, 40:1, and 80:1. The vials were incubated for 4 days at 20±1°C in a 12:12 light:dark cycle of white fluorescent light at 90 µmol photons m^2 s^1. This incubation time was sufficient to allow the formation of mature sporangia but was shorter than the time needed to detect infected cells from a second round of infection. The samples were fixed with formaldehyde (1% final concentration) and examined by inverted light microscopy (Leica–Leitz DMIRB) in a Sedgwick-Rafter chamber. Parasitoid prevalence was calculated as the percentage of infected cells and was determined by scoring at least 300 cells per sample as infected or uninfected. The data were fitted to a single two-parameter exponential increase to the maximum following the method of Coats and Park (2002). The equation used in curve fitting was $y = a(1–e^{-bx})$, where a is the maximum infection level (I_{max}) and b is α/I_{max}. The slope of the initial linear portion of the fitted curve is represented by α, which reflects the potential of the zoospores to infect host cells. Alpha was estimated as I_{max}*b.

RESULTS

Using different methodologies, we followed the complete evolution of infection of *Parvilucifera sinerae* in *Alexandrium minutum* and were able to identify and define the various stages of parasitoid infection. A theoretical scheme of dinoflagellate infection by *P. sinerae* is presented in Fig-

ure 1. The life cycle of *P. sinerae* can be divided into three stages: a free-living motile stage, in which the parasitoids search for a susceptible host cell to invade (Fig. 1A, B), an intracellular stage in which the parasitoids develop inside the host (Fig. 1C, D) and a free-living non-motile stage in which the sporangium detaches from the host and divides to produce hundreds of new infective zoospores (Fig. 1E, F). This sporangium remains dormant (Fig. 1F) until it is activated by an appropriate signal, at which time the zoospores abandon the sporocyte (sporangium) via one or several opercula in its wall (Fig. 1G).

Life cycle of *P. sinerae*

Light, epifluorescence, confocal, and transmission electron microscopy techniques were used to visually follow the stages of *P. sinerae* infection of *A. minutum* and to compare the features of the infected and non-infected cells (Fig. 2 and 3).

The cytoplasm of non-infected cells of *A. minutum* (Fig. 2A) contained numerous chloroplasts interspersed with small lipid bodies (Fig. 2G). The nucleus was elongated and horseshoe-shaped (Fig. 3A). Infection was initiated by a zoospore that actively penetrated a host cell (Fig. 3J), which immediately lost its flagella, stopped swimming, and sank. Once inside its dinoflagellate host, the parasitoid localized in the cytoplasm, close to the nucleus (Fig. 3B, C). Early stages of infection were recognizable based on the presence of a round body in the host, referred to as the trophocyte, which is the intracellular feeding stage (Fig. 2B and 3D). In this early stage of infection, the parasitoid grew until it occupied most of the host cytoplasm, by digesting all of the host's organelles (Fig. 3D, K). Infected cells therefore showed a decrease in autofluorescence due to the degradation of host chlorophyll by the growing trophont, which harbored lipid droplets (Fig. 2H, I).

During the late trophocyte stage, the parasitoid round body filled the host cytoplasm (Fig. 2C and 3E). This structure was transparent whereas in the host cytoplasm chlorophyll was almost totally degraded and large lipid-containing vacuole-like structures predominated (Fig 2I). After feeding on the cytoplasm of its host, the trophocyte underwent schizogony to form new zoospores (Fig. 2D, J, and 3F). Trophocyte nucleus (Fig. 3L) divided first through several rounds of mitosis without cytokinesis, resulting in the formation of a multinucleated stage (Fig. 3M–P) arranged around the periphery of the parasitoid round body (Fig. 3F, G). During schizogony, there was a gradual reduction in nuclear size and hundreds of new zoospores developed. After cytokinesis, the zoospore nuclei acquired the same size and shape observed in free-living infective zoospores (Fig. 3R). This structure filled with fully developed zoospores is called the sporangium (mature sporocyte) (Fig. 3H, Q), and under light microscopy was dark and spherical in appearance (Fig. 2E). In the case of *A. minutum* as the host, the sporangium may contain 250–300 zoospores. In parallel with the initiation of zoospore division inside the developing sporangium, the number of lipid droplets decreased (Fig. 2J, K). After release of the zoospores, the sporangium (Fig. 2F), now empty except for a few residual lipid bodies (Fig. 2L), was colonized by bacteria (Fig. 3I, small blue nuclei).

Infection process at the ultrastructural level

Trophocyte and sporocyte development

Parasitoid intracellular stages at the ultrastructural level are shown in Figure 4. A comparison between infected and healthy host cells (Fig. 4A) revealed the evolution of parasitoid infection and

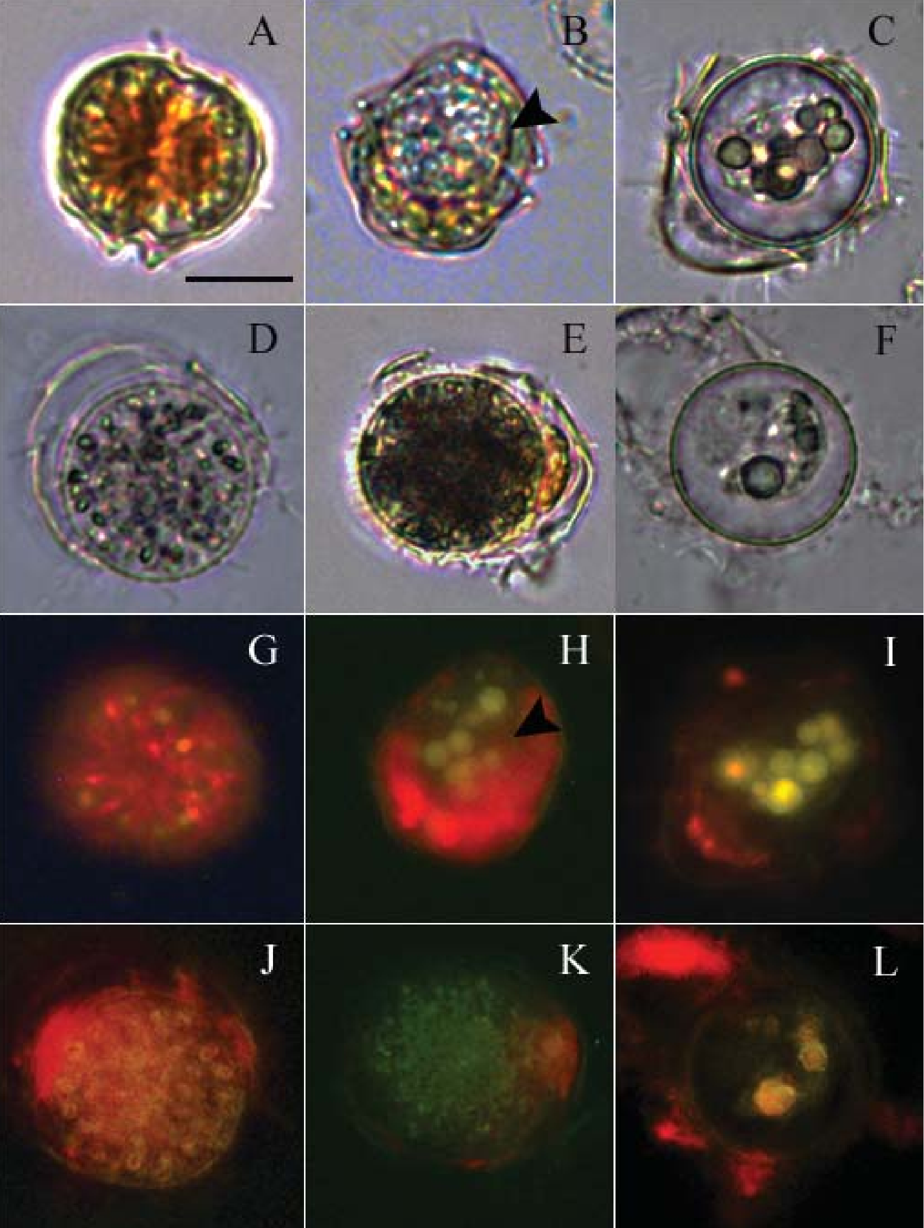
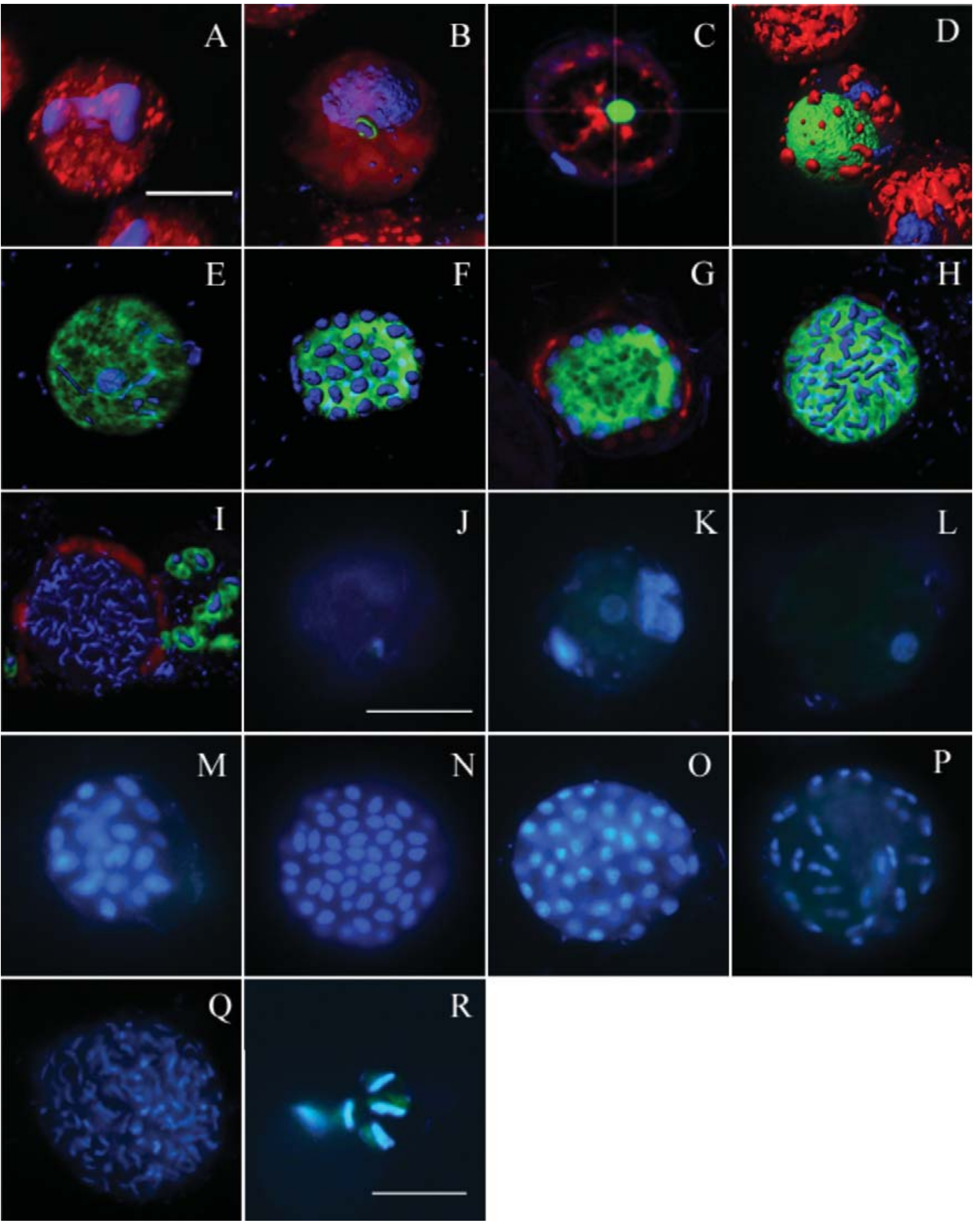


Figure 2. Infection of *A. minutum* (strain P4) by *P. sinerae* as observed by optical microscopy (A–F), and by epifluorescence microscopy of cells stained with the hydrophobic stain Nile red (G–L). Yellow and red fluorescence indicates lipids and chlorophyll, respectively. **A, G**. Healthy cell of *A. minutum*. **B, H.** Early trophocyte. The parasitoid develops inside the parasitophorous vacuole (arrowhead). Lipid globules appear inside the parasitoid body. **C, I.** The transparent, spherically shaped late trophocyte containing larger lipid globules. **D, J**. Early sporocyte containing zoospores inside the parasitoid structure. **E, K**. Late sporocyte (sporangium) filled with fully developed zoospores. **F, L.** Empty sporangium (after zoospore release) containing residual bodies of a lipid nature. Note that A and G is the same cell, as well as for the other pairs of images. Scale bar = 10 µm.

the changes it produced. After parasitoid penetration, trophocytes developed inside the parasitophorous vacuole (Fig. 4B), which grew separated from the host cytoplasm. Among the contents of the parasitophorous vacuole were starch granules, lipid droplets, and vacuoles that

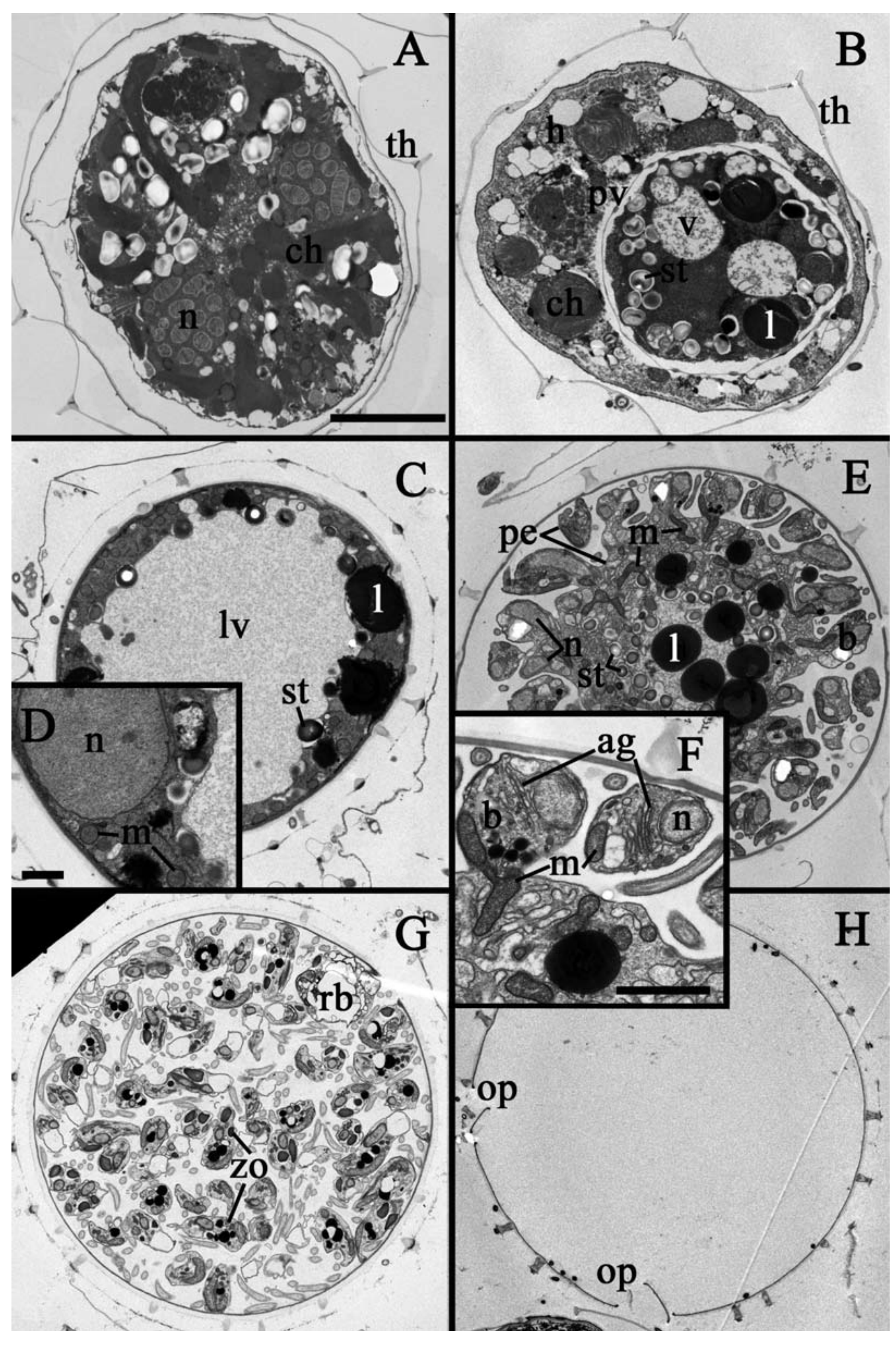


formed during the degradation of the host cytoplasm. A few host cellular organelles still remained intact. In the late trophocyte stage, the parasitoid occupied the entire host body, transforming its cellular content into a large vacuole which pushes the nucleus and some cellular organelles to the periphery of the cell (Fig. 4C and D). At this stage, a thick early sporangial wall was observed, and the host theca was usually disrupted. In the early sporocyte (Fig. 4E), the division and synthesis of cellular organelles were observed. The large vacuole was filled with these organelles which were contained within a mass of mitochondria, starch granules, the Golgi apparatus, lipid globules, and nuclei. *P. sinerae* preassembled its zoospores as buds, with the scaffold for zoospore assembly provided by the pellicle. The zoospore nuclei were arranged along the periphery of the parental mass (Fig. 4E). Cellular organelles were directed into the growing buds (Fig. 4F), thus providing each daughter cell (zoospores) with all the necessary cellular components prior to cytokinesis. After the completion of cytokinesis, the sporangium (Fig. 4G), now filled with zoospores, was surrounded by a membrane that separated it from the parental mass. Each fully formed zoospore had two flagella. At this stage, residual bodies from zoospore formation were seen inside the sporangium. With the onset of germination, the zoospores abandoned the sporangium via opercula-covered orifices in its wall. Finally, only the empty structure, with open opercula, remained (Fig. 4H).

Development of the sporangium wall

After the zoospore had settled inside its host, membrane formation and transformation were initiated and continued until the sporocyte, with its characteristic three-layered sporangial wall, had developed (Fig. 5). During the feeding stage, the trophocyte grew inside the parasitophorous vacuole, which was separated from the host cytoplasm by the parasitophorous vacuole membrane (Fig. 5A). Under the parasitophorous vacuole membrane, a heterogeneous intermediate layer composed of amorphous material accumulated electron-dense material to form the processes and finally, the sporangium wall. Membranous vesicles (alveoli) were located beneath the plasma membrane, at the top of the folded surface of the trophocyte. In the next stage, the amorphous material disappeared, such that the intermediate layer became homogeneous and

← **Figure 3.** Infection of *A. minutum* (strain P4) by *P. sinerae* as detected with FISH-TSA followed by confocal (**A–I**) and epifluorescence (**J–R**) microscopy. Green fluorescence indicates the Parvi-2R probe, which targets the parasitoid. Red and blue fluorescence reveals host chlorophyll and the host/parasitoid nuclei, respectively. **A.** Healthy *A. minutum* cell with its typical U-shaped nucleus. **B.** *A. minutum* infected by a parasitoid zoospore located close to the host nucleus. **C.** Orthogonal section of an infected host cell showing the location of the parasitoid. **D.** Early trophocyte growing inside the host. The chloroplasts and nucleus of the host are degraded. **E.** Late trophocyte occupying the entire body of the host. Note that the parasitoid structure contains only a single, large nucleus. **F.** Early sporocyte, Nuclei division by schizogony. **G.** Orthogonal section of an early sporocyte. The formation of new zoospores begins in the periphery of the early sporangium; rounded nuclei can be seen along the margin of the parasitoid structure. **H.** Late sporocyte (sporangium). The nuclei of the fully formed zoospores are elongated. **I.** After zoospore release, the empty sporangium is colonized by bacteria. Free zoospores are seen outside the sporangium. **J.** Zoospore attached to the flagellar pore of the dinoflagellate theca. **K.** Early infection. The host nucleus is partially degraded. **L.** Late trophocyte. The host nucleus is completely degraded; the single, large parasitoid nucleus is located in the center of the trophocyte, as in E. **M–Q.** Consecutive nuclei divisions. The nuclei become smaller and more elongated as the sporangium produces new zoospores. **R**. Free-living zoospores with their elongated nuclei. Scale bars A–I = 10 µm; J–Q = 10 µm; R = 5 µm.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electron-translucent with exception of the early processes embedded in it and the outer membrane connecting the outer end of those processes. This stage was marked by both endocytosis and exocytosis (Fig. 5B, arrowheads). At the late trophocyte stage (Fig. 5C), the pellicle was composed of plasma membrane, which later served as a scaffold for the developing zoospores. In addition, the three layers of the sporangium wall began to differentiate, forming an innermost, an intermediate, and the outer layer (Fig 5C). By the early sporocyte stage, the differentiation of these layers was completed giving rigidity to the processes of the wall (Fig. 5D). At the same time, invagination of the pellicle, the innermost, and the medium layer of the sporangium wall (Fig. 5D, arrowheads) resulted in the formation of several orifices (opercula), which, as noted above, allowed the zoospores to abandon the sporangium. Once the zoospores had formed, during the sporangium stage, the pellicle had become the plasma membrane of the zoospores and the well-developed sporangium wall, with its three layers and processes, was evident (Fig. 5E). In the abandoned sporangium, the opercula covering the orifices of the sporangium wall were open (Fig. 4G and 5F).

Parasitoid infection kinetics and time for intracellular development

The kinetics of *P. sinerae* infection in *A. minutum* is presented in Figure 6. *P. sinerae* completed its life cycle, from the addition of parasitoid zoospores until sporangium development, in 3–4 days. The first infected cells were observed 6 h after a parasitoid was added to a healthy host culture. By 54 h, the peak of the early trophocyte stage was reached; 18 h later (72 h post-infection) > 80% of the host population was infected. By 75 h post-infection, the parasitoid population had reached the late trophocyte stage and within the next 3 h underwent schizogony (early sporocyte stage), initiating the formation of new zoospores. By 96 h post-infection, the majority of the parasitoid population had reached the late sporocyte stage (sporangium). Based on the frequencies of these stages (Fig. 6), *P. sinerae* spent 1 day searching for and then invading the host cell and required 2–3 days to develop intracellularly. The trophont stage was the longest (approx. 50 h) whereas the replicative stage was relatively short (approx. 20 h). The 24 h that *P. sinerae* spent searching and invading its host was consistent with an infection rate of 1.07±0.026 day^{-1} (R^2=0.82; $p < 0.01$) (Fig. 7). Parasitoid prevalence increased exponentially over time, reaching a maximum (85%) 2 days after infection was initiated by the addition of the parasitoid to the host culture. The host population decreased by one order of magnitude (from 10^4 to 10^3 cells·mL^{-1}), corresponding to a host mortality rate of -0.82±0.015 d^{-1} (R^2=0.98; $p < 0.01$) (Fig. 7).

← **Figure 4.** Transmission electron microscopy study of the ultrastructure of a *P. sinerae* infection of *A. minutum* (strain P4). **A.** Healthy *A. minutum* cell. **B.** Early trophozoite. The parasitoid growing inside the host is covered by the parasitophorous vacuole. **C.** Late trophocyte. The contents of the host cell are almost completely degraded and form a large vacuole. **D.** Detailed picture of late trophocyte stage. Note that there is only one big nucleus located in the periphery of the cell. **E.** Early sporocyte. The parasitoid forms new zoospores as buds, located along the margins of the sporangial wall. **F.** Detail of zoospore formation (buds). Note the segregation and division of the cellular organelles. **G.** Late sporocyte (sporangium) containing fully developed zoospores and the residual body. **H.** Empty sporangium after zoospore release. Scale bars A–C, E, G and H = 5 µm; D and F = 1 µm. Abbreviations: buds (b), chloroplast (ch), Golgi apparatus (ag), host (h), large vacuole (lv), lipid body (l), mitochondria (m), nucleus (n), operculum (op), parasitophorous vacuole (pv), pellicle (pe), residual body (rb), starch granule (st), theca (th), vacuole (v), and zoospore (zo).

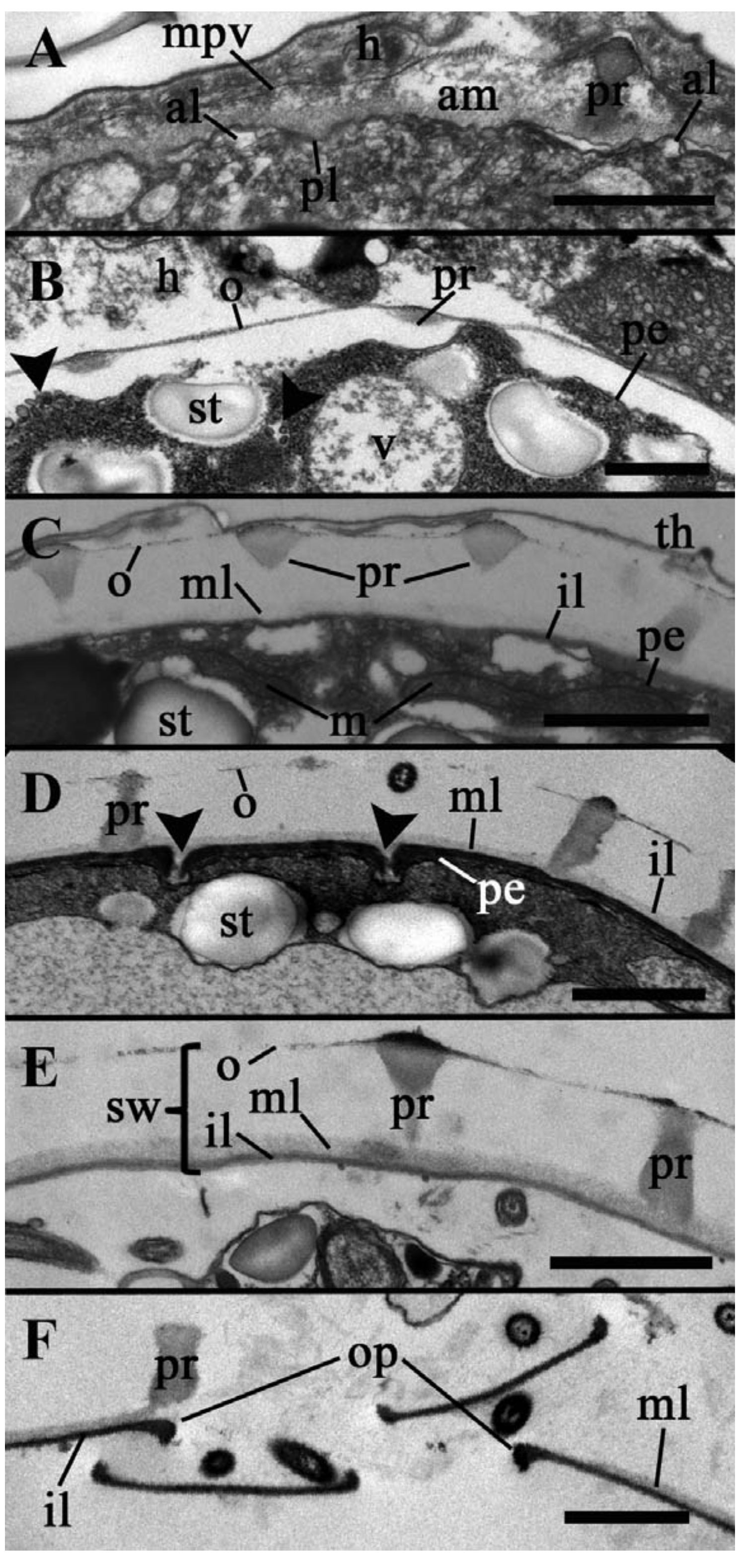
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Table 1. Percentage of losses (*k*) between consecutive life stages of *Parvilucifera sinerae* during an infection of *Alexandrium minutum* at a zoospore: host ratio of 1:1. *k*-1 is the intensity of parasitoid losses during invasion; *k*-2 represents parasitoid losses during invasion of the trophocyte (feeding stage); *k*-3 represents parasitoid losses during the transition from trophocyte to sporocyte (sporangium). K is the total parasitoid losses between the zoospore free-living infective stage and the sporocyte stage.

	Zoospores	*k*-1	Invasion	*k*-2	Trophocyte	*k*-3	Sporocyte	*K*
Abundance	34225		23501		23260		20117	
Log_{10}	4.53		4.37		4.37		4.30	
K values		0.16		0.004		0.06		0.23
Loss percentage		31.3%		1%		13.5%		41.2%

Parasitoid mortality during infection and life-stage transitions

We estimated the percentage of parasitoid losses that occurred during the transition between *P. sinerae* life stages (Table 1). The greatest loss of parasitoids took place during zoospore invasion, as > 30% of free-living zoospores were unable to invade a host cell. After invasion, only 1% of the established infections did not result in a trophocyte. After trophont development, 13.5% of the trophocytes failed to differentiate into sporocytes. For the infection process as a whole, total parasitoid mortality, estimated from the free-living infective stage to the sporangium stage, was about 41.2% of the parasitoid population, assuming a zoospore:host ratio of 1:1.

Prevalence as a function of inoculum size

Parasitoid prevalence increased exponentially, reaching a maximum that was proportional to the inoculum size (Fig. 8). The estimated maximum infection level (I_{max}) was 82.6±2.68% and the initial slope of the fitted curve (α) was 16.7 (r^2=0.98). The prevalence of *P. sinerae* increased to reach near maximum levels at a zoospore:host ratio of 20:1. Inoculations above this ratio consistently resulted in the infection of > 80% of host cells, but 100% infection levels were never reached, even at a zoospore:host ratio of 80:1. In that experiment, the initial host concentration was 10^3 cells·mL^{-1} and the prevalence obtained for a 1:1 ratio was much lower than the prevalence obtained when the initial host concentration was one order of magnitude higher (10^4 cells·mL^{-1}), as was the case in the

Figure 5. Transmission electron micrographs of sporangial wall maturation in *P. sinerae*. **A.** The folded surface of the parasitoid is covered by the parasitophorous vacuole membrane that is separated from the parasitoid plasma membrane by an intermediate layer of amorphous material where the developing wall processes are embedded. Alveoli at the top of the folded surfaces. **B.** During the next stage of sporangium wall maturation, processes appear from under the outer layer. Vesicles of endo/exocytotic activity (arrowheads) under the pellicle. **C.** The first stages in the differentiation of the sporangium wall into its three typical layers. Developing processes embedded in the sporangium wall. **D.** Processes completely formed. Formation of the operculum (arrowheads). **E.** Detail of the three layers of the sporangium wall at the sporangium stage. **F.** Structure of the operculum after zoospore release. Scale bars = 0.5 µm. Abbreviations: Alveoli (al), amorphous material (am), host (h), innermost layer (il), medium layer (ml), membrane of parasitophorous vacuole (mpv), mitochondria (m), opercula (op), outer layer (o), pellicle (pe), plasma membrane (pl), processes (pr), sporangium wall (sw), starch granule (st), theca (th), and vacuole (v).

previous experiment. Thus, prevalence appears to be highly dependent on the host density at the time of zoospore addition.

DISCUSSION

Eukaryotic parasitoids of dinoflagellates have evolved in accordance with the life-cycle strategies of their hosts to successfully invade, feed, and reproduce within them. In the case of *P. sinerae*, an understanding of its life cycle and of its infection kinetics provides the basis for addressing both the role played by this parasitoid in its natural host populations and its broader ecological effects.

Comparison of the life cycles of *Parvilucifera* species and its parasitic relatives within the alveolates

By monitoring a *P. sinerae* infection over time using different microscopy techniques we were able to discern previously unknown morphological and ultrastructural features of the parasitoid's life cycle. Here we discuss the *P. sinerae* life cycle in detail and compare its characteristics with those described for the three other *Parvilucifera* species recognized to date: *P. infectans, P. rostrata*, and *P. prorocentri*. All *Parvilucifera* species are classified as endoparasitoids able to infect a broad range of dinoflagellate species, the exception being *P. prorocentri* which specifically infects *Prorocentrum fukuyoi* (Leander and Hoppenrath 2008). The four species have largely similar life-cycle strategies, with only minor differences (Table 2). Thus, a free-living motile and infective stage (the zoospore), an intracellular stage, and a free-living non-motile stage are basic properties of *Parvilucifera*. The intracellular stage, called trophocyte, is defined as the feeding stage in which

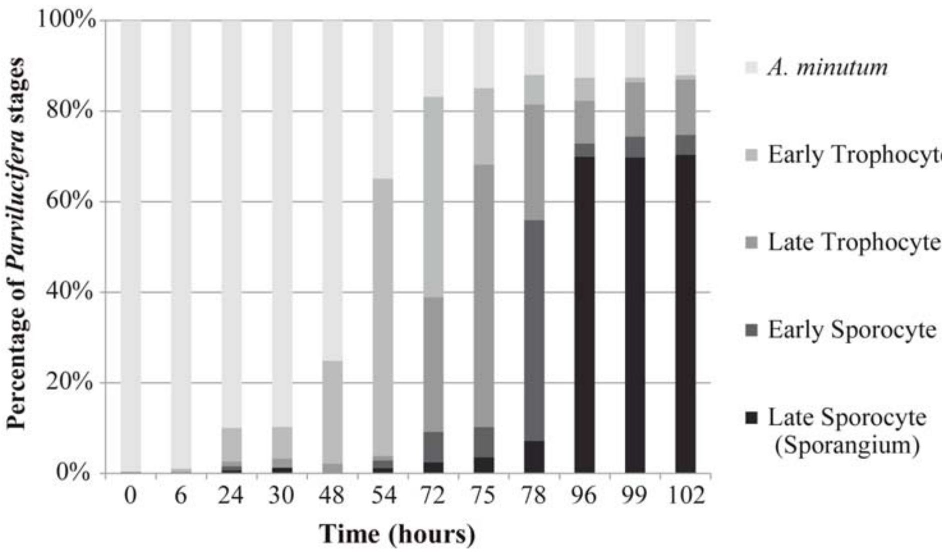

Figure 6. Time-frequency of *P. sinerae* infection stages during the infection of a culture of *A. minutum* at a zoospore:host ratio of 1:1. The accumulative bars (%) show *A. minutum* healthy cells, Early Trophocyte (stage 1), Late Trophocyte (stage 2), Early Sporocyte (stage 3), and Late Sporocyte (Sporangium).

the parasitoid grows while devouring the host cytoplasm. The non-motile free-living stage, called sporocyte or sporangium, is the replicative stage in which the parasitoid multiplies to yield an enormous number of new zoospores that are subsequently released into the marine environment, where after encountering a new host, another round of infection is initiated. *P. sinerae* actively penetrates its host and in thecate species enters the host cell through the flagellar pores as reported for *A. catenella* (Delgado 1999) and *A. minutum* (Erard-Le Denn et al. 2000). Leander and Hoppenrath (2008) suggested the same penetration mechanism by *P. prorocentri*. Norén et al. (1999) observed that dinoflagellates infected by *P. infectans* or *P. sinerae* (personal observation) lose their swimming ability and then sink. How *Parvilucifera* zoospores are able to recognize, attach to, and invade its host is largely unknown. However, all *Parvilucifera* species have an apical complex containing rhoptry-type extrusive organelles (Garcés and Hoppenrath 2010; Leander and Hoppenrath 2008; Lepelletier et al. 2014; Norén et al. 1999) and their involvement in the above-mentioned processes and in the formation of the parasitophorous vacuole membrane has been demonstrated in the apicomplexan parasites *Plasmodium* and *Toxoplasma* (Carruthers and Boothroyd 2007; Cowman and Crabb 2006) which are the causatives agents of the human diseases malaria and toxoplasmosis, respectively. Figueroa et al. (2008) described two routes of infection with respect to the subsequent localization of the trophocyte in the host. The cytoplasmic route is used for athecate host species and the nuclear route in the case of thecate host species. By combining confocal and fluorescence in situ hybridization-tyramide signal amplification (FISH-TSA) techniques, we observed that the *P. sinerae* trophocyte was located close to, but not within the nucleus of *A. minutum* (thecate), as described in reports of infections by the three other *Parvilucifera* species of dif-

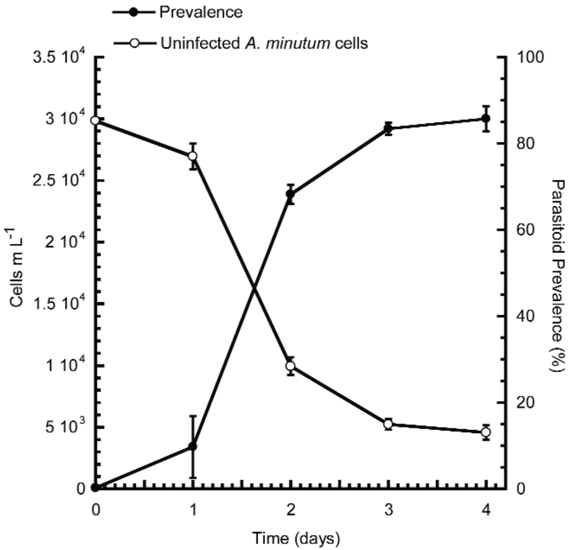

Figure 7. Dynamics of *P. sinerae* infection of a population of *A. minutum* and host mortality due to parasitism. The prevalence is the percentage of host cells infected each day. Host mortality shows the daily decrease in the number of uninfected cells of *A. minutum* population.

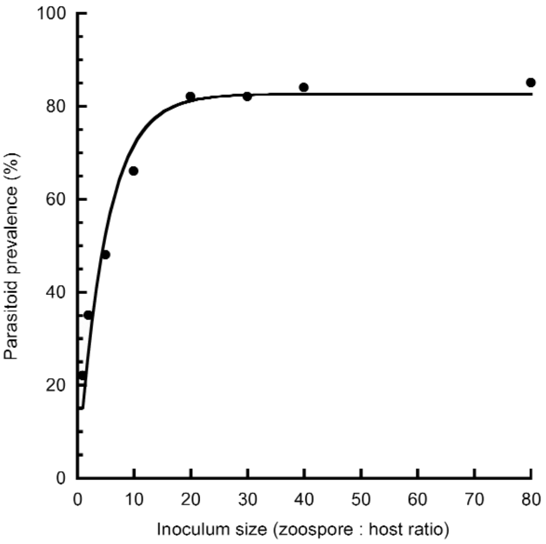

Figure 8. *P. sinerae* prevalence in an infection of *A. minutum* as a function of inoculum size. Host density was maintained at 10^3 cells·mL^{-1}, while parasitoid density varied to yield zoospore:host ratios of 1:1, 2:1, 5:1, 10:1, 20:1, 30:1, 40:1, and 80:1.

Table 2. Morphological and structural features of the life cycle of several parasitic alveolate lineages, including Perkinsozoans (*Parvilucifera* species, *Perkinsus* and *Rastrimonas*), *Colpodella*, *Amoebophrya* (Syndiniales), and *Plasmodium* (Apicomplexa).

Genus	Species	Type of parasite	Host range	Life cycle	Generation time	Host penetration	Trophocyte location	Feeding mode	Reproduction	Number of zoospores/sporocyte	Zoospore release	References
Parvilucifera	***infectans***	Endoparasitoid; causes host death	Wide range of dinoflagellates	Free-living infective stage (zoospores). Intracellular stage: trophont (feeding stage) undergoes division to form a resting sporangium inside the host cell	*Alexandrium*: 1–2 days *Dinophysis*: 2 days average (1–6 days)	Active, penetration of the host not observed	Within the host cytoplasm, inside a parasitophorous vacuole	Massive endocytosis of the host cytoplasm	Asexual; multiple fission: schizogony	Hundreds (depends on host size)	Several opercula in the sporangial wall	Delgado 1999; Norén et al. 1999
	sinerae	Endoparasitoid; causes host death	Wide range of dinoflagellates	Free-living infective stage (zoospores). Intracellular stage: trophont (feeding stage) undergoes division to form a resting sporangium inside the host cell	*Alexandrium*: 3–4 days; time increases with increasing host size	Active, through the flagellar pore of the host	Within the host cytoplasm, inside a parasitophorous vacuole	Massive endocytosis of the host cytoplasm	Asexual; multiple fission: schizogony	Hundreds to thousands (depends on host size)	Several opercula in the sporangial wall	Garcés et al. 2013a; Garcés and Hoppenrath 2010; This study
	prorocentri	Endoparasitoid; causes host death	*Prorocentrum fukuyoi*	Free-living infective stage (zoospores). Intracellular stage: trophont (feeding stage) undergoes division to form a resting sporangium inside the host cell	-	Probably through the flagellar pore	Within the host cytoplasm, inside a parasitophorous vacuole	Massive endocytosis of the host cytoplasm	-	-	Through the germ tube	Leander and Hoppenrath 2008
	rostrata	Endoparasitoid; causes host death	Wide range of dinoflagellates	Free-living infective stage (zoospores). Intracellular stage: trophont (feeding stage) undergoes division to form a resting sporangium inside the host cell	*Alexandrium*: 5 days	Active, not observed	Within the host cytoplasm, inside a parasitophorous vacuole	Massive endocytosis of the host cytoplasm	Not observed	Hundreds	Several opercula in the sporangial wall	Lepelletier et al. 2014
Perkinsus	**sp.**	Endoparasite; causes tissue necrosis that inhibits host growth and reproduction; causes death in heavily infected hosts	Marine mollusks	Free-living motile stage: the zoospore develops into a trophozoite, which yields hypnospores (pre-zoosporangia). Then hypnospores undergo zoosporulation. Once into host tissue trophont divides into daughter cells, which, when mature are released into the marine environment	4–5 days	Passive, by host ingestion during filter-feeding; trophozoite are phagocytised by hemocytes in the alimentary canal	Within all types of host tissues (most often in the connective tissue) inside the phagosome of the host hemocyte. A parasitophorous vacuole develops	Host hemolymph, but poorly understood	Sexual and asexual. Trophozoites divide by multiple fission: schizogony. Whilst less frequent, binary fission has also been observed	Trophonts divide into 4–32 (often 8–16) trophozoites in host tissue. Trophozoites that undergo zoosporulation produce hundreds of zoospores	In the case of zoosporulation: discharge/germ tube present in the zoosporangium wall.	Bushek et al. 2002; Coss et al. 2001; Perkins 1996; Sunila et al. 2001;

Table 2 (cont).

Genus	Species	Type of parasite	Host range	Life cycle	Generation time	Host penetration	Trophocyte location	Feeding mode	Reproduction	Number of zoospores/sporocyte	Zoospore release	References
Rastrimonas	***subtilis***	Endoparasitoid; causes host death	Freshwater cryptophytes	Free-living infective stage (zoospore). Intracellular replicative stage: trophont grows and divides directly within the host cytoplasm and it is not surrounded by an envelope. A resting cyst-like phase, which is surrounded by a cyst wall, has sometimes been observed under certain conditions, such as during the decline of the host populaiton	Hours	Active, zoospore attaches or adheres to the base of the host flagella, where the plasma membrane lacks a subpellicular cytoskeleton	Within the host, free in the cytoplasm, and it is not enclosed in a parasitophorous vacuole	The trophont probably feeds on host cytoplasm by pinocytosis, as micropores were observed, and by osmotrophy	Asexual. Multiple fission. Reproduction occurs by separation of the protoplast into zoospores.	Hundreds	The host cell burst and the zoospores or the cyst are released	Brugerolle 2002b; Brugerolle 2003; Ettl and Moestrup 1980
Colpodella	**sp.**	Predator or ectoparasite	Free-living protists (chlorophytes, kinetoplastids, cryptophyceae)	Free-living infective stage (trophozoite). Extracellular replicative stage Trophozoite attaches to and feeds on the host and then detaches. The trophont and subsequent sporozoites develop enclosed in a cyst-like envelope lying on the substrate. Sexual phase: the conjugation of two cells was observed, but the complete fusion and development could not be followed to completion	5-6 days (in 26.5% w/v NaCl) 3 days (in 24% w/v NaCl).	Adheres to but never enters the host. Attaches to any point of the host surface by an anterior pointed tip, which enlarges	Outside but attached to the host	Prey ingestion or Myzocytosis: The cytoplasm and organelles of the host are aspirated, become engulfed within the parasite, and are then channelled into the posterior large food vacuole	Asexual: binary fission of free-living vegetative cells. Multiple fission (four-way fision or palintomy) from a parental mass was observed in the cyst stage, followed by the formation of new trophozoites	2 (binary fission) or 4 (multiple fission)	The flagellates begin to move within the cystic envelope before it opens, and then exit. The cystic aperture has not been characterized	Brugerolle 2002a; Mylnikov 2009; Simpson and Patterson 1996
Amoebophrya	***'ceratii' complex***	Endoparasitoid; causes host death	Wide range of dinoflagellates	Free-living infective stage: dinospores. Intracellular feeding/replicative stage: trophont. Extracellular reproductive stage: vermiform	2–3 days	Zoospore attaches to the host surface and penetrates the host cytoplasm. - Within the host cytosol, the parasite crosses the nuclear envelope through the host pellicle	Host nucleus or cytoplasm and enclosed by a parasitophorous vacuole	Trophont located in the nucleus feeds by phagotrophy with a cytopharynx that serves as a gullet. Food passes from the cytosol to the cell interior and is collected in the food vacuole, where it is digested	Asexual: division yields multiple nuclei but without cytokinesis. Nuclei are arranged in numerous curving rows	Hundreds	Vermiform stage emerges from the host cell, completes cytokinesis, and ruptures into small pieces, releasing dinospores	Coats and Bockstahler 1994; Fritz and Nass 1992; Kim et al. 2004; Miller et al. 2012

Table 2 (cont).

Genus	Species	Type of parasite	Host range	Life cycle	Generation time	Host penetration	Trophocyte location	Feeding mode	Reproduction	Number of zoospores/sporocyte	Zoospore release	References
Plasmodium	**sp.**	Endoparasite; causes host death by obstruction of the microcirculation and results in the dysfunction of multiple organs	Mosquito (definitive host) and humans; poultry and rodents (secondary hosts)	Infection begins with sporozoites released by the *Anopheles* mosquito into human blood and arriving in hepatocytes, where they produce merozoites that are released into the blood and grow as trophocytes. This stage can be transferred to the mosquito when it bites the infected host. Some trophocytes become gametocytes that produce gametes. Gamete fusion produces a motile zygote that becomes a sporozoite in the mosquito salivary glands, and the cycle starts again	Varies within and among *Plasmodium* species. *P. falciparum* (human malaria) development takes 15–30 days in the mosquito depending on the temperature. In humans, erythrocytic stages may last 5 days and gametogenesis up to 3 days	Sporozoites are injected passively via the mosquito salivary glands during a bite or transferred to mosquitoes by blood feeding on infected humans, rodents or poultry. Once in the host blood. Merozoites actively attach and invade erythrocytes using secretory organelles of the apical complex	Trophocytes and merozoites develop into erythrocytes inside a parasitophorous vacuole	Trophont within red blood cells feeds on small aliquots of haemoglobin by phagocytosis through its cytostome	Asexual: sporozoite and merozoites divides by schizogony. Sexual: merozoites develop into gametocytes that produce male and female gametes. These fuse in the gut of the mosquito after an infected human is bitten and then mature into a zygote	During asexual reproduction, thousands of merozoites are produced. During sexual reproduction, hundreds of zygotes are formed	Egression of sporozoites, merozoites, and gametocytes is due to proteases that cause the disruption and disintegration of the parasitophorous vacuole and erythrocyte membranes	Bannister and Mitchell 2003; Baton and Ranford-Cartwright 2005; Day et al. 1998; Lambros and Vandenberg 1979; Vaughan 2007

ferent dinoflagellates hosts. All four parasitoid species, once inside their hosts, develop a trophocyte that feeds on the host cytoplasm via massive endocytosis. The trophocyte is surrounded by a parasitophorous vacuole that confers protection against host-cell defenses and allows its unfettered growth (Plattner and Soldati-Favre 2008). The process how the trophocyte enlarges, consumes and digests the host is unknown in *Parvilucifera* species and, it would be a very interesting issue to be addressed. Once the host cytoplasm has been completely consumed, the parasitoid undergoes schizogony to form daughter cells. In this form of cell division, nuclei multiply by asynchronous rounds of mitosis, resulting in a multinucleate stage. As differentiation proceeds, daughter cells are formed by synchronous budding, accompanied by the segregation of the newly synthesized nuclei and other cellular organelles. This study is the first to fully demonstrate zoospore formation in the genus *Parvilucifera*. Norén et al. (1999) also described the intracellular differentiation of *P. infectans* (Figure 9 of that study), but what those authors referred to as the mature sporangium at the time of zoospore release was instead an immature sporangium with budding zoospores. The number of zoospores produced per sporangium in *P. sinerae* depends on the size of the infected host (Garcés et al. 2013a) and ranges from hundreds to thousands, as also reported for *P. infectans* (Norén et al. 1999). Data on the other two species within this genus are not available. While *P. infectans, P. sinerae*, and *P. rostrata* release zoospores via the operculum in the sporangial wall, in *P. prorocentri* zoospores are discharged through the germ tube, similar to members of the genus *Perkinsus* (Table 2).

Parvilucifera species, like many other parasitic genera belonging to the alveolates, such as *Colpodella*, *Rastrimonas*, and *Perkinsus*, possess features of dinoflagellates and apicomplexans, the other two main groups of this superphylum. Therefore, phylogenetically,

these parasitic lineages do not fit neatly within either group, which, in terms of their life cycle, raises several interesting evolutionary questions (Brugerolle 2002b, 2003; Mignot and Brugerolle 1975; Moore et al. 2008; Norén et al. 1999; Perkins 1976, 1996; Siddall et al. 1997; Siddall et al. 2001). In the following, we examine the features of the *Parvilucifera* life cycle with respect to those of the above-mentioned parasitic genera and the dinoflagellate and apicomplexan parasites *Amoebophrya* and *Plasmodinium*, respectively. The comparison is summarized in Table 2.

Although *Perkinsus* and *Rastrimonas* are the organisms most closely related to *Parvilucifera* known at date, their life cycles share only a few features. All three are endoparasitoids that cause the death of their hosts, but the nature of those hosts differs. During infection, zoospores of *Parvilucifera* and *Rastrimonas* actively penetrate their hosts through their flagellar pores. By contrast, *Perkinsus* zoospores are ingested by their hosts during filter-feeding. However, once inside the host *Perkinsus* grows inside a parasitophorous vacuole, just as *Parvilucifera* and apicomplexan parasites do, whereas *Rastrimonas* grows free in the host cytoplasm. *Colpodella,* the early divergent sister group of apicomplexans, shares some ultrastructural features with perkinsozoans, mainly in the zoospore stage, presenting a pseudoconoid in the apical complex rather than a conoid, typical of apicomplexans (Brugerolle 2002a). However *Colpodella* is an ectoparasitoid such that its life cycle has little in common with that of *Parvilucifera*, including their target hosts (Table 2). Moreover, there are remarkable differences between the life cycle of *Colpodella* species, as for example the type of cell division to produce the offspring or the feeding mode on their hosts. These differences, added to the lack of molecular data suggested that some species could be erroneously classified within this genus (Okamoto et al. 2012). Comparisons of the life cycles and hosts of *Parvilucifera* and *Plasmodium falciparum* are also limited since the latter parasite, which cause malaria in humans, has a very complex life cycle that involves intermediate hosts. Nonetheless, several of the ultrastructural features that characterize *P. sinerae* infections, such as zoospore budding (schizogony) and parasitoid development within a parasitophorous vacuole, are also seen in *P. falciparum* (Francia and Striepen 2014). These two characteristics and the presence at the infective stage of an apical complex involved in host recognition and invasion (Garcés and Hoppenrath 2010), are traits typical of apicomplexans (Levine 1973). Dinoflagellates, another Alveolata lineage, comprise the parasitic group *Amoebophrya* (Syndiniales). Despite its phylogenetic distance to *Parvilucifera*, *Amoebophrya* has the same host range. It is also an endoparasitoid and is able to infect > 75 dinoflagellate host taxa (see Table 1 of the review of Park et al. (2013)). Like *Parvilucifera* species, it has a free-living infective stage but once inside the host, the trophont grows by feeding on the host cytoplasm and nucleus. However, its feeding mode is different since *Amoebophrya* feeds by phagotrophy, via a cytopharynx typical of mixotrophic dinoflagellates (Miller et al. 2012). A further similarity with *Parvilucifera* is that the large trophont, occupying most of the host cell, undergoes a series of nuclear and flagellar replications without completing cytokinesis (Fritz and Nass 1992). But unlike *Parvilucifera*, this short-lived vermiform stage is highly motile and is able to emerge from the host cell and swim freely in the marine environment. Moreover, swimming is accompanied by the completion of cytokinesis, thus yielding many infective dinospores (Coats and Park 2002).

Dynamics of parasitoid infection

Because they are lethal to their hosts, parasitoids such as *Parvilucifera* reduce the size of their natural populations. Thus, insights into parasitoid-host interactions, including the dynamics of infection and the kinetic parameters that increase parasitoid transmission, provide important contributions to ecological models of phytoplankton bloom dynamics.

The transmission of *P. sinerae* is based on the successful development of the sporocyte (sporangium), which contains the infective zoospores. Sporangium development involves a sequence of morphologically distinct life stages that is the same in all *Parvilucifera* species and, as discussed above, similar to those of *Amoebophrya*. The infection cycle of *P. sinerae* can be divided into three general stages. In the first, the free-living zoospore actively searches out and then penetrates its host. This brief stage is completed within 6–24 h after zoospores formed in the previous infective cycle have left the sporangium. It is also the stage in which parasitoid mortality is highest. The short life of zoospores outside the host has also been suggested in *Amoebophrya* and *Parvilucifera*-like parasitoids (Coats and Park 2002; Delgado 1999). The feeding stage is the longest, lasting roughly 2 days. During this time the trophocyte grows until it fills its host (Garcés et al. 2013a), as also occurs in apicomplexan parasites (Plattner and Soldati-Favre 2008). The lengthy trophocyte stage implies a cascade of processes that result in the degradation of the host cytoplasm and the use of its nutrients to synthesize parasitoid structures and ultimately new parasitoids. The reproductive stage is the third and final stage of sporangium development. It lasts for about 20 h and consists of the formation of new zoospores. However, zoospore ability to respond to the appropriate chemical signal, which induces zoospore release, requires more than 1 day of a zoospore final maturation (data not shown). In summary, the life cycle of *P. sinerae* infecting *A. minutum* requires 3–4 days, with a single infection resulting in the release of 250–300 or even thousands of new zoospores, depending on the size of the host (Garcés et al. 2013a). The short generation times and high reproductive rates typical of microparasites have also been described for other *Parvilucifera* species (Lepelletier et al. 2014; Norén et al. 1999), such that the rates of asexual reproduction by this parasitic group are higher than those of their host species (Stolte and Garcés 2006).

Under laboratory conditions, *P. sinerae* can become highly prevalent at high host cell densities, which in turn will result in high host mortality rates. However prevalence is also strongly dependent on the zoospore:host ratio and varies with host density. This density dependence increases the probability of a successful infection because high ratios and/or high host densities increase the likelihood of an encounter between parasitoid and host. The infection kinetics and parasitoid parameters of other *Parvilucifera* species have not been reported, but the infectivity of *Parvilucifera* strains is known to vary, as does host susceptibility (Råberg et al. 2014). Moreover, infection parameters may also depend on the host species; for example, the dinospore:host ratio needed to reach a 100% prevalence of *Amoebophrya* depends on the host species (Coats and Park 2002). Thus, not only does a single parasitoid species exert different effects in different host species, but within the same host species the dynamics of parasitoid infection varies. Therefore, variations in infection levels and prevalences in different host species and in different strains of the same species should translate to varying population-level effects.

Nonetheless, even at very high zoospore:host ratios, the prevalence of *P. sinerae* never reached 100%, as 10–20% of the cells consistently remained uninfected. Thus, some cells of an *A. minutum* clonal culture are apparently resistant to infection. The source of this possible resistance is unknown but is presumably related to cell-cycle events, genetic mutations accumulated over the time, or host-cell states. Host cells stressed by the presence of the parasite or those with a suboptimal metabolic rate could render the host unattractive to an infective zoospore, which requires host resources for its development and reproduction. In the literature there are many examples of parasites that mostly infect healthy hosts instead of unhealthy ones. For instance, the study of Pulkkinen and Ebert (2004) demonstrated that *Daphnia*'s parasites reached higher prevalences

either higher spore loading when infecting non-stressed hosts (non-starved). Therefore, it would be of great interest to study the source of *A. minutum* resistance to *Parvilucifera* infection, and to know if this resistance is permanent or temporarily caused by the cell state, as well as to explore the parasitoid prevalence reached in the different growth phases of the dinoflagellate.

Ecological perspectives

In the marine environment, outbreaks of *P. sinerae* coincide with seasonal peaks of *A. minutum* (Figueroa et al. 2008) as also reported for the spatial distribution of *P. infectans* along the Swedish coast (Johansson et al. 2006). The co-occurrence of host and parasitoid in the field and the dynamics of *Parvilucifera* infection, as determined in this study, suggest that *P. sinerae* is perfectly adapted to the biology of its blooming dinoflagellate hosts. The high rate of *P. sinerae* reproduction and the density dependence of parasitoid prevalence allow a rapid increase in the size of the parasitoid population at high host concentrations. After the bloom reaches its peak, the sporangia remain dormant in the sediments until host density reaches a threshold—defined as either a sufficient number of hosts or high concentrations of their exudates (dimethylsulfide)—at which time the zoospores abandon the sporangium and seek out new hosts (Garcés et al. 2013b). That suggests that sporangium formation allows *Parvilucifera* to survive under low host densities, as also proposed by Lepelletier et al. (2014) for *P. rostrata*. A life strategy based on an infective zoospore-containing sporangium that remains in the sediments distinguishes *Parvilucifera* from *Amoebophrya* parasitoids in terms of their ecological niches and may allow their coexistence despite having the same host range, since the dinospore-containing vermiform stage of *Amoebophrya* is motile and remains in the water column. Genetic libraries obtained from environmental studies support this hypothesis as they showed that while Syndiniales (*Amoebophrya*) is one of the most important eukaryotic lineages represented in samples from the water column, perkinsids (*Parvilucifera*) are rarely reported in that environment (Guillou et al. 2008) and are instead more active in marine sediment (Chambouvet et al. 2014). In addition, although *Parvilucifera* species are classified as generalist parasitoids, whether they exhibit host preferences among dinoflagellates that allow them to coexist with other *Parvilucifera* with respect to shared host species (Lepelletier et al. 2014) remains to be determined. Confirmation of this possibility would have ecological implications at community level, since parasitoid virulence depends on parasitoid specificity, and therefore also on the size of their host populations.

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Chapter 2

"Host-released dimethylsulphide activates the dinoflagellate parasitoid *Parvilucifera sinerae*"

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Host-released dimethylsulphide activates the dinoflagellate parasitoid *Parvilucifera sinerae*

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ABSTRACT

Parasitoids are a major top-down cause of mortality of coastal harmful algae, but the mechanisms and strategies they have evolved to efficiently infect ephemeral blooms are largely unknown. Here we show that the generalist dinoflagellate parasitoid *Parvilucifera sinerae* (Perkinsozoa, Alveolata) is activated from dormancy not only by *Alexandrium minutum* cells but also by culture filtrates. We unequivocally identified the algal metabolite dimethylsulphide (DMS) as the density-dependent cue of the presence of potential host. This allows the parasitoid to alternate between a sporangium-hosted dormant stage and a chemically-activated, free-living virulent stage. DMS-rich exudates of resistant dinoflagellates also induced parasitoid activation, which we interpret as an example of coevolutionary arms race between parasitoid and host. These results further expand the involvement of dimethylated sulphur compounds in marine chemical ecology, where they have been described as foraging cues and chemoattractants for mammals, birds, fish, invertebrates and plankton microbes.

INTRODUCTION

Marine harmful algal blooms (HABs) are dense ephemeral proliferations typically of dinoflagellates, cyanobacteria or diatoms, that can directly cause illness and death in humans and marine life through the production of toxins, or cause ecosystem alterations affecting food provision and recreational activities (Zingone *et al.*, 2000; *MEA*, 2005). Even though HABs have been recognized as a major environmental challenge (*MEA*, 2005), little is known about what makes them thrive and wane. In the case of dinoflagellates, which account for 75% of HAB-forming phytoplankton species, bottom-up factors (including man-enhanced eutrophication, climate shifts and species dispersal) are usually invoked as triggers (Zingone *et al.*, 2000; Heisler *et al.*, 2008; Anderson, 2009), but the causes and mechanisms of termination remain obscure.

Parasitoids have been identified as a main cause of mortality of harmful dinoflagellates (Taylor, 1968; Chambouvet *et al.*, 2008), to the extent that their deliberate use has been suggested as a biological mitigation of HABs (Taylor, 1968), in the same manner it is done in agricultural applications on land. The suggestion has faced opposition on the basis of the lack of knowledge on their specificity, the mechanisms of infection, and the potential side effects (Anderson, 2009). The de-

bate has prompted the need for a better understanding of parasitoid-host interactions, along the same lines as the increasing interest in chemical ecology and interspecific communication in oceanic plankton (Ianora *et al.*, 2011).

Parvilucifera sinerae (Perkinsozoa, Alveolata) is a flagellate parasitoid that efficiently infects and kills a taxonomically broad variety of dinoflagellates, including harmful bloom forming species within the genera *Alexandrium*, *Dinophysis*, *Gambierdiscus*, *Gymnodinium, Ostreopsis* and *Protoceratium* (Garcés *et al.*, 2012). The infection cycle proceeds as follows (Fig. 1a): a flagellate zoospore penetrates the host cell, destroys its content, forms a spherical sporangium the size of the host, and divides to fill up the sporangium with dormant zoospores. They remain dormant until a wakeup call signals the presence of a sufficient density of host cells; then the zoospores activate into an apparently random motion and eventually leave the sporangium through one or several opercula opened in the wall. The objective of the present study is to identify the nature and origin of the signal involved in sporangia activation, and quantify the parasitoid activation response to a signal concentration gradient.

MATERIAL AND METHODS

Host and parasitoid strains and culture maintenance

Experiments were conducted with strains of *Alexandrium minutum* (AMP4 and AMP13), *Karlodinium veneficum* (K24) and *Amphidinium carterae* (ACRN03) of the culture collection of the Centro Oceanográfico de Vigo (CCVIEO), Spain, and the culture collection of the Institut Ciències del Mar, Barcelona, Spain. Non-axenic culture stocks were grown in L1 medium (Guillard, 1995) at 20±1°C, 90 µmol photons m^{-2} s^{-1} and a 12:12 hour photoperiod.

The culture of the *Parvilucifera sinerae* parasitoid (strain ICMB 852; Garcés & Hoppenrath, 2010) was propagated by transferring aliquots of mature sporangia (1 mL) every 5–7 days into an uninfected host stock culture of *A. minutum* AMP4 in sterile polystyrene Petri dishes. The time needed for the formation of a mature sporangium in the *A. minutum* culture is 5 days. Asynchrony was observed in the formation and maturation of sporangia after induced infection. To assure complete and synchronic maturation of all the sporangium population before the experiments, sporangia were kept at 4ºC in the dark. In the range of 12-15 days, all infected cells were in the mature sporangium stage (i.e., the stage at which sporangia are completely full of dormant zoospores, and there are no host cells left). Samples were used for experiments always within this 12-15 days period after infection. Therefore, the stage of the sporangia was always the same.

Mature sporangia were transferred from 4ºC to fresh medium at 20±1°C in the light one day before experiments were conducted. No significant opening of sporangia was observed with medium only, i.e., in the absence of host cells or exudates or DMS solution.

Experiments and sporangia activation counts

For chemical signalling experiments, the *A. minutum* AMP4 culture in exponential growth was diluted with L1 medium to a concentration that, once mixed with the sporangia in the experimental chambers, gave a concentration of 5000 host cells mL^{-1}. Exudates were prepared by filtering 10 mL of the culture through 0.22-µm pore size Swinnex filters (Millipore) right before the

experiment. Exudates of *A. minutum* AMP13 were prepared in the same way as AMP4. In the case of *A. carterae* and *K. veneficum* exudates, they were prepared from cultures with total cell biovolumes equivalent to the 5000 cells mL^{-1} of *A. minutum*.

Experimental mixtures were prepared triplicate 2-mL phytoplankton chambers by pipetting aliquots of 0.5 mL of mature *P. sinerae* sporangia stock at 20ºC, with no host cells (see first response), and adding 1.5-mL aliquots of potential host, exudates, chemical solutions or control medium. Initial *P. sinerae* sporangium and host concentrations in the chambers were 100-1000 sporangia mL^{-1} and 5000 host cells mL^{-1}, respectively. *P. sinerae* activation rates were determined in simultaneous triplicates by counting mature inactive (full) sporangia every 5 minutes during 30 minutes, and every 10 minutes until completing 60 minutes, under a Leica–Leitz DM IRB inverted microscope, a Leica–Leitz DM IL inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) and a Nikon DIAPHOT inverted microscope, respectively. Data were normalized to the initial concentrations of inactive sporangia, and activation rate constants were calculated as the slope of the logarithm-converted numbers over the first 30 minutes. Negative controls consisted of L1 medium additions to the parasitoid suspensions.

Chemical solutions and analyses

Stock solutions of dimethylsulphoniopropionate (DMSP·HCl, TCI America) and acrylate ($C_3H_3O_2Na$, Aldrich) were prepared by weighing and dissolution in MilliQ water. The stock solution of DMS was prepared by adding a pellet of NaOH to 10 mL of the stock solution of DMSP; the base hydrolyzed DMSP into equimolar amounts of dimethylsulphide (DMS) and acrylate. A few µL of the stock solutions were added to L1 medium immediately before the addition to the *P. sinerae* suspension.

DMS concentrations were measured in GF/F-filtered aliquots of exudates by a purge and trap gas chromatographic method described elsewhere (Galí *et al.*, 2011). DMSP concentrations in exudates were measured after alkaline hydrolysis into DMS.

RESULTS AND DISCUSSION

While studying the infection of the toxic dinoflagellate *Alexandrium minutum* by *P. sinerae*, we observed that the presence of filtered exudates of the dinoflagellate was enough to activate the dormant zoospores into motion and induce their release from the sporangia as efficiently as the presence of host cells did (Fig. 1b), pointing towards the involvement of a chemical signal. *A. minutum* is a strong producer of the osmolyte dimethylsulphoniopropionate (DMSP), which occurs at intracellular concentrations as high as 0.3 M, or 7% of total cell carbon (Berdalet *et al.*, 2011; Caruana *et al.*, 2012). Considering that *A. minutum* exudates contained DMSP concentrations in the order of 100-800 nM, and that this compound has been shown to induce positive chemotaxis in a variety of plankton microorganisms (Seymour *et al.*, 2010), it stood as a good candidate for the activation of the parasitoid. Additions of a lab-prepared solution of 270 nM DMSP, however, did not give any activation response significantly different from the negative control (Fig. 1b).

A. minutum also harbours high activity of DMSP lyases (Caruana *et al.*, 2012), the enzymes that cleave DMSP into equimolar amounts of acrylate and dimethylsulphide (DMS). Indeed, *A. minutum* blooms and cultures have the characteristic seafood smell of DMS, and exudates used in

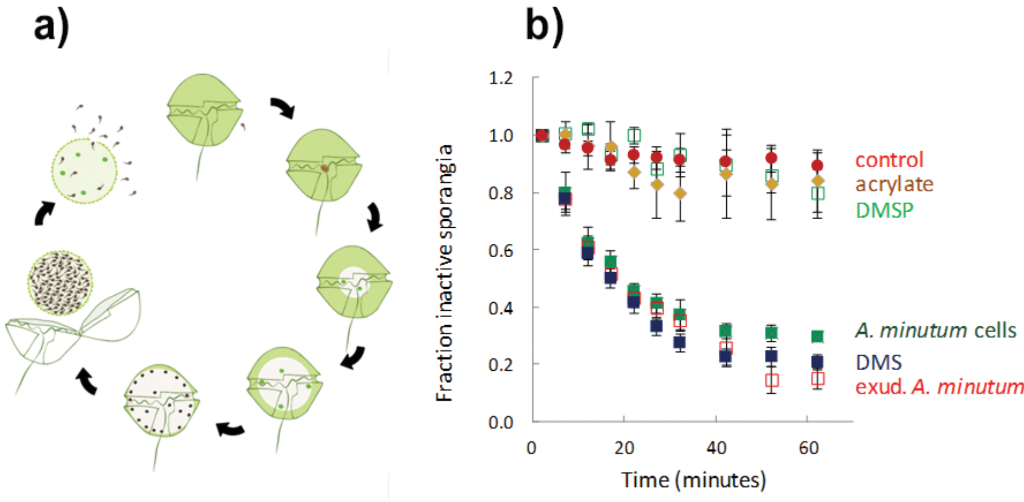

Figure 1. Response of *P. sinerae* sporangia to chemical cues. (a) The infection cycle of *A. minutum*: a zoospore enters the host, destroys its content, forms and sporangium and fills it with dormant zoospores; host-signalling cues cause the activation of *P. sinerae* zoospores and their release from the sporangium. (b) Decay in the number of inactive sporangia upon exposure to L1 medium (control), *A. minutum* AMP4 cells and exudates (which contain 300 nM of DMS), 270 nM DMSP in L1, 270 nM acrylate in L1, and 270 nM DMS in L1. Data are expressed as mean±s.e. of 3–11 replicates.

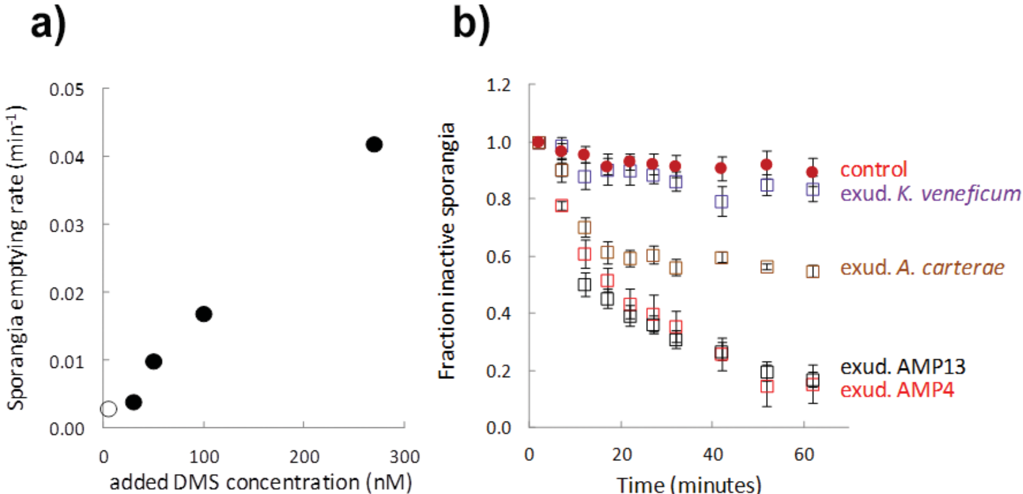

Figure 2. DMS-dependent parasitoid activation. (a) Rate constants of the emptying of *P. sinerae* sporangia upon exposure to L1 medium (open circle) and increasing concentrations of DMS in medium (filled circles). (b) Decay in the number of inactive *P. sinerae* sporangia upon exposure to L1 medium (control) and exudates of *A. minutum* AMP4 (susceptible host, 300 nM DMS), *A. minutum* AMP13 (resistant host, 270 nM DMS), *Amphidinium carterae* (resistant host, 140 nM DMS), and *Karlodinium veneficum* (resistant host, 1 nM DMS). Data are expressed as mean±s.e. of 3–11 replicates.

this study had DMS concentrations of ca. 300 nM. Additions of an acrylate solution did not induce any response, but additions of DMS at a concentration similar to that in the exudates did (Fig. 1b). Moreover, the response rate was proportional to the added DMS concentration down to a threshold close to 30 nM (Fig. 2a).

We showed that DMS alone was enough for parasitoid activation, i.e., for the necessary step prior to infection. Since many dinoflagellates produce DMS (Stefels *et al.*, 2007; Caruana *et al.*, 2012), including susceptible and resistant strains, how host-specific is this mechanism? Could it be that DMS acts in concert with other chemicals contained only in the exudates of the susceptible dinoflagellates? Assays with culture filtrates of non-susceptible *A. minutum*, *Amphidinium carterae* and *Karlodinium veneficum* strains showed parasitoid activation rates proportional to their DMS content (Fig. 2b). In other words, exudates of dinoflagellates caused parasitoid activation as long as they had enough DMS, independently of the strains' susceptibility to infection.

Our experiments demonstrated that *P. sinerae* perceives DMS as the wakeup call for activation. In the coastal ocean, background DMS concentrations are typically 0.5-10 nM (Lana *et al.*, 2011), while a confined coastal bloom of *A. minutum* (ca. 1000 cells mL^{-1}) on 7 March 2012 had a DMS concentration of 217 nM. Since DMS is a short-lived substance in seawater (Pinhassi *et al.*, 2005), where it is consumed by bacteria and photolysis, the threshold found here for the activation of the dormant zoospores (a few tens of nM) allows the parasitoid to activate only in the presence of relatively high densities of potential host cells, and do it more rapidly within denser blooms. This alternation between a sporangium-hosted dormant stage and a chemically-activated, free-living virulent stage stands as an efficient strategy for success in the maintenance of the parasitoid population. How do they survive between host blooms, either by serially infecting a sequence of dinoflagellate hosts, as shown for other parasites (Chambouvet *et al.*, 2008) or by sporangium sinking to the sediments along with host cysts, remains unknown.

DMS is a by-product of both algal physiology (Stefels *et al.*, 2007) and food web interactions, including herbivore grazing and bacterial catabolism (Simó, 2001). In dense monospecific microalgal blooms under some degree of physiological stress due to nutrient scarcity or high sunlight exposure, DMS leakage from the algal cell is suggested to occur as part of an overflow of excess of energy and sulphur (Stefels *et al.*, 2007) and/or as part of a protection mechanism against oxidative stress (Sunda *et al.*, 2002). *P. sinerae* has evolved a sensory response to this by-product and, because of its chemotactic characteristics for protists (Seymour *et al.*, 2010), it is conceivable that the zoospores further use DMS gradients for an oriented swimming towards the potential host. However, the occurrence of an eventual infection depends on host resistance mechanisms that are still unknown.

Alexandrium species are known to produce allelochemicals with deleterious (lytic) effects on autotrophic and heterotrophic protists (Tillman *et al.*, 2008) as a mechanism to overcome competition and grazing. Rather, we show that DMS behaves as a 'kairomone', i.e., a chemical signal released by the dinoflagellate, which mediates an interspecific interaction that benefits the receiving organism (the parasitoid) without benefiting the producer (Pohnert *et al.*, 2007). In this case, the kairomone is even disadvantageous to the producer, as it induces infection and subsequent death. Therefore, its release must be unavoidable or its costs must be outweighed by the aforementioned physiological benefits. In any case, this stands as one of the scarce examples of a chemically- mediated arms race in the coevolution of plankton microbes (Smetacek, 2001; Ianora *et al.*, 2011).

Our study further expands the importance of ubiquitous dimethylated sulphur compounds (DMSP and DMS) in the chemical ecology of the oceans. These compounds have been described as foraging cues for seals, turtles, penguins, procellariiform birds, fishes, some macroinvertebrates and copepods (Van Alstyne, 2008; Nevitt, 2011; Endres and Lohmann, 2012; and refs. therein), and chemotactic attractants for protists, microalgae and bacteria (Seymour *et al.*, 2010, and refs. therein), in what possibly stands as a unique case amidst the infochemical landscape of the biosphere. Here we discover their involvement in planktonic host-parasitoid interactions.

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Chapter 3

"*Parvilucifera sinerae* (Alveolata, Myzozoa) is a generalist parasitoid of dinoflagellates"

Protist, 2013

Parvilucifera sinerae (Alveolata, Myzozoa) is a generalist parasitoid of dinoflagellates

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ABSTRACT

This study begins with a description of the infective process in the dinoflagellate type host *Alexandrium minutum* by a strain of the parasitoid, *Parvilucifera sinerae*, including the morphologies of the various dinoflagellate and parasitoid stages during the infection. Then, the susceptibility of 433 microalgal strains to *P. sinerae* infection was studied. The parasitoid was found to be capable of infecting several dinoflagellate species of the genera *Alexandrium*, *Coolia*, *Dinophysis*, *Fragilidium*, *Gambierdiscus*, *Gymnodinium*, *Gyrodinium*, *Heterocapsa*, *Kryptoperidinium*, *Lepidodinium*, *Ostreopsis*, *Pentapharsodinium*, *Protoceratium*, *Scrippsiella*, and *Woloszynskia*. Intra-strain variability was observed as well, such that within the same dinoflagellate species some strains were infected whereas others were not. Likewise, species of other dinoflagellate genera were not infected, such as *Akashiwo*, *Amphidinium*, *Barrufeta*, *Bysmatrum*, *Karenia*, *Karlodinium*, *Prorocentrum*, and *Takayama*. Moreover, *P. sinerae* was not able to infect any of the tested haptophyte, diatom, and chlorophyte species. In natural samples screened for *P. sinerae* infectivity, several dinoflagellates species of the genera *Alexandrium*, *Coolia*, *Gonyaulax*, *Gymnodinium*, *Phalacroma*, *Protoperidinium*, and *Scrippsiella* were identified as susceptible. Sporangia size was found to be proportional to the size of the host, and variations in the sporangia size were observed to influence the maturation time of it.

INTRODUCTION

Interactions among parasites and planktonic protists constitute a complex food web of particular relevance given the high proportion of parasites in the biota (Lefèvre et al. 2008) and the wide variety of organisms, including prokaryotes and eukaryotes susceptible to infection (see the review of Park et al. (2004)). Indeed, for protists of marine planktonic ecosystems, the important role played by parasitism has long been underestimated, despite the enormous range of parasite life cycles and life styles.

Parasites differ in their relative specificity; some are specialists, with a limited host range, while others are generalists, able to parasitize a wide variety of hosts (Agosta et al. 2010). The specificity of infection reflects the mutual evolution of parasites and host(s), with most parasites being specialists. Evolutionary models and empirical studies predict a high selectivity of parasites for their hosts, including numerous cases in which the relationship between the two is unique. However, this relationship is not necessarily fixed since changes in parasite behaviour can occur and host

shifts are common, with specialists able to become generalists and vice versa (Agosta et al. 2010). Several studies have concluded that generalized lineages are often derived from specialists, in what is referred to as the "parasite paradox," i.e., how do highly specialized parasites shift to accept novel, multiple hosts? Host-parasite interactions and the processes that drive them must be understood in order to address ecological issues, such as host-parasite evolution, and the dynamics of biological introductions and invasions.

What is currently known about specialist or generalist parasites of planktonic protists? Among marine parasites and parasitoids, chytrids and their infections of planktonic algae are an example of a highly host-specific parasite interaction (Kagami et al. 2007). Among Syndiniales, *Amoebophrya* spp. is representative of strains that vary from extremely species-specific to rather unspecific. The infection of a unique host strain by a single, genetically distinct parasite in situ has been described by (Chambouvet et al. 2008) while as in *Amoebophrya* some strains have a broader host range in vitro (Kim 2006). For members of the genus *Parvilucifera*, the infection of several dinoflagellate strains under laboratory conditions has been shown (Figueroa et al. 2008b; Norén et al. 1999) but, their host specificity has yet to be characterized. Nonetheless, the ecology of the parasites of planktonic protists and investigations into specialist versus generalist parasites are of significant economic and social interest, since eukaryotic parasites have long been considered as potential agents for controlling the noxious and/or toxic episodes regularly caused by dinoflagellate blooms in marine environments.

In the case of *Parvilucifera*, early efforts were successful in describing the stages of infection (Garcés and Hoppenrath 2010) but other aspects, including the parasite's behaviour in its interactions with susceptible hosts and the stages of its life cycle, have yet to be elucidated. Additionally, parasites are likely to play a prominent role in shaping the structure of microalgal communities, by modifying host population. For example, evidence has been presented for the influence of *Parvilucifera sinerae* on genotype pools within the dinoflagellate blooming population. The parasitoid - host interaction results in different degrees of infection tolerance in host strains (Figueroa et al. 2008, Llavería et al. 2010) and/or promotes the generation of new host genotypes by favouring genetic recombination (Figueroa et al. 2010). However, the actual relevance of the modulation of population structure and microalgal succession will also depend on the range of host species infected and the within-species genotype infection rate of the parasite. While neither has been extensively studied so far, previous work suggests that within a given infectible species distantly related strains may exhibit higher levels of parasite resistance (Figueroa et al. 2008, Llavería et al. 2010), perhaps indicative, according to the authors, of local adaption by the parasite.

In this study, we describe the infective process in the dinoflagellate type host *Alexandrium minutum* by a strain of the parasitoid *Parvilucifera sinerae*. The parasitoid was isolated during a bloom of *A. minutum*, and is routinely maintained in culture with this species, which is thought to be the primary host. Details of the active infection process, in particular the morphologies of the various dinoflagellate and parasitoid stages, are presented. In addition, the host specificity of the parasitoid *P. sinerae* under culture conditions is examined, based on the results of a comprehensive survey of 433 microalgal strains, including an assessment of the infection process and the morphology of the stages within each non-type host. We also focus on biological characteristics, such as body size of the parasitoid, in the sporangium stage, the number of zoospores produced in the sporangium, and the time needed for sporangium maturation. Finally, we characterized parasitoid infection of microalgal species in field samples.

MATERIAL AND METHODS

Laboratory cultures of host and parasitoid, culture maintenance, growth and infection

Experiments were conducted with strains of several microalgal taxa obtained from the culture collection of the Centro Oceanográfico (CCVIEO) in Vigo, Spain, and the culture collection of the Institut Ciències del Mar, Barcelona, Spain (Table S1 in Supplementary Material). Cultures were maintained in 50-mL polystyrene tissue culture flasks filled with 20 mL of L1 medium (Guillard, 1995) without silica. The medium was prepared with filtered (0.2-µm pore size), autoclaved seawater, adjusting the salinity to 31 by the addition of sterile MilliQ water. Cultures were grown at 20±1 ºC with a photoperiod of 12:12 h (light:dark) cycle. Illumination was provided by fluorescent tubes with a photon irradiance of about 90 µmol photons m^{-2} s^{1}.

Parvilucifera sinerae culture (strain ICMB 852) was established from an almost monospecific bloom of *Alexandrium minutum* that took place in Vilanova Harbor (Mediterranean Sea, Spain) in March 2009, as explained in Garcés and Hoppenrath (2010). Briefly, mature sporangium was individually isolated, washed in several drops of filtered seawater, and used to infect a clonal culture of *A. minutum* strain P4. The established parasitoid culture was propagated by transferring a 1-ml aliquot of mature sporangium every 6–7 days into an uninfected host stock culture of exponentially growing *A. minutum* strain P4 in sterile polystyrene Petri dishes (Iwaki, Japan, 16-mm diameter). The cultures were incubated at 20±1°C with an irradiance of about 90 µmol photons m^{2} s^{1} in a L:D cycle of 12:12 h.

Infection dynamics on *Alexandrium minutum*

The infection of *A. minutum* strain P4 by *P. sinerae* strain ICMB 852 was followed twice daily (at hours 2 and 8 of the light cycle) over 4 days under the same culture conditions as described above. To identify the infection stages (from early stages to mature sporangium), the samples were fixed in formaldehyde (1% final concentration), and the cells counted and photographed using a Leica–Leitz DMIRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) and the ProgRes CapturePro image analysis software (JENOPTIK Laser, Optik, Systeme GmbH).

Screening microalgal strains for parasitoid infection

The susceptibility of 433 microalgae strains to parasitoid infection was tested *(*S1, supplementary data). Strains belong to the division of Dinophyta (78 species), Chlorophyta (1 species), Haptophyta (11 species), Raphidophyta (6 species), and Diatoms (5 species). In the division of Dinophyta, five orders were tested (Dinophysiales, Gonyaulacales, Gymnodiniales, Peridiniales, Prorocentrales) for a total of 38 genera. The strains were originally obtained from 116 different locations worldwide (Argentina, Australia, Belize, Brazil, Chile, China, Croatia, Denmark, Finland, France, Germany, Greece, Indonesia, Italy, Malaysia, Mauritius, Mexico, Netherlands, Portugal, Reunion, Spain, Tunis, UK and USA). Among all the strains tested, those belonging to Dinophyta predominated (n=407) with a large number of strains from the Mediterranean Sea (n= 187) and the remainder from other locations (n=220).

Recently formed mature sporangia of *P. sinerae* (day 6 post-infection of the host at 20ºC) were added to exponentially growing cells of each microalgal strain. The infections were carried out in

sterile polystyrene Petri dishes (Iwaki, Japan, 22mm diameter) at a zoospore:host ratio of 10:1 in a total volume of 3 mL. Host-infected cultures were examined daily under an inverted light microscope, initially to monitor infection of the cells and later to follow the development of the infection process. The detection of the various stages of infection until the formation of a mature sporangium unequivocally confirmed strain infection. In case of a negative result, a second round of *P. sinerae* inoculation was conducted by adding mature sporangia to the same Petri dish. In several susceptible strains, stages of the infection were followed daily to quantify the maximum occurrence of each stage. Time differences between two successive maxima was took as an estimation of time required for the mode moving from stage $_{n}$ to stage $_{n+1}$.

Host cell size and sporangium size in microalgal strains

The widths of healthy cells of 28 strains and their mature sporangia (n=30 cells for each strain) were measured using a Leica–Leitz DMIRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with ProgRes CapturePro image analysis software (JENOPTIK Laser, Optik, Systeme GmbH). Sporangial biovolume was estimated assuming a spherical form. When the mature sporangia of the infected microalgal strains had reached the point just prior to zoospores release, the number of zoospores that had formed were counted by video camera (SONY NEX-3 recording) at low velocity (0.3×). The entire process was accordingly followed in three sporangia per species, beginning with the initial movements of the zoospores until their complete release from the sporangium body. In this experiment, the following species were examined: *Heterocapsa niei*, *Scrippsiella* sp., *Protoceratium reticulatum*, *Coolia monotis*, *Alexandrium ostenfeldii*, and *Gambierdiscus excentricus*.

Parasitoid infections on natural microalgal communities

Live natural samples obtained from different localities along the Catalan coast, NW Mediterranean Sea (Vilanova, Cambrils, Estartit, Arenys, Blanes), during different seasons, were tested for infectivity by *Parvilucifera sinerae* strain ICMB 852. Five mL of the concentrated natural samples were transferred to sterile polystyrene Petri dishes (Iwaki, Japan, 32-mm diameter) and then inoculated with 1 ml of cultured sporangia of *P. sinerae* strain ICMB 852 at zoospore:host of 30:1. Live cells were observed daily for 7 days using a Leica–Leitz DMIRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) days. The detection of the various stages of infection confirmed cell infection.

RESULTS

Observations of the infection of *Alexandrium minutum*

Live examination by inverted light microscopy of *Alexandrium minutum* cells allowed identification of the various stages of infection (Fig. 1) and the comparison of the infected cells to their healthy counterparts (Fig. 1A, B). Early stages of infection were recognized based on the presence of the parasitoid's round body, containing vacuole-like structures (stage 1), that grew until it occupied most of the host cytoplasm (Fig. 1C-F). Occasionally, the development of two sporangia, indicative of a double infection, was observed (Fig. 1E). Late-stage infection was confirmed based on the detachment of the round body from the theca of the cell (in thecated hosts), at which point the parasitoid had a pronounced spherical shape (stage 2, Fig. 1G). This transparent immature sporangium con-

tained several peripheral lipid-vacuole-like structures (Fig. 1 G, H) and gradually filled with flagellated cells (zoospores), beginning at peripheral sites and progressing inwards (stage 3, Fig. 1I). During this late stage of infection, the mature sporangium became very dark, and full of zoospores (Fig. 1J-L). Zoospore release by this mature sporangium initiated a second round of infection process, leaving behind the empty sporangium (Fig. 1M). In *A. minutum* under a parasitoid: host ratio of 2:1 and the above-described culture conditions, the entire infection cycle lasted 4 days. Stage 1 was observed 2 days after the addition of parasitoid to a healthy culture, followed over the remaining 2 days by stages 2, and 3 such that by day 4 mature sporangia had formed. The subsequent emptying of the mature sporangia released zoospores and thus initiated another round of infection.

By identifying and defining the various stages of infection in *A. minutum* we were able to follow the evolution of *P. sinerae* infection in the microalgal strains tested. The morphology of the mature sporangium was similar in all species examined except with respect to size, as described below.

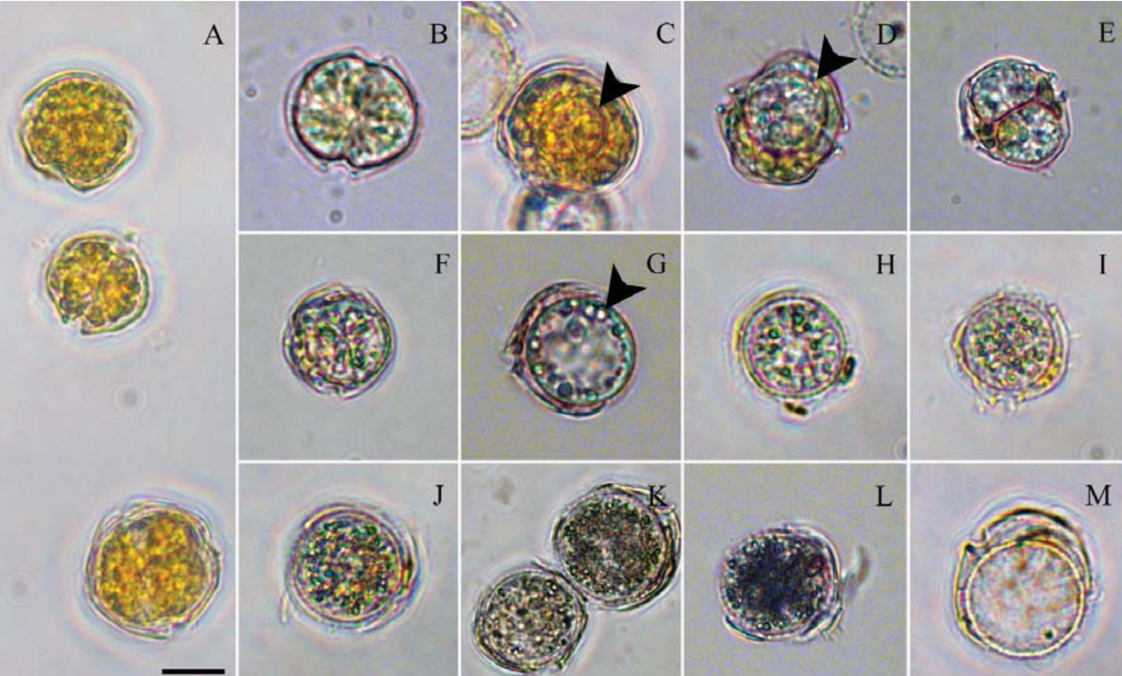

Figure 1. Infection of *Alexandrium minutum* strain P4 with *Parvilucifera sinerae*. Vegetative healthy cells (A and B). (C and D) Stage 1 of the infection is identified based on the presence of a round body in the cytoplasm, arrowheads point to the round body. (E) The development of two infections due to double infection, and (F) a round body occupying the complete cytoplasm. In stage 2 of the infection, (G) the round body is completely formed and contains peripheral like lipid-drops and it is usually surrounded by the broken theca of *A. minutum*, arrowhead point to a lipid-drop. (H) The round body starts a process of ornamentation and the peripheral lipid drops advances to the center, still partly surrounded by the broken theca. Late stage 2 and early stage 3 are marked by (I) growth of the zoospore stage inside of the sporangium, (J) the immature sporangium, and growth of the zoospore stage. In stage 3, (K) the immature sporangium is occupied by several zoospores, leading to the formation of the mature sporangium, (L) which is recognized by its dark color. (M) Release of the zoospores leaves behind an empty sporangium, surrounded by the broken *A. minutum* theca. Scale bar = 10 µm.

Table 1. Strains of the genus *Alexandrium* infected by *Parvilucifera sinerae*. yes/no indicate infection. In the case of an infected strain, if > 3 strains were tested, the percentage of infected strains is shown; n = number of tested strains. The *A. tamarense* complex formed a monophyletic clade subdivided into five groups, Groups I, II, III, IV, and V following Scholin et al. 1994, John et al. 2003 and Lilly et al. 2005.

	Genus	Species	Infected	Infected strains (%)	n
Gonyaulacales	*Alexandrium*	*affine*	yes		2
		andersoni	yes	40	5
		tamarense complex (Group I)	yes	100	7
		tamarense complex (Group II)	yes	100	12
		tamarense complex (Group III)	yes	85	13
		tamarense complex (Group IV)	yes	100	56
		margalefi	yes	100	4
		minutum	yes	94	86
		ostenfeldii	yes	100	6
		peruvianum	yes		1
		tamarense	yes	92	13
		tamutum	yes	83	6
		taylori	no		1

Screening microalgal strains for parasitoid infection

Among the dinoflagellate species known to be infected by *P. sinerae*, the following were included in this study: *Alexandrium, Coolia, Dinophysis, Fragilidium, Gambierdiscus, Gymnodinium, Gyrodinium, Heterocapsa, Kryptoperidinium, Lepidodinium, Ostreopsis, Pentapharsodinium, Protoceratium, Scrippsiella* and *Woloszynskia* (Tables 1 and 2, Figs. 2 and 3). The dinoflagellate genera that, at least under the conditions of this study, were not infected (mature sporangia not detected) were: *Akashiwo, Amphidinium, Barrufeta, Bysmatrum, Karenia, Karlodinium, Prorocentrum*, and *Takayama* (Tables 1, 2). In addition, *P. sinerae* did not infect haptophyte species, chlorophytes, and diatoms (Table 3).

Figure 2A shows stages 1 and 2 of a parasitic infection of *Heterocapsa niei* (completely and incompletely round bodies with lipid drops), contrasting the appearance of infected cells with healthy one. Fig. 2B-E, 2G and 2H shows stage 1 of the infection, identified by the presence of a round body in the cytoplasm in *Gymnodinium catenatum, Alexandrium* cf. *catenella, Gymnodinium litoralis, Gymnodinium nolleri, A. margalefi*, and *A. kutnerae*. Mature zoospores-filled sporangia of in *Pentapharsodinium thyrrenicum* and, for comparison, healthy cells of the same species are shown in Fig. 2F.

The infection process in *Fragilidinium subglobosum* is shown in Fig. 2I-K. The early stage of infection (stage 1, Fig. 2I; stage 2, Fig. 2J) as well as the appearance of mature sporangia within the host cells (Fig. 2K) proceeded as described above for *A. minutum*. Stage 1 and mature sporangia in *A. ostenfeldii* (Fig 2L), stage 1 in *Scrippsiella trochoidea* (Fig 2M), and stage 1 and mature sporangia in *A. peruvianum* (Fig 2N, O) also resembled the respective stages in *A. minutum*.

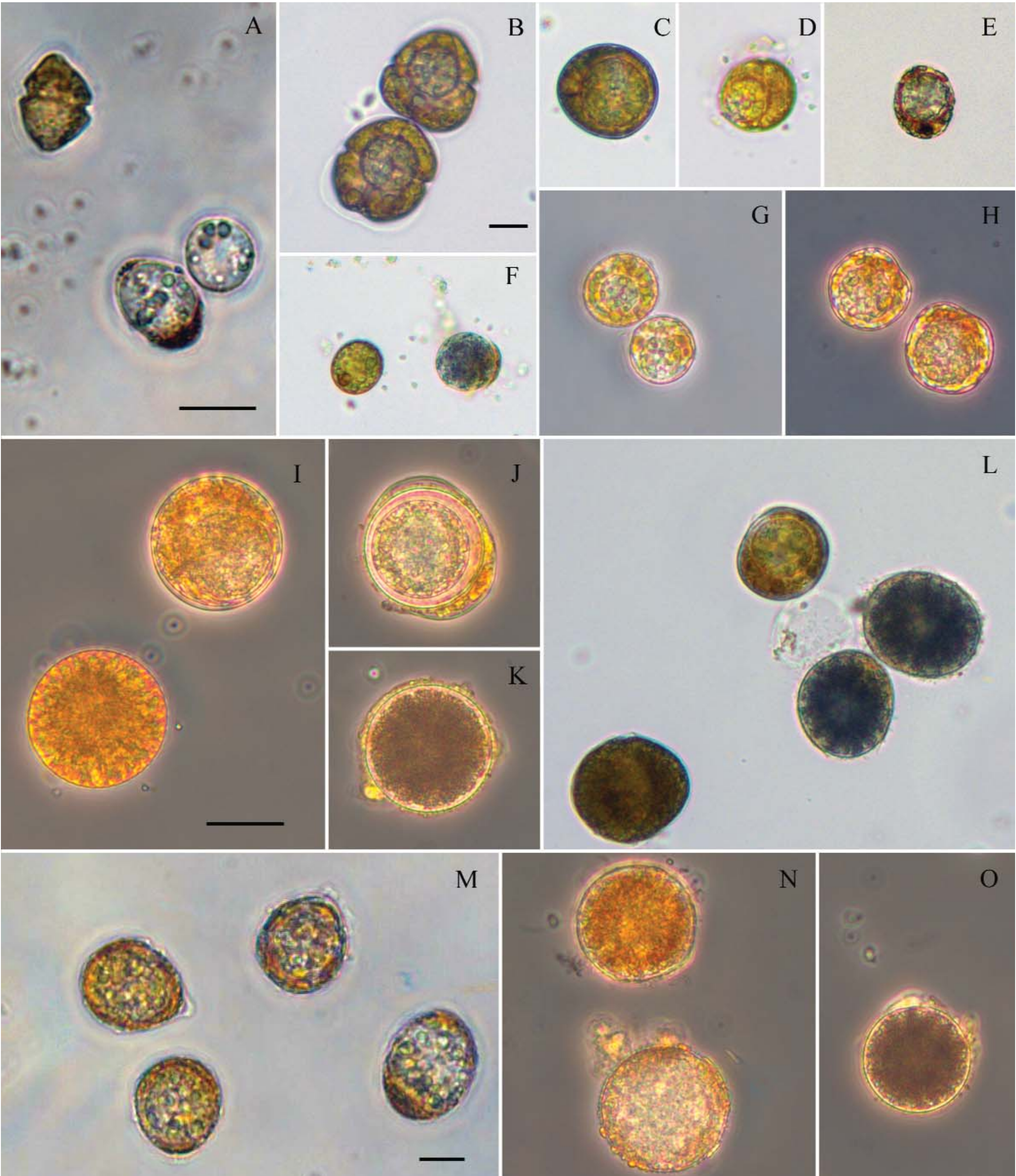

Figure 2. Phytoplankton strains infected with the parasite *Parvilucifera sinerae* under laboratory conditions: (A) *Heterocapsa niei,* (B) *Gymnodinium catenatum,* (C) *Alexandrium catenella,* (D) *Gymnodinium litoralis,* (E) *Gymnodinium nolleri,* (F) *Pentapharsodinium thyrrenicum*, (G) *Alexandrium margalefi*, (H) *Alexandrium kutnerae*, (I-K) *Fragilidinium subglobosum* (I-K). (L) *Alexandrium ostenfeldii*, (M) *Scrippsiella trochoidea,* (N and O) *Alexandrium peruvianum.* Figs A-H, M: Scale bar = 10 µm; Figs. I-L, N, O: scale bar = 20 µm.

Table 2. Strains of the dinoflagellate genera infected by *Parvilucifera sinerae*. yes/no indicate infection. In the case of an infected strain, if > 3 strains were tested, the percentage of infected strains is shown; n =number of tested strains.

	Genus	Species	Infected	Infected strains (%)	n
Dinophisiales	*Dinophysis*	*acuminata*	yes		1
		acuta	yes		1
		caudata	yes		1
		tripos	yes		1
Gonyaulacales	*Coolia*	*canariensis*	yes	33	3
		malayensis	no		1
		monotis	yes	50	14
		sp.	yes		1
		tropicalis	no		1
	Fragilidium	cf. *dupolocampanaeforme*	yes	33	3
		subglobosum	yes		1
	Gambierdiscus	cf. *pacificus*	no		2
		australes	yes	67	3
		excentricus	yes	25	4
		sp. 1	no		1
		sp. 2	yes		1
		sp. 3 Ribotype1	yes		1
	Ostreopsis	cf. *ovata*	yes	74	35
		cf. *siamensis*	yes	100	3
		sp. 1	yes	86	7
		sp. 2	yes	50	2
		sp. 3	yes		1
		sp. 4	yes		1
	Protoceratium	*reticulatum*	yes	90	10
Gymnodiniales	*Akashiwo*	*sanguinea*	no		2
	Amphidinium	*carterae*	no		4
		sp.	no		1
	Barrufeta	*bravensis*	no		2
	Gymnodinium	*catenatum*	yes	80	5
		cf. *simplex*	yes		2
		impudicum	no		7
		instriatum	no		1
		litoralis	yes		1
		microreticulatum	no		1
		nolleri	yes		1
	Gyrodinium	*dominans*	no		1
		sp.	yes		1
	Karenia	*brevis*	no		1
		selliformis	no		1
	Karlodinium	*armiger*	no		1
		veneficum	no		9
	Lepidodinium	*chlorophorum*	yes	100	3
	Takayama	sp.	no		1

Table 2 (cont.)

	Genus	Species	Infected	Infected strains (%)	n
Peridiniales	*Bysmatrum*	sp.	no		1
	Heterocapsa	*niei*	yes	100	2
		triquetra	no		2
	Kryptoperidinium	*foliaceum*	yes	50	2
	Pentapharsodinium	*thyrrenicum*	yes		1
	Scrippsiella	*trochoidea*	yes		1
		sp.	yes	75	4
	Woloszynskia	*cincta*	yes		1
Prorocentrales	*Prorocentrum*	*arenarium*	no		1
		balticum	no		1
		belizeanum	no		1
		cassubicum	no		3
		cf. *belizeanum*	no		4
		levis	no		1
		lima	no		32
		micans	no		1
		minimum	no		3
		rathymum	no		1
		rostratum	no		1
		sp.	no		2
		triestinum	no		5

Mature sporangia in the species *Dinophysis acuminata* (Fig. 3A), *D. acuta* (Fig. 3B), *D. caudata* (Fig. 3C) and *D. tripos* (Fig. 3D) were observed after *Parvilucifera* inoculation. In most cases, the newly formed sporangia completely filled the central part of the cell and had apparently digested nearly all of the host cytoplasm. Although a single sporangium per cell in the genus *Dinophysis* was generally the rule, two and three sporangia, all of them capable of producing produced effective zoospores, were also occasionally observed (Fig. 3E, F). Empty sporangia in these different species are shown in Fig. 3E, F, and H. Zoospores release from the sporangium occurred rapidly, within minutes (Fig. 3G). Frequently, the sporangial diameter exceeded the thickness of the host cell, thus forcing open the two valves of the *Dinophysis* theca.

Benthic dinoflagellate species infected with *P. sinerae* are shown in Fig. 4. Figure 4A-C shows stage 1, stage 2, and the mature sporangia in *Coolia canariensis*, respectively, with a healthy cell included for comparison. The infection of *Gambierdiscus toxicus* is shown in Fig. 4D-I, including a single, double, and triple infection (Fig. 4D-F, respectively). Mature sporangia with a part of *Gambierdiscus* theca are shown in Fig. 4G. Following release of the flagellated zoospores, residual lipid-like droplets and occasional dead zoospores were seen inside the sporangium (Fig. 4H). Sporangia with diameter exceeding the thickness of the host, thus forcing open of the two valves of the *Gambierdiscus* theca are shown in Fig. 4I. As in the genus *Dinophysis*, cells of *Gambierdiscus* also occasionally contained two or three simultaneous infections per host. Double infections were seen in 26.3 ± 1.9 % and triple infections in 6.0 ± 1.1 %, in both cases higher than the percentages determined in *Alexandrium* in which the majority (99.7 ± 1.9 %) of the infections involved a single sporangium per cell.

The *Ostreopsis* cf. *ovata* infection process and a comparison to healthy cells is shown in Fig. 4J-L. In this species, stage 3 and the mature sporangia (Fig. 4J) as well as empty sporangia (Fig. 4K, L) were essentially the same as described above for the other species.

In general terms, the thecate dinoflagellate orders Dinophysiales, Gonyaulacales, and Peridiniales were found to susceptible to infection (1 genus, 6 genera, 5 genera, respectively) whereas no infection occurred in strains of the order Prorocentrales. Among athecate species (Gymnodiniales),

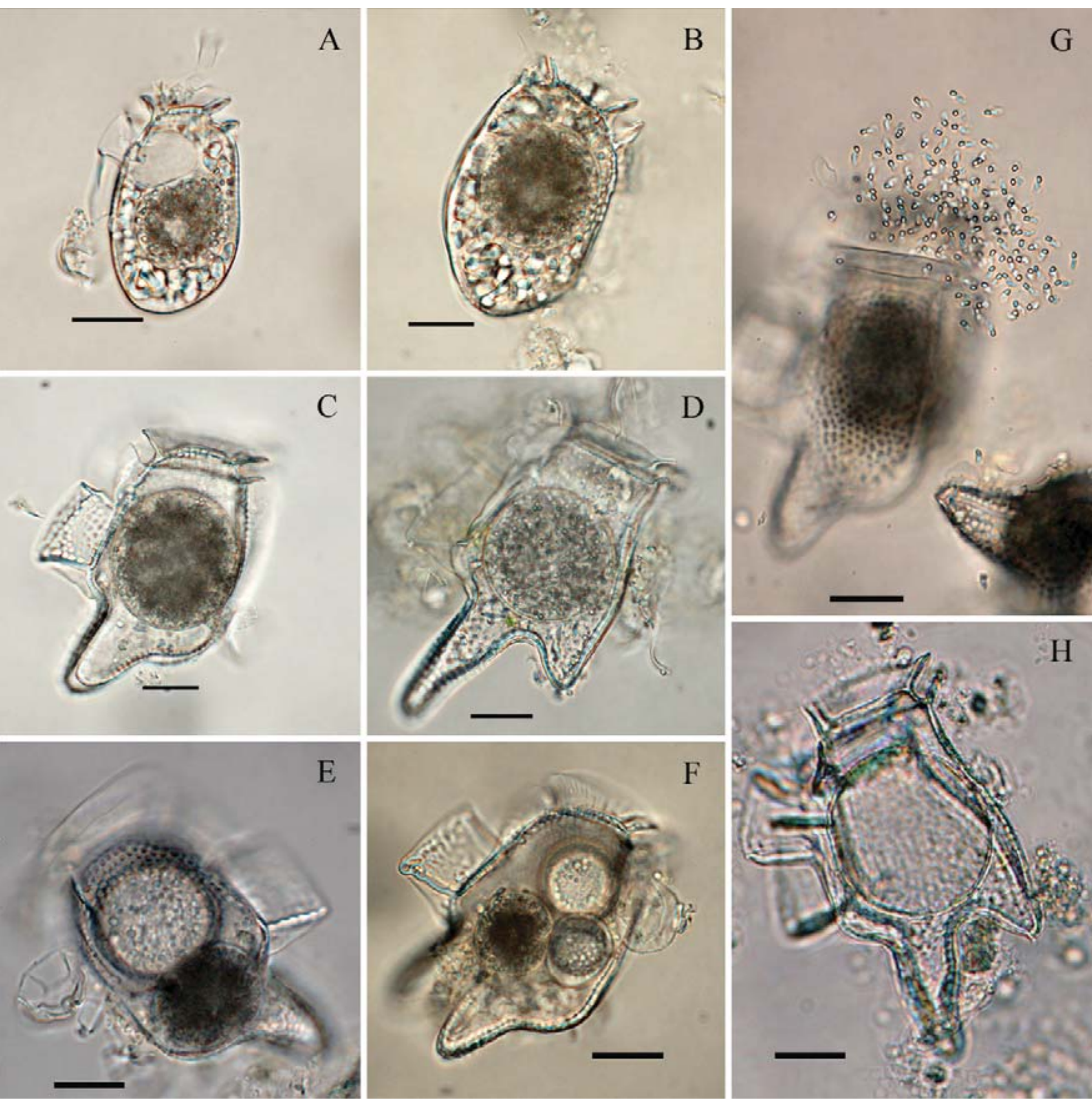

Figure 3. Phytoplankton strains infected with the parasite *Parvilucifera sinerae.* Mature sporangia in (A) *Dinophysis acuminata,* (B) *D. acuta,* (C) *D. caudata,* (D) *D. tripos.* (E) double and (F) triple infection in *D. caudata.* (G) Zoospores release in *D. caudata* infection. (H) Empty sporangium in *D. tripos.* All scale bars = 10 µm.

infectivity was low (3 genera) and none of the members of the family Kareniaceae was infected. Intra-species variability was sometimes observed in strains of *Alexandrium minutum, A. andersoni, A. tamarense, A. tamutum, Coolia canariensis, C. monotis, Gambierdiscus australes, G. excentricus, Gymnodinium catenatum*, *Ostreopsis ovata, Ostreopsis* sp. 1 and *Ostreopsis* sp. 2, *Protoceratium reticulatum, Fragilidium* cf. *dupolocampanaeforme, Scrippsiella* sp., and *Kryptoperidinium foliaceum* (Tables 1, 2).

Relation between host size, sporangium size, sporangium maturation, number of zoospores per sporangium and zoospore release process

The body size of the mature sporangium stage was positively and significantly related to host size (Fig. 5) (mature sporangium diameter = 0.7341 host size + 7.7664, $R^2 = 0.8623$, $p<0.001$). The largest host, *G. excentricus* (81.2 μm diameter), contained the largest sporangium (67.3 μm). Excluding cells > 30 μm in size and non-spherical cells, such as those of the *Dinophysis* and *Gambierdiscus* genera, an 1:1 relationship between host body size and the size of the mature

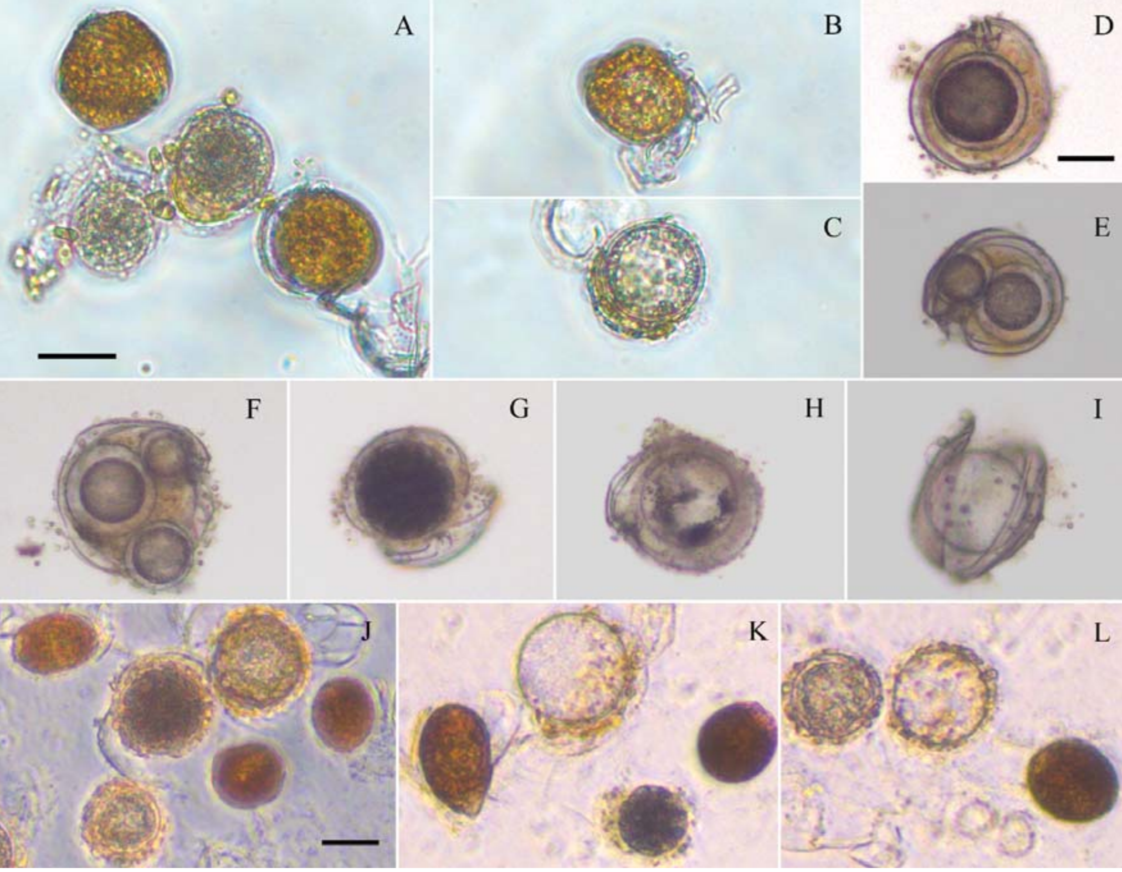

Figure 4. Phytoplankton strains infected with the parasite *Parvilucifera sinerae.* (A-C) *Coolia canariensis,* (D-I) *Gambierdiscus toxicus*, (J-L) *Ostreopsis ovata.* All scale bars = 20 μm.

Table 3. Strains, belonging to different planktonic orders that were not infected by *Parvilucifera sinerae*. n =number of tested strains.

Phylum	Genus	Species	n
Chlorophyta	*Pyramimonas*	sp.	1
Diatoms	*Coscinodiscus*	cf. *radiatus*	1
	Phaeodactylum	*tricornutum*	2
	Pseudo-nitzschia	*pungens*	1
	Skeletonema	*costatum*	1
	Thalassiosira	*weissflogii*	1
Haptophyta	*Chattonella*	*antiqua*	1
		verruculosa	1
		subsalsa	1
	Emiliania	*huxleyi*	1
	Fibrocapsa	*japonica*	1
	Heterosigma	*akashiwo*	3
	Olisthodiscus	*luteus*	1
	Pavlova	*girans*	1
	Phaeocystis	*globosa*	1
	Prymnesium	*faveolatum*	1
	Prymnesium	sp.	1

sporangium was determined (mature sporangium diameter = 0.9322 host size + 3.4964, R^2 = 0.8858, $p<0.001$, data not shown).

The time needed for sporangial maturation (from stage 1 to stage 3) ranged from 5 days (*Heterocapsa*, *Alexandrium, Scrippsiella*) to 13 days (*Gambierdiscus*) and was related to host body size (Fig. 6). Time differences between successive maxima of stages 1, 2, and 3 differed between species. The time for stage 1 was significantly longer in *G. excentricus* than in the other genera examined. In this early stage of infection, recognized by the presence of the parasitoid's round body containing vacuole-like structures, the parasitoid grows until its body occupies most of the host cytoplasm, at which point the host cytoplasm is consequently destroyed. Among the species studied, time differences in stage 2 was longer in *Coolia monotis*, *Gymnodinium catenatum*, and *G. excentricus,* and stage 3 in *G. excentricus* and *Fragilidium subglobosum*.

The number of zoospores per sporangium was positively and significantly related to sporangium size (number of zoospores per sporangium = 0.0443 sporangium biovolume in μm^3 + 207.02, R^2 = 0.996, $p<0.001$, data not shown). The number of zoospores liberated per sporangium ranged from 170 (± 8) in *A. minutum* and *Heterocapsa niei* to > 6000 (± 582) in *G. excentricus.* The duration of zoospore release varied according to the host species. More time was needed in sporangia of greater size, such as those seen in *A. ostenfeldii* (357 ± 30 s) or *G. excentricus* (460 ± 104 s), than for smaller sporangia, such as those in *Heterocapsa niei* (130 ± 45 s). In addition, the number of zoospores was positively related to the time of zoospore release from the sporangium, (time in seconds) = 91.306 ln (number of zoospores) - 369.52, R^2 = 0.8325, $p<0.001$). In larger sporangium such as those that formed in *Gambierdiscus excentricus*, while the majority of zoospores were released, a few aggregates of dead zoospores remained inside the sporangium at the end of the process (Fig. 4H).

Host susceptibility in field samples

Natural samples from different localities were tested for *P. sinerae* infectivity. Species successfully infected in the lab were also infected when inoculated with the parasitoid, including: *Alexandrium minutum* (Fig. 7A, B), *A.* cf. *catenella, Coolia monotis,* and *Gymnodinium litoralis* (Fig. 7H) (Table 4).

The parasitoid strain was also able to infect dinoflagellate species in natural populations that were

not tested in the lab due to the lack of the cultured representatives such as: *Gonyaulax spinifera, G. polygramma, Scrippsiella* sp. (Fig. 7C), *Protoperidinium* sp. (2 species, Fig. 7D, E), *Phalacroma* sp. (Fig. 7F), *Gymnodinium* sp. (Fig. 7G), and, *Phalacroma oxytoxoides* (Fig. 7I) (Table 4). Other dinoflagellates genera, i.e., *Akashiwo, Ceratium* (4 species), *Dinophysis* (3 species), *Ornithocercus, Ostreopsis, Polykrikos, Prorocentrum* (4 species), and *Protoperidinium*, were not infected nor were nine species belonging to the Diatoms group (Table 4). Coincident non-infected species in both studies were *Akashiwo sanguinea, Prorocentrum micans, P. triestinum, P. rathymum,* and *P. lima*.

DISCUSSION

Parasites cause varying amounts of harm to their host species while at the same time their performance greatly depends on them. Moreover, since generalist parasites are supported by multiple hosts differing in their susceptibility to infection, they alter the competitive interactions

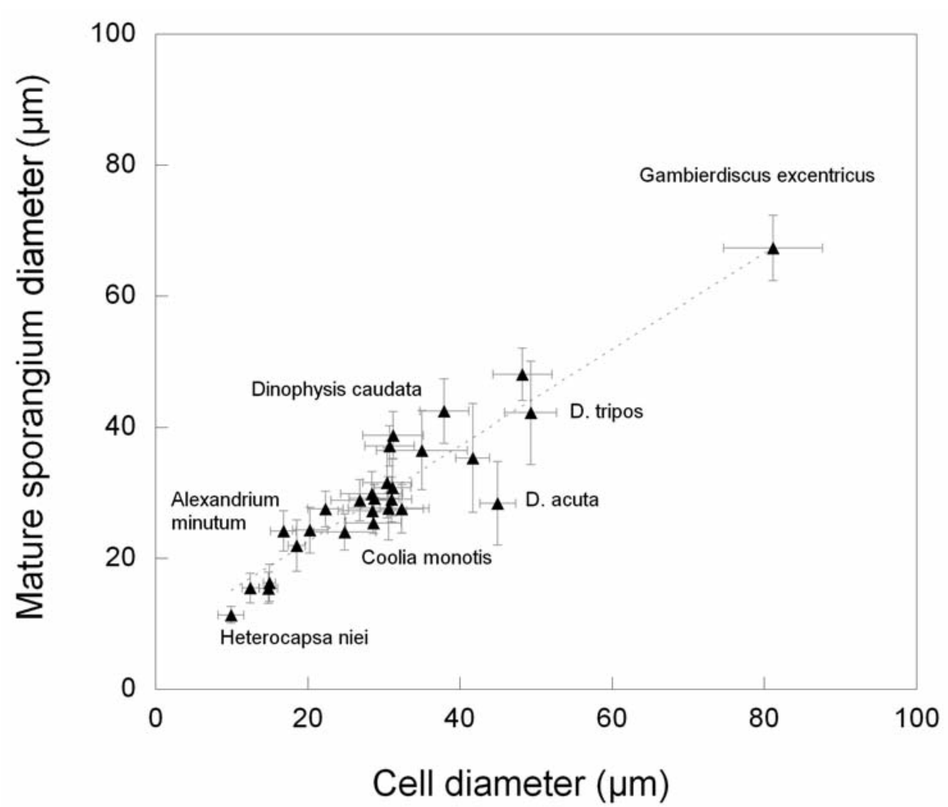

Figure 5. The sizes of the mature sporangia in different species in relation to the sizes of the host cells. The analyzed species were: *Alexandrium catenella* , *A. minutum*, *A. kutnerae*, *A. margalefi*, *A. peruvianum*, *A. ostenfeldii*, *A. tamutum*, *Coolia monotis*, *Dinophysis acuminata*, *D. acuta*, *D. caudata*, *D. tripos*, *Fragilidinium subglobosum*, *Gambierdiscus excentricus*, *Gymnodinium catenatum*, *G. nolleri*, *G. simplex*, *Heterocapsa niei*, *L. chlorophorum*, *Protoceratium reticulatum*, and *Scrippsiella trochoidea*. Two or more infections occurred in the larger species but the size of the sporangia in these multiple infections was not taken into account in the determinations. Values are means ± SD.

among their hosts. For these reasons, parasites likely play an important role in determining the structure of microalgal communities, directly as well as indirectly. The *P. sinerae* strain used in the present study is a generalist parasitoid of dinoflagellates and it is unable to infect haptophytes, diatoms, and chlorophytes species. The culture and field experiments described herein showed that: i) under lab conditions the parasitoid infects several genera in the order of Dinophysiales, Gonyaulacales, and Peridiniales as well as athecate species (of the order Gymnodiniales); ii) some dinoflagellate genera, such as *Alexandrium, Gymnodinium, Protoceratium, Ostreopsis*, and *Scrippsiella*, are more susceptible to infection than others. These observations provide evidence of the parasitoid's ability to differentially impact populations of potential host taxa, in this case, exclusively dinoflagellates, to drive changes in community composition and, possibly, microalgal successions in natural communities.

In the screening experiment, the absence of infection was distinguished from the formation of mature sporangia (susceptible), with the term "resistant strain" purposely avoiding when the results of the infection studies were negative. This was done for several reasons, but especially because discussions of parasite resistance are complicated by interpretation such that the term "resistant" lacks clear limits. The experimental design used in the strain screening was unable to demonstrate "true resistance to the parasitoid" because the absence of infection following exposure of the host strain to the parasitoid strain does not mean that the same result would be achieved with other *P. sinerae* strains. Moreover, even if several parasitoid strains had been tested

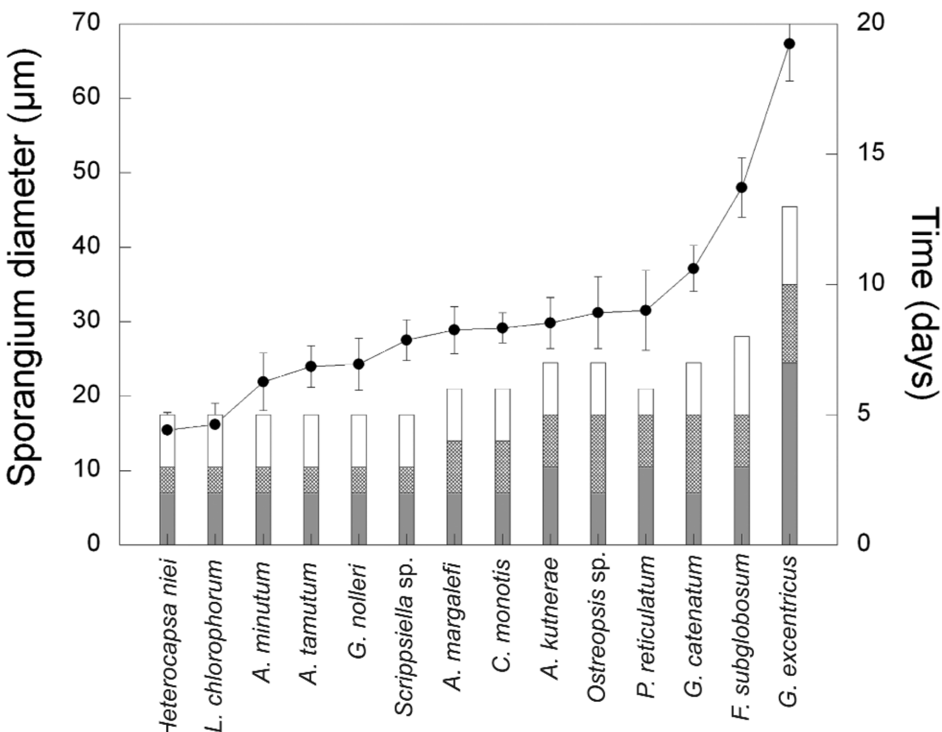

Figure 6. Sporangium body size (line) from different species and strains and the time differences between two successive maxima of stages 1, 2, and 3 (accumulative bars).

and negative results obtained in all cases, it could not be ruled out that other, untested parasite strains are able to infect the host. Importantly, it must also be noted that the lack of infection (or sporangium formation) under particular laboratory condition does not mean that infections (or mature sporangia) would not occur under other conditions, either in the laboratory or in the field. If an intra-specific variability among *P. sinerae* strains in terms of their virulence is indeed the case, as is expected, further experiments would be needed to determine virulence as well as host susceptibility and resistance. Essentially nothing is known about the genotypic diversity of *Parvilucifera* within blooms or between geographical locations, and therefore about the implications for host susceptibility to infection by parasites differing in genotype. In parallel to this biological intra-specific variability in virulence, abiotic factors that modulate infection can also be presumed. Again, however, little is known about these factors, except for the effects of temperature, light, and salinity in lab (Figueroa et al., 2008).

Host genetic variability is just as important, as intra-specific variability in the virulence of *P. sinerae,* and it can be hypothesized that, as generalist, the performance of this parasitoid among hosts increases with their increasing genetic similarity as well as the parasitoid's ability to distinguish

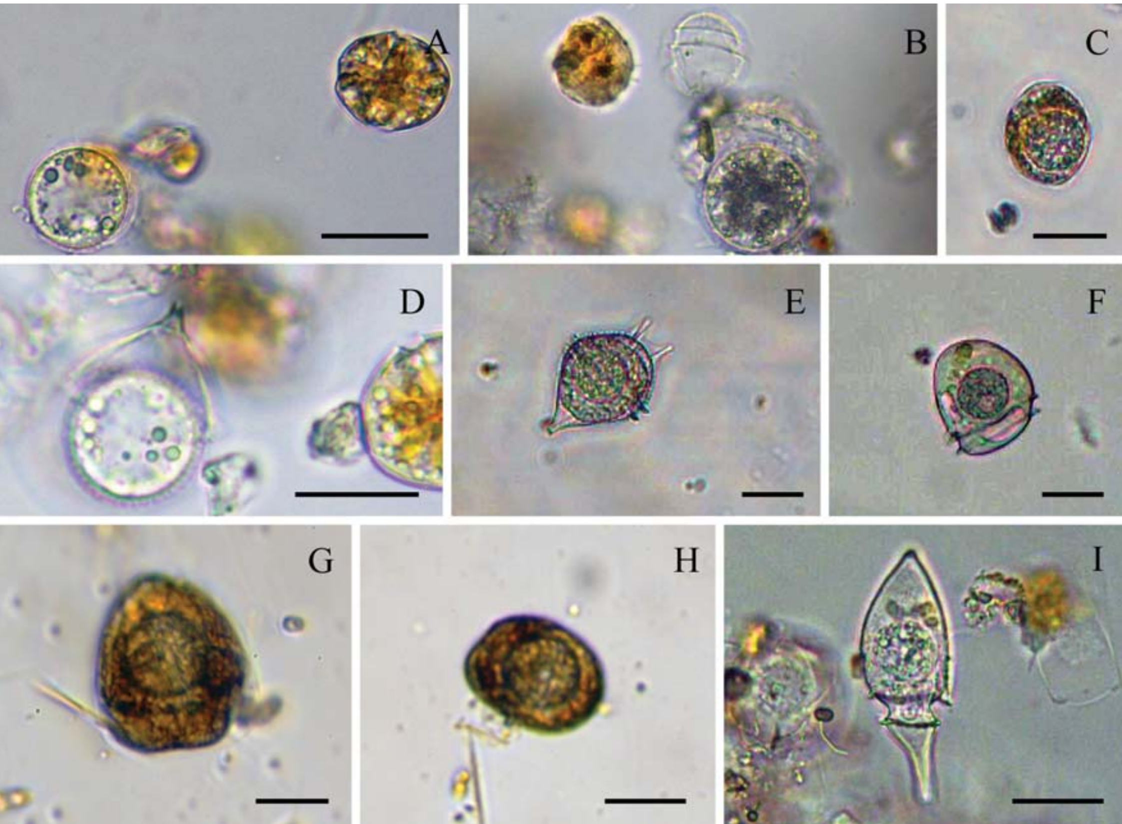

Figure 7. Infected cells from field samples with the parasite *Parvilucifera sinerae.* (A and B) *Alexandrium minutum*, (C) *Scrippsiella* sp., (D and E) *Protoperidinium* sp., (F) *Phalacroma* sp., (G) *Gymnodinium* sp., (H) *Gymnodinium litoralis*, (I) *Phalacroma oxytoxoides.* All scale bars = 20 µm.

Table 4. Phytoplankton species from natural samples obtained from different localities along the Catalan coast (NW Mediterranean Sea) tested for *Parvilucifera sinerae* infectivity. yes/no indicate infection in field and lab if strains were available. n= number of observations of infected organisms.

Phylum	Genus	Species	Infected	n	Infected in lab
Dinophyta	*Akashiwo*	*sanguinea*	no		no
	Alexandrium	*minutum*	yes	9	yes
		cf. *catenella* ME clade	yes	2	yes
	Ceratium	*pentagonum*	no		
		ranipes	no		
		furca	no		
		fusus	no		
	Coolia	*monotis*	yes	1	yes
	Dinophysis	*sacculus*	no		
		ovum	no		
		caudata	no		yes
	Gonyaulax	*spinifera*	yes	1	
		polygramma	yes	1	
	Gymnodinium	*litoralis*	yes	1	yes
		sp.	yes	1	
	Ornithocercus	sp.	no		
	Ostreopsis	cf. *ovata*	no		yes
	Oxyphysis	sp.	yes	1	
	Phalacroma	*rotundata*	yes	1	
	Polykrikos	sp.	no		
	Prorocentrum	*micans*	no		no
		triestinum	no		no
		rathymum	no		no
		lima	no		no
	Protoperidinium	*divergens*	no		
		sp.1	yes	1	
		sp.2	yes	1	
	Scrippsiella	sp.	yes	2	
Diatoms	*Chaetoceros*	cf. *simplex*	no		
	Guinardia	*striata*	no		
	Leptocylindrus	*danicus*	no		
	Lichmophora	*sp.*	no		
	Nitszchia	*longuissima*	no		
	Pseudo-nitszchia	sp.	no		
	Skeletonema	*costatum*	no		
	Thalassionema	sp.	no		
	Thalassiossira	*weisflogii*	no		

among them. In fact, the diversity of hosts and strains parasitized by a single *Parvilucifera* clonal strain is intriguing. How does *Parvilucifera* (and other generalists) exploit this wide host variety? Thus far, two hypotheses have been proposed in the parasitology literature, one centred on the host and the other on the parasite. In the former, the novel host may share important characteristics with current hosts or might have previously acted as a host (Futuyma & Mitter, 1996). In the latter, the parasite's capabilities are assumed to include the use of novel resources (Agosta & Klemens, 2008) which implies the previous existence of the parasitoid in other host since following a novel infection the parasite will be able to survive or "fit" based on traits that it already possesses. Evidence for ecological fitting among hosts and parasites, thereby supporting both theories, is abundant (Agosta et al., 2010) but confirmation is difficult because host shifts are difficult to observe in nature.

In our study, *Alexandrium* was assumed to be the primary host since the majority of field observations of the parasitoid have been made under bloom conditions of *A. minutum*. According to the screening results, it can be hypothesized that in the field *Parvilucifera* is able to complete its life cycle not only in *Alexandrium* blooms but also in blooms of other dinoflagellate species. In general terms, there are several closely related genera that are susceptible, mainly the thecated dinoflagellates, Dinophysiales, Gonyaulacales, and Peridiniales. Nonetheless, the specificity of *P. sinerae* infection for dinoflagellate strains remains unresolved

Similarities and differences between the three known species of *Parvilucifera*

Based on phylogenetic relationships among hosts, Gonyaulacales (*Gambierdiscus, Alexandrium, Coolia,* and *Ostreopsis*) are infected by *Parvilucifera sinerae* while members of Prorocentrales are not. In fact, one species of *Parvilucifera, P. prorocentri* Leander and Hoppenrath infects the marine, benthic, non-toxic dinoflagellate *Prorocentrum fukuyoi* Murray and Nagahama (Hoppenrath & Leander, 2009, Leander & Hoppenrath, 2008). *P. prorocentri* is morphologically divergent from the other two species described in the genus, *P. sinerae* and *P. infectans*, but whether it is a generalist parasitoid is currently unknown.

As in *P. sinerae*, *P. infectans* may infect a broad range of dinoflagellate genera under laboratory conditions, such as *Alexandrium fundyense*, *A. tamarense*, *A. ostenfeldii*, *Dinophysis acuta*, *D. norvegica*, *D. dens*, and *D. acuminata* (Norén et al., 1999), but it is likewise unable to infect diatoms. Similarly, *P. infectans* infect a broad range of dinoflagellate species, observed in mixed plankton samples containing *Protoperidinium, Diplopsalis*, *Scrippsiella*, *Prorocentrum*, *Heterocapsa*, and *Ceratium*. As in *P. infectans,* an active penetrating mechanism of infection characterizes *P. sinerae*. Other parasitoid life-history traits, specifically, the time needed for the development of new mature sporangia, are difficult to compare with those described in other studies involving *Parvilucifera* species due to differences in experimental equipment and design (e.g., different host: ratio).

Body size

Body size analysis both for species of microalgae and for the parasitoid (mature sporangium stage), as determined in the laboratory, revealed a close 1:1 (host: parasitoid) relationship, which agrees with parasitoid development under host influence. A smaller sporangia size was observed in small hosts, with the two increasing in parallel, as reported in other studies of parasite–host

systems (Holfeld, 2000). Ecology studies predict that the average body size of a species is an adequate approximation of the size of the individuals involved in a particular trophic interaction (Cohen, Jonsson et al., 2005, Cohen, Pimm et al., 1993). Thus, animal consumers are often considerably larger than their prey whereas parasites and pathogens are generally much smaller than their resources (Memmott, Martinez et al., 2000). In the case of *Parvilucifera*, which depends on its host for completion of its development, parasitoid sporangium and host body sizes are similar and thus well suited to each other. The observed exceptions in this study involved large cells (>30 μm), such as those of *Gambierdiscus* in which *Parvilucifera sinerae* was unable to occupy the entire cytoplasm. This finding implies an upper size limit for sporangium. The possibility of double infection, evidenced by the formation of two mature sporangia, only in large hosts, such as *Gambierdiscus* and *Dinophysis*, is of interest, since it occurred very infrequently in small cells, although this may instead reflect the probability of encounter. Also, if the parasitoid is chemically attracted to its hosts, then larger hosts, with their larger surface area and/or the ability to release larger amounts of a chemical attractant into the water, would have a greater likelihood of zoospore infection.

Variations in an organism's body size influence several of its biological characteristics. Sporangium formation is correlated with host size such that in larger hosts both sporangium formation and zoospore reproduction (number of zooids per sporangium) are delayed, probably because of the longer time needed to achieve destruction of the host cytoplasm. Factors such as these can modulate the evolution of parasite life-history traits. Thus, larger hosts provide more energy, favour higher parasitoid abundance, and may imply an increase of infection rates in natural populations. Large sporangia sizes accommodate more zoospores, in turn, and ensuring a higher infectivity. Although *Parvilucifera* seems to be a generalist parasitoid of dinoflagellates, its various host species will likely differ in their degree of fitness.

An interesting point arises from the relationship between the mass of *P. sinerae* and the size of its host. Since the host is the parasitoid's source of nutrients, *P. sinerae* must be highly dependent on its host and must efficiently use its resources. The consequences for the parasitoid of a deficient host nutrient supply, host starvation, or a host with a suboptimal metabolic rate, and therefore the impact on the population dynamics of the infecting species, are unclear. Presumably, host nutritional status determines parasitoid growth (that of individuals as well as populations), with implications for parasite fitness (e.g., zoospore number and release). Moreover, host nutritional status and environmental conditions may act synergistically. While a number of studies in freshwater microalgae have assessed the impact of host nutritional status in parasite fitness (Bruning, 1991), little is known about the further effects on marine parasites.

Field experiments

One of the most important aspects of the field experiments was the verification of species able to be parasitized among those infected in the laboratory, although the rates of parasitism were not quantified. The results of our laboratory and field experiments were congruent both generally, i.e. for the observations in the diatom group, and with respect to the susceptibility of several genera, including *Alexandrium, Coolia*, and *Scrippsiella.* However, some species were infected in culture but not infected in mixed plankton samples. This was the case for the *Dinophysis* genus, in which *Dinophysis sacculus*, *D. ovum*, and *D. caudata* were not infected in the natural population whereas *Dinophysis* cultures were consistently infected. Moreover, in the field, infections of

some dinoflagellate species were detected sporadically, mostly in *Alexandrium*, and were highly variable in other hosts. These differences in laboratory and field results can be explained by to the preferences of the parasitoid in a mixed host population. In cultures of *Parvilucifera*, if the zoospores did not infect the host they died within hours. In field samples, however, the parasitoid is presented with a mixture of species and thus is less likely to die if it does not find its preferred host as it can instead infect another. In fact, parasitoid zoospores are highly motile and assuming that can be chemically attracted to their hosts (Garcés et al. submitted) are capable of host selection.

Interestingly, the prevalence of the parasitoid in the field was quite low. Although *Parvilucifera sinerae* is an efficient parasitoid in culture experiments, this low prevalence in natural samples (Figueroa et al., 2008) suggests that endemic infections are the rule in natural populations, even in the case of a very abundant host. A similar conclusion was previously reached regarding the incidence of diatom infection by parasites (Kuhn & Hofmann, 1999). Many factors hamper successful infections in the field to explore, such as host concentration, encounter rates, predation of the parasitoids, and genotypic variability within host species, although their relative importance remains to be explored.

***Parvilucifera* as a biological agent in the control of harmful algal blooms?**

Based on the ability of *Parvilucifera* to infect several toxic dinoflagellate species (Figueroa et al., 2008, Norén et al., 1999), its use as a biological agent in the control of harmful algal blooms (HABs) has been proposed (Norén et al., 1999). However, the effective use of *Parvilucifera* in controlling HABs in natural environments will rely on detailed knowledge of the parasite's ecology, both under natural occurrences and as a biological control agent. Generalist parasites are poorly efficient in natural host population and are therefore not suitable for minimizing or preventing HABs.

In conclusion, *Parvilucifera* seems to be a generalist parasitoid of dinoflagellates and its various host species are likely to provide it with different benefits. Generalism has several advantages, including the maintenance of infections in different host reservoir and a more abundant and reliable food supply in a highly changeable niche such as the marine environment. The maintenance of infection is dependent upon the efficiency of acquisition and transmission between host and parasite, but much remains to be learned about this interaction, including the successful transmission of the marine parasite between species. A better understanding of these aspects of infectivity may help to explain the observed differences between laboratory and field infection rates, the intra-species variability in the virulence, and the ecological relevance of *Parvilucifera* in phytoplanktonic bloom successions and control.

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Table S1. Supplementary material. 1 of 10

Division	Order	Genus	Specie	Strain	Location	Infection
Dinophyta	Dinophisiales	*Dinophysis*	*acuminata*	VGO1063	Ría de Vigo, Galicia, Spain	yes
	Dinophisiales	*Dinophysis*	*acuta*	VGO1065	Ría de Pontevedra, Galicia, Spain	yes
	Dinophisiales	*Dinophysis*	*caudata*	VGO1064	Ría de Pontevedra, Galicia, Spain	yes
	Dinophisiales	*Dinophysis*	*tripos*	VGO1062	Ría de Vigo, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*affine*	PA3V	Ría de Vigo, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*affine*	PA8V	La Linea de la Concepción, Spain	yes
	Gonyaulacales	*Alexandrium*	*andersonii*	ICMB222	Alfacs, Delta l'Ebre, Catalunya, Spain	no
	Gonyaulacales	*Alexandrium*	*andersonii*	SZN12	Napoli, Italy	no
	Gonyaulacales	*Alexandrium*	*andersonii*	VGO664	Elefsis Bay, Saronikos Gulf, Greece	no
	Gonyaulacales	*Alexandrium*	*andersonii*	CCMP1718	Town Cove, Eastman, MA, USA	yes
	Gonyaulacales	*Alexandrium*	*andersonii*	clon CCMP1597-9A2	Town Cove, Eastham, MA, USA	yes
	Gonyaulacales	*Alexandrium*	*catenella* (Group I)	AL10	Monterey Bay, CA, USA	yes
	Gonyaulacales	*Alexandrium*	*catenella* (Group I)	AL52	Pacifica Pier, CA, USA	yes
	Gonyaulacales	*Alexandrium*	*catenella* (Group I)	AL78	Morro Bay, CA, USA	yes
	Gonyaulacales	*Alexandrium*	*catenella* (Group I)	ACQ06	Quellón, X Región, Chile	yes
	Gonyaulacales	*Alexandrium*	*catenella* (Group I)	ACSD01	Bahía Sto. Domingo, XI Región, Chile	yes
	Gonyaulacales	*Alexandrium*	*fundyense* (Group I)	CCMP1719	Portsmouth, New Hampshire, USA	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group I)	MDQ1096	Mar del Plata, Argentina	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group II)	CNRATAA1	Mar Piccolo di Taranto, Ionian Sea, Italy	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group II)	VGO654	Paguera, Mallorca, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group II)	OLFA-B5	Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group II)	VGO1042	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group II)	BT 30	Bay of Tunis, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group II)	BT 31	Bay of Tunis, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group II)	BT 32	Bay of Tunis, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group II)	BT33	Bay of Tunis, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group II)	BT34	Bay of Tunis, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group II)	BT36	Bay of Tunis, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group II)	BT 37	Bay of Tunis, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *kutnerae* (Group II)	VGO714	Port Vilanova, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamarense* (Group III)	CCAP1119/1	Tamar Estuary, United Kingdom	yes
	Gonyaulacales	*Alexandrium*	*tamarense* (Group III)	PE1V	Ría de Vigo, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamarense* (Group III)	VGO926	Praia de Carnota, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamarense* (Group III)	VGO927	Praia de Carnota, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamarense* (Group III)	VGO928	Praia de Carnota, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamarense* (Group III)	VGO1082	Praia de Carnota, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamarense* (Group III)	VGO1083	Praia de Carnota, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamarense* (Group III)	VGO1084	Praia de Carnota, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamarense* (Group III)	VGO1085	Praia de Carnota, Galicia, Spain	no
	Gonyaulacales	*Alexandrium*	*tamarense* (Group III)	VGO1086	Praia de Carnota, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamarense* (Group III)	VGO1087	Praia de Carnota, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamarense* (Group III)	SA1	Fangar, Delta l'Ebre, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	sp. (Group III)	VGO1078	Porto de Baiona, Galicia, Spain	no
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	AC1C	Port de Barcelona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	AC2C	Port de Barcelona, Catalunya, Spain	yes

Table S1. Supplementary material (cont.). 2 of 10

Division	Order	Genus	Specie	Strain	Location	Infection
Dinophyta	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	C6	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	C7	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	AC6T	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO561	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO562	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO563	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO564	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO565	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO566	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO567	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO570	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO571	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO573	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO574	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO583	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO584	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO585	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO587	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO588	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO589	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO710	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO593	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO594	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO595	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO596	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO598	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO599	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO600	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO601	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO603	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO604	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO606	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO607	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO608	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO609	Port de Tarragona, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	AT01	Etang de Thau, France	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO675	Etang de Thau, France	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO676	Etang de Thau, France	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	AT02	Etang de Thau, France	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO673	Etang de Thau, France	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO814	Etang de Thau, France	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO815	Etang de Thau, France	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO816	Etang de Thau, France	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO817	Etang de Thau, France	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO818	Etang de Thau, France	yes

Table S1. Supplementary material (cont.). 3 of 10

Division	Order	Genus	Specie	Strain	Location	Infection
Dinophyta	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	VGO819	Etang de Thau, France	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	BZ3	Lac de Bizerte, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	BZ7	Lac de Bizerte, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	BZ9	Lac de Bizerte, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	BZ8	Lac de Bizerte, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	BZ10	Lac de Bizerte, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	BZ11	Lac de Bizerte, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *catenella* (Group IV)	BZ14	Lac de Bizerte, Tunis	yes
	Gonyaulacales	*Alexandrium*	cf. *tamarense* (Group IV)	CCMP1493	Bahia de Da Yia, China	yes
	Gonyaulacales	*Alexandrium*	*margalefi*	VGO 763	Port Vilanova, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*margalefi*	VGO 661	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*margalefi*	VGO 794	Port Palamós, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*margalefi*	661-A10	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	A.MIN	-	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO707	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO756	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AL8C	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AL9C	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min5	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min6	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min7	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min8	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min9	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min10	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min11	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min16	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min17	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min18	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min19	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min21	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min22	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min23	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min1	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min2	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min3	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Min4	Arenys, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO874	Boughrara, Tunis	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO929	Boughrara, Tunis	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO930	Boughrara, Tunis	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO722	Cambrils, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO723	Cambrils, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	GH min 04	Denmark	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AL10C	Estartit, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AL12C	Estartit, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AL13C	Estartit, Catalunya, Spain	yes

Table S1. Supplementary material (cont.). 4 of 10

Division	Order	Genus	Specie	Strain	Location	Infection
Dinophyta	Gonyaulacales	*Alexandrium*	*minutum*	Clon Startit A10	Estartit, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Clon Startit A7	Estartit, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	CCFWC417	Florida, USA	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AMAD21	Jervois Bridge, Port River, SA. Australia	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO577	La Fosca, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	18A	Lagoa d'Óbidos, Portugal	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AL6V	Lorbé, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AL7V	Lorbé, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO942	Mar Adriático, Italy	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AMITA	Mar Adriático, Italy	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Palmira 1	Palmira, Mallorca, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Palmira 2	Palmira, Mallorca, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Palmira 3	Palmira, Mallorca, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Palmira 4	Palmira, Mallorca, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	Palmira 5	Palmira, Mallorca, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AL5V	Ponte de Toralla, Ría de Vigo, Spain	no
	Gonyaulacales	*Alexandrium*	*minutum*	AL4V	Ponte de Toralla, Ría de Vigo, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AMP13	Port de Palma, Mallorca, Spain	no
	Gonyaulacales	*Alexandrium*	*minutum*	AMP4	Port de Palma, Mallorca, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AMP10	Port de Palma, Mallorca, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	P4	Port de Palma, Mallorca, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	P4 Clon C6(8)	Port de Palma, Mallorca, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AMAD06	Port River, SA, Australia	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AMAD01	Port River, SA. Australia	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO650	Port Saint Hubert, Brittany, France	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO651	Port Saint Hubert, Brittany, France	no
	Gonyaulacales	*Alexandrium*	*minutum*	VGO 651(5)	Port Saint Hubert, Brittany, France	no
	Gonyaulacales	*Alexandrium*	*minutum*	VGO652	Port Saint Hubert, Brittany, France	no
	Gonyaulacales	*Alexandrium*	*minutum*	VGO653	Port Saint Hubert, Brittany, France	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO657	Port Saint Hubert, Brittany, France	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO 650(4)	Port Saint Hubert, Brittany, France	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO712	Port Vilanova, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO713	Port Vilanova, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO716	Port Vilanova, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO717	Port Vilanova, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO718	Port Vilanova, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO719	Port Vilanova, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO720	Port Vilanova, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO721	Port Vilanova, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO1074	Porto de Baiona, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO1075	Porto de Baiona, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO1076	Porto de Baiona, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO1079	Porto de Baiona, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO1089	Porto de Baiona, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO1090	Porto de Baiona, Galicia, Spain	yes

Table S1. Supplementary material (cont.). 5 of 10

Division	Order	Genus	Specie	Strain	Location	Infection
Dinophyta	Gonyaulacales	*Alexandrium*	*minutum*	VGO1091	Porto de Baiona, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO1080	Porto de Baiona, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO1081	Porto de Baiona, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO1088	Porto de Baiona, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AL1V	Ría de Vigo, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AL2V	Ría de Vigo, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	AL3V	Ría de Vigo, Galicia, Spain	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO663	Sardinia, Italy	yes
	Gonyaulacales	*Alexandrium*	*minutum*	VGO746	Saronikos Gulf, Greece	yes
	Gonyaulacales	*Alexandrium*	*ostenfeldii*	FAL50	Falmouth, United Kingdom	yes
	Gonyaulacales	*Alexandrium*	*ostenfeldii*	FAL50 9.06.11 301	Falmouth, United Kingdom	yes
	Gonyaulacales	*Alexandrium*	*ostenfeldii*	AOTV-B4	Tvärminne, Baltic Sea, Finland	yes
	Gonyaulacales	*Alexandrium*	*ostenfeldii*	AOTV-A1	Tvärminne, Baltic Sea, Finland	yes
	Gonyaulacales	*Alexandrium*	*ostenfeldii*	AOTV-A4	Tvärminne, Baltic Sea, Finland	yes
	Gonyaulacales	*Alexandrium*	*ostenfeldii*	AOTV-B3	Tvärminne, Baltic Sea, Finland	yes
	Gonyaulacales	*Alexandrium*	*peruvianum*	VGO956	Palamós, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamutum*	SZN029	Golfo de Nápoles, Italy	no
	Gonyaulacales	*Alexandrium*	*tamutum*	VGO615	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamutum*	VGO616	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamutum*	VGO617	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Gonyaulacales	*Alexandrium*	*tamutum*	E6Q2 Sibling 12x10	-	yes
	Gonyaulacales	*Alexandrium*	*tamutum*	A8	-	yes
	Gonyaulacales	*Alexandrium*	*taylori*	VGO703	Alfacs, Delta l'Ebre, Catalunya, Spain	no
	Gonyaulacales	*Coolia*	*canariensis*	VGO780	Pta. Hidalgo, Tenerife, Canary Islands, Spain	no
	Gonyaulacales	*Coolia*	*canariensis*	VGO786	Pta. Hidalgo, Tenerife, Canary Islands, Spain	no
	Gonyaulacales	*Coolia*	*canariensis*	VGO775	Pta. Hidalgo, Tenerife, Canary Islands, Spain	yes
	Gonyaulacales	*Coolia*	*malayense*	CCMP1345	Florida, USA	no
	Gonyaulacales	*Coolia*	*monotis*	CM6V	Almería, Spain	yes
	Gonyaulacales	*Coolia*	*monotis*	VGO831	Almerimar, Almería, Spain	no
	Gonyaulacales	*Coolia*	*monotis*	VGO832	Almerimar, Almería, Spain	yes
	Gonyaulacales	*Coolia*	*monotis*	VGO833	Almerimar, Almería, Spain	yes
	Gonyaulacales	*Coolia*	*monotis*	VGO858	Charca del Conde, La Gomera, Canary Islands, Spain	no
	Gonyaulacales	*Coolia*	*monotis*	VGO941	Llavaneres, Catalunya, Spain	no
	Gonyaulacales	*Coolia*	*monotis*	RIKZ4	North Sea, Yerseke, Netherlands	no
	Gonyaulacales	*Coolia*	*monotis*	CM7C	Pixavaques, Girona, Catalunya, Spain	yes
	Gonyaulacales	*Coolia*	*monotis*	CM1V	Ría de Vigo, Galicia, Spain	no
	Gonyaulacales	*Coolia*	*monotis*	CM2V	Ría de Vigo, Galicia, Spain	no
	Gonyaulacales	*Coolia*	*monotis*	CM3V	Ría de Vigo, Galicia, Spain	yes
	Gonyaulacales	*Coolia*	*monotis*	CM4V	Ría de Vigo, Galicia, Spain	yes
	Gonyaulacales	*Coolia*	*monotis*	VGO782	Saronikos Gulf, Greece	yes
	Gonyaulacales	*Coolia*	*monotis*	SZN43	Tyrrhenian sea, Italy	no
	Gonyaulacales	*Coolia*	sp.	VGO1055	Belize	yes
	Gonyaulacales	*Coolia*	*tropicalis*	VGO923	Manado, Indonesia	no
	Gonyaulacales	Fragilidium	cf. *dupolocampanaeforme*	VGO692	Elefsis Bay, Saronikos Gulf, Greece	yes
	Gonyaulacales	Fragilidium	cf. *dupolocampanaeforme*	VGO1120	Ría de Vigo, Galicia, Spain	no

Table S1. Supplementary material (cont.). 6 of 10

Division	Order	Genus	Specie	Strain	Location	Infection
Dinophyta	Gonyaulacales	*Fragilidium*	cf. *dupolocampanaeforme*	VGO1121	Ría de Vigo, Galicia, Spain	no
	Gonyaulacales	*Fragilidium*	*subglobosum*	I091-01	Cascais, Portugal	yes
	Gonyaulacales	*Gambierdiscus*	cf. *pacificus*	GPSi	Malaysia	no
	Gonyaulacales	*Gambierdiscus*	cf. *pacificus*	G10DC	Malaysia	no
	Gonyaulacales	*Gambierdiscus*	*australes*	VGO1046	Honolulu, Hawaii, USA	no
	Gonyaulacales	*Gambierdiscus*	*australes*	CBA1a	Honolulu, Hawaii, USA	yes
	Gonyaulacales	*Gambierdiscus*	*australes*	CBA1b	Honolulu, Hawaii, USA	yes
	Gonyaulacales	*Gambierdiscus*	*excentricus*	VGO790	Pta. Hidalgo, Tenerife, Canary Islands, Spain	no
	Gonyaulacales	*Gambierdiscus*	*excentricus*	VGO791	Pta. Hidalgo, Tenerife, Canary Islands, Spain	no
	Gonyaulacales	*Gambierdiscus*	*excentricus*	VGO1035	Playa Las Cabras, La Palma, Canary Islands, Spain	no
	Gonyaulacales	*Gambierdiscus*	*excentricus*	VGO792	Pta. Hidalgo, Tenerife, Canary Islands, Spain	yes
	Gonyaulacales	*Gambierdiscus*	sp. 1	KC82G2	Creta, Greece	no
	Gonyaulacales	*Gambierdiscus*	sp. 2	VGO917	Manado, Indonesia	yes
	Gonyaulacales	*Gambierdiscus*	sp. 3 (Ribotype 1)	VGO1022	La Puntilla, Las Palmas de Gran Canaria, Spain	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO898	Ancona, Italy	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO900	Ancona, Italy	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	CBA-0	Ancona, Italy	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO614	Bahia de Abra, Madeira, Portugal	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO1070	Croatia	no
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO1072	Croatia	no
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO1073	Croatia	no
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	261009aA3	Croatia	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO822	Es Codolar, Tossa de Mar, Catalunya, Spain	no
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO820	Es Codolar, Tossa de Mar, Catalunya, Spain	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	CBA 0203 R	Honolulu, Hawaii, USA	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	CNR-D1	La Spezia, Italy	no
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO883	Lanzarote, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO884	Lanzarote, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO886	Lanzarote, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO887	Lanzarote, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO1052	Llavaneres, Catalunya, Spain	no
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO1069	Llavaneres, Catalunya, Spain	no
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	CBA 6ACB3	Manado, Indonesia	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO1019 R	Playa Las Cabras, La Palma, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO889	Portnovo, Italy	no
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO899	Portnovo, Italy	no
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO769	Pta. Hidalgo, Tenerife, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	OS01BR	Río de Janeiro, Brasil	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	OS04BR	Río de Janeiro, Brasil	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	OS05BR	Río de Janeiro, Brasil	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	OS10BR	Río de Janeiro, Brasil	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	OS11BR	Río de Janeiro, Brasil	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	OS14BR	Río de Janeiro, Brasil	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	OS15BR	Río de Janeiro, Brasil	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	OS16BR	Río de Janeiro, Brasil	yes

Table S1. Supplementary material (cont.). 7 of 10

Division	Order	Genus	Specie	Strain	Location	Infection
Dinophyta	Gonyaulacales	*Ostreopsis*	cf. *ovata*	OS18BR	Río de Janeiro, Brasil	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	OS20BR	Río de Janeiro, Brasil	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	OS19BR	Río de Janeiro, Brasil	yes
	Gonyaulacales	*Ostreopsis*	cf. *ovata*	VGO693	Sousse, Tunis	yes
	Gonyaulacales	*Ostreopsis*	cf. siamensis	VGO978	La Fosca, Catalunya, Spain	yes
	Gonyaulacales	*Ostreopsis*	cf. siamensis	VGO983	La Fosca, Catalunya, Spain	yes
	Gonyaulacales	*Ostreopsis*	cf. siamensis	VGO985	La Fosca, Catalunya, Spain	yes
	Gonyaulacales	*Ostreopsis*	sp. 1	VGO1016	Playa Las Cabras, La Palma, Canary Islands, Spain	no
	Gonyaulacales	*Ostreopsis*	sp. 1	VGO1011	Playa Las Cabras, La Palma, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	sp. 1	VGO1013	Playa Las Cabras, La Palma, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	sp. 1	VGO1014	Playa Las Cabras, La Palma, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	sp. 1	VGO1015	Playa Las Cabras, La Palma, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	sp. 1	VGO1017	Playa Las Cabras, La Palma, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	sp. 1	VGO1019	Playa Las Cabras, La Palma, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	sp. 2	VGO1000	Famara, Lanzarote, Canary Islands, Spain	no
	Gonyaulacales	*Ostreopsis*	sp. 2	VGO882	Lanzarote, Canary Islands, Spain	yes
	Gonyaulacales	*Ostreopsis*	sp. 3	VGO1058	Florida, USA	yes
	Gonyaulacales	*Ostreopsis*	sp. 4	VGO1061	Florida, USA	yes
	Gonyaulacales	*Protoceratium*	*reticulatum*	VGO758	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Gonyaulacales	*Protoceratium*	*reticulatum*	VGO757	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Gonyaulacales	*Protoceratium*	*reticulatum*	VGO764	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Gonyaulacales	*Protoceratium*	*reticulatum*	CCMP1720	Biscayne Bay, Miami, Florida, USA	no
	Gonyaulacales	*Protoceratium*	*reticulatum*	VGO903	Bueu, Ría de Pontevedra, Galicia, Spain	yes
	Gonyaulacales	*Protoceratium*	*reticulatum*	VGO904	Bueu, Ría de Pontevedra, Galicia, Spain	yes
	Gonyaulacales	*Protoceratium*	*reticulatum*	VGO905	Bueu, Ría de Pontevedra, Galicia, Spain	yes
	Gonyaulacales	*Protoceratium*	*reticulatum*	CCMP1889	Friday Harbor, WA, USA	yes
	Gonyaulacales	*Protoceratium*	*reticulatum*	GG1AM	La Atunara, Cádiz, Spain	yes
	Gonyaulacales	*Protoceratium*	*reticulatum*	CCMP404	Salton Sea, California, USA	yes
	Gymnodiniales	*Akashiwo*	*sanguinea*	VGO626	Kavala Harbour, Greece	no
	Gymnodiniales	*Akashiwo*	*sanguinea*	ICMB233	Vilanova i la Geltrú, Catalunya, Spain	no
	Gymnodiniales	*Amphidinium*	*carterae*	A01BR	Brasil	no
	Gymnodiniales	*Amphidinium*	*carterae*	ACMK03	Mauritius	no
	Gymnodiniales	*Amphidinium*	*carterae*	ACRN02	Reunion	no
	Gymnodiniales	*Amphidinium*	*carterae*	ACRN03	Reunion	no
	Gymnodiniales	*Amphidinium*	sp.	VGO781	Pta. Hidalgo, Tenerife, Canary Islands, Spain	no
	Gymnodiniales	*Barrufeta*	*bravensis*	VGO864	La Fosca, Catalunya, Spain	no
	Gymnodiniales	*Barrufeta*	*bravensis*	VGO866	La Fosca, Catalunya, Spain	no
	Gymnodiniales	*Gymnodinium*	*catenatum*	GC12V	Ría de Vigo, Galicia, Spain	no
	Gymnodiniales	*Gymnodinium*	*catenatum*	GC11V	Ría de Vigo, Galicia, Spain	yes
	Gymnodiniales	*Gymnodinium*	*catenatum*	GC13V	Ría de Vigo, Galicia, Spain	yes
	Gymnodiniales	*Gymnodinium*	*catenatum*	GC40AM	La Aturana, Cádiz, Spain	yes
	Gymnodiniales	*Gymnodinium*	*catenatum*	EST 2 D6	Galicia, Spain	yes
	Gymnodiniales	*Gymnodinium*	cf. *simplex*	VGO671	Kervoyal, Damgan, Bretaña, France	yes
	Gymnodiniales	*Gymnodinium*	cf. *simplex*	VGO690	Elefsis Bay, Saronikos Gulf, Greece	yes
	Gymnodiniales	*Gymnodinium*	*impudicum*	10B	Italy	no

Table S1. Supplementary material (cont.). 8 of 10

Division	Order	Genus	Specie	Strain	Location	Infection
Dinophyta	Gymnodiniales	*Gymnodinium*	*impudicum*	VGO665	Kervoyal, Damgan, Bretaña, France	no
	Gymnodiniales	*Gymnodinium*	*impudicum*	GY6V	La Línea de la Concepción, Spain	no
	Gymnodiniales	*Gymnodinium*	*impudicum*	VGO1054	Tunis	no
	Gymnodiniales	*Gymnodinium*	*impudicum*	OLFA B6	Tunis	no
	Gymnodiniales	*Gymnodinium*	*impudicum*	GY3VA	Valencia, Spain	no
	Gymnodiniales	*Gymnodinium*	*impudicum*	GY4VA	Valencia, Spain	no
	Gymnodiniales	*Gymnodinium*	*instriatum*	ICMB234	Port Arenys, Catalunya, Spain	no
	Gymnodiniales	*Gymnodinium*	*litoralis*	ICMB226	Desembocadura de la Muga, Spain	yes
	Gymnodiniales	*Gymnodinium*	*microreticulatum*	VGO328	Praia Panxón, Galicia, Spain	no
	Gymnodiniales	*Gymnodinium*	*nolleri*	922I	Kattegat, Denmark	yes
	Gymnodiniales	*Gymnodinium*	sp.	GY	-	yes
	Gymnodiniales	*Gyrodinium*	*dominans*	AC	Catalunya, Spain	no
	Gymnodiniales	*Karenia*	*brevis*	CCMP2281	Florida, USA	no
	Gymnodiniales	*Karenia*	*selliformis*	VGO876	Boughrara, Tunis	no
	Gymnodiniales	*Karlodinium*	*armiger*	AC	Catalunya, Spain	no
	Gymnodiniales	*Karlodinium*	*veneficum*	ICMB256	Alfacs, Delta l'Ebre, Catalunya, Spain	no
	Gymnodiniales	*Karlodinium*	*veneficum*	k3	Alfacs, Delta l'Ebre, Catalunya, Spain	no
	Gymnodiniales	*Karlodinium*	*veneficum*	k4	Alfacs, Delta l'Ebre, Catalunya, Spain	no
	Gymnodiniales	*Karlodinium*	*veneficum*	k17	Alfacs, Delta l'Ebre, Catalunya, Spain	no
	Gymnodiniales	*Karlodinium*	*veneficum*	k24	Alfacs, Delta l'Ebre, Catalunya, Spain	no
	Gymnodiniales	*Karlodinium*	*veneficum*	VGO872	Boughrara, Tunis	no
	Gymnodiniales	*Karlodinium*	*veneficum*	K1	Boughrara, Tunis	no
	Gymnodiniales	*Karlodinium*	*veneficum*	K6	Boughrara, Tunis	no
	Gymnodiniales	*Karlodinium*	*veneficum*	K10	Boughrara, Tunis	no
	Gymnodiniales	*Lepidodinium*	*chlorophorum*	BAHME100	List, Sylt Island, Germany	yes
	Gymnodiniales	*Lepidodinium*	*chlorophorum*	RCC 1489	-	yes
	Gymnodiniales	*Lepidodinium*	*chlorophorum*		Basque Country, Spain	yes
	Gymnodiniales	*Takayama*	sp.	VGO341	Baiona, Galicia, Spain	no
	Peridiniales	*Bysmatrum*	sp.	SA2	Fangar, Delta l'Ebre, Catalunya, Spain	no
	Peridiniales	*Heterocapsa*	*niei*	VGO399	Lorbé, Galicia, Spain	yes
	Peridiniales	*Heterocapsa*	*niei*	VGO623	Lorbé, Galicia, Spain	yes
	Peridiniales	*Heterocapsa*	*triquetra*	VGO1053	Alfacs, Delta l'Ebre, Catalunya, Spain	no
	Peridiniales	*Heterocapsa*	*triquetra*	241105C2	Alfacs, Delta l'Ebre, Catalunya, Spain	no
	Peridiniales	*Kryptoperidinium*	*foliaceum*	BAIONA06A1	Baiona, Galicia, Spain	no
	Peridiniales	*Kryptoperidinium*	*foliaceum*	AR	Muga, Catalunya, Spain	yes
	Peridiniales	*Pentapharsodinium*	*thyrrenicum*	SA3	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Peridiniales	*Scrippsiella*	*trochoidea*	S3V	Ría de Vigo, Galicia, Spain	yes
	Peridiniales	*Scrippsiella*	sp.	OLFA C9	Tunis	yes
	Peridiniales	*Scrippsiella*	sp.	OLFA C7	Tunis	yes
	Peridiniales	*Scrippsiella*	sp.	SA4	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Peridiniales	*Scrippsiella*	sp.	071005E5	Alfacs, Delta l'Ebre, Catalunya, Spain	no
	Peridiniales	*Woloszynskia*	*cincta*	SA4	Alfacs, Delta l'Ebre, Catalunya, Spain	yes
	Prorocentrales	*Prorocentrum*	*arenarium*	VGO776	Pta. Hidalgo, Tenerife, Canary Islands, Spain	no
	Prorocentrales	*Prorocentrum*	*balticum*	VGO365	Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*belizeanum*	VGO867	Charca del Conde, La Gomera, Canary Islands, Spain	no

Table S1. Supplementary material (cont.). 9 of 10

Division	Order	Genus	Specie	Strain	Location	Infection
Dinophyta	Prorocentrales	*Prorocentrum*	*cassubicum*	VGO834	Almerimar, Almería, Spain	no
	Prorocentrales	*Prorocentrum*	*cassubicum*	VGO835	Almerimar, Almería, Spain	no
	Prorocentrales	*Prorocentrum*	*cassubicum*	VGO836	Almerimar, Almería, Spain	no
	Prorocentrales	*Prorocentrum*	cf. *belizeanum*	VGO1028	La Puntilla, Las Palmas, Canary Island, Spain	no
	Prorocentrales	*Prorocentrum*	cf. *belizeanum*	VGO1029	La Puntilla, Las Palmas, Canary Island, Spain	no
	Prorocentrales	*Prorocentrum*	cf. *belizeanum*	VGO1030	La Puntilla, Las Palmas, Canary Island, Spain	no
	Prorocentrales	*Prorocentrum*	cf. *belizeanum*	VGO1031	La Puntilla, Las Palmas, Canary Island, Spain	no
	Prorocentrales	*Prorocentrum*	*levis*	VGO777	Pta. Hidalgo, Tenerife, Canary Islands, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL10V	Bueu, Ría de Pontevedra, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL11V	Praia Canido, Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL12V	Praia Canido, Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL13V	Praia Canido, Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL14V	Praia Canido, Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL15V	Praia Canido, Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL16V	Lago Cíes, Cies Island, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL17V	Lago Cíes, Cies Island, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL18V	Lago Cíes, Cies Island, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL19V	Praia Canido, Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL1V	Lago Cíes, Cies Island, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL20V	Lago Cíes, Cies Island, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL21V	Praia Canido, Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL22V	Lago Cíes, Cies Island, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL23V	Praia Canido, Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL24V	Lago Cíes, Cies Island, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL26V	Praia Areas, Ría de Pontevedra, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL27V	Praia Areas, Ría de Pontevedra, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL28V	Praia Areas, Ría de Pontevedra, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL29V	Praia Canelas, Ría de Pontevedra, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL2V	Lago Cíes, Cies Island, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL30V	Praia Areas, Ría de Pontevedra, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL3V	Ría de Aldan, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL4V	Ría de Aldan, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL5V	Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL6V	Bueu, Ría de Pontevedra, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL7V	Bueu, Ría de Pontevedra, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL8V	Bueu, Ría de Pontevedra, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PL9V	Bueu, Ría de Pontevedra, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*lima*	PLEU02	-	no
	Prorocentrales	*Prorocentrum*	*lima*	PLMA01	Mayotte Island, France	no
	Prorocentrales	*Prorocentrum*	*lima*	PLRN02	Reunion	no
	Prorocentrales	*Prorocentrum*	*micans*	PM1V	Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*minimum*	AND1V	Río San Pedro, Cádiz, Spain	no
	Prorocentrales	*Prorocentrum*	*minimum*	AND3V	Río San Pedro, Cádiz, Spain	no
	Prorocentrales	*Prorocentrum*	*minimum*	VGO367	Ponte de Toralla, Ría de Vigo, Spain	no
	Prorocentrales	*Prorocentrum*	*rathymum*	VGO680	Crique de l'Angle, Étang de Thau, France	no

Table S1. Supplementary material (cont.). 10 of 10

Division	Order	Genus	Specie	Strain	Location	Infection
Dinophyta	Prorocentrales	*Prorocentrum*	*rostratum*	PR1V	Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	sp.	VGO995	Lanzarote, Canary Island, Spain	no
	Prorocentrales	*Prorocentrum*	sp.	VGO761	Alfacs, Delta l'Ebre, Catalunya, Spain	no
	Prorocentrales	*Prorocentrum*	*triestinum*	PT	Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*triestinum*	PT2V	Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*triestinum*	PT3V	Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*triestinum*	PT5V	Ría de Vigo, Galicia, Spain	no
	Prorocentrales	*Prorocentrum*	*triestinum*	VGO672	Kervoyal, Damgan, Brittany, France	no
Chlorophyta		Pyramimonas	sp.	PY01V	Lorbé, Galicia, Spain	no
Haptophyta		*Emiliania*	*huxleyi*	EH02V	Cabo Estay, Galicia, Spain	no
		Pavlova	*girans*	CCMP 608	Helford River, nr. Falmouth, Cornwall, United Kingdom	no
		Phaeocystis	*globosa*	PH01	-	no
		Prymnesium	*faveolatum*	VGO557	Pixavaques, Girona, Catalunya, Spain	no
		Prymnesium	sp.	VGO1040	-	no
Raphidophyta		*Chattonella*	*antiqua*	VGO1037	-	no
		Chattonella	*subsalsa*	VGO1039	-	no
		Chattonella	*verruculosa*	VGO1038	-	no
		Fibrocapsa	*japonica*	VGO1043	-	no
		Heterosigma	*akashiwo*	HA1V	Ría de Arousa, Galicia, Spain	no
		Heterosigma	*akashiwo*	HA2V	Ría de Arousa, Galicia, Spain	no
		Heterosigma	*akashiwo*	HA3V	Ría de Arousa, Galicia, Spain	no
		Olisthodiscus	*luteus*	VGO1036	-	no
Diatoms		*Coscinodiscus*	cf. *radiatus*	CCMP313	Baja California, Mexico	no
		Phaeodactylum	*tricornutum*	CCMP632	Balckpool, United Kingdom	no
		Phaeodactylum	*tricornutum*	PHAEO2	-	no
		Pseudo-nitzschia	*pungens*	CCAP1061	United Kingdom	no
		Skeletonema	*costatum*	CCMP2092	Gulf of Trieste, Italy	no
		Thalassiosira	*weissflogii*	CCAP 1085/1	USA	no

Chapter 4

“A game of Russian roulette for a generalist dinoflagellate parasitoid: host susceptibility is the key to success”

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A game of Russian roulette for a generalist dinoflagellate parasitoid: host susceptibility is the key to success

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ABSTRACT

Marine microbial interactions involving eukaryotes and their parasites play an important role in shaping the structure of phytoplankton communities. These interactions may alter population densities of the main host, which in turn may have consequences for the other concurrent species. The effect generalist parasitoids exert on a community is strongly dependent on the degree of host specificity. *Parvilucifera sinerae* is a generalist parasitoid able to infect a wide range of dinoflagellates, including toxic-bloom-forming species. A density-dependent chemical cue has been identified as the trigger for the activation of the infective stage. Together these traits make *Parvilucifera*-dinoflagellate hosts a good model to investigate the degree of specificity of a generalist parasitoid, and the potential effects that it could have at the community level. Here, we present for the first time, the strategy by which a generalist dinoflagellate parasitoid seeks out its host and determine whether it exhibits host preferences, highlighting key factors in determining infection. Our results demonstrate that in its infective stage, *P. sinerae* is able to sense potential hosts, but does not actively select among them. Instead, the parasitoids contact the host at random, governed by the encounter probability rate and once encountered, the chance to penetrate inside the host cell and develop the infection strongly depends on the degree of host susceptibility. As such, their strategy for persistence is more of a game of Russian roulette, where the chance of survival is dependent on the susceptibility of the host. Our study identifies *P. sinerae* as a potential key player in bloom community ecology, where in mixed dinoflagellate communities consisting of hosts that are highly susceptible to infection, parasitoid preferences may mediate coexistence between host species, reducing the dominance of the superior competitor. Alternatively, it may increase competition, leading to species exclusion. If, however, highly susceptible hosts are absent from the community, the parasitoid population could suffer a dilution effect maintaining a lower parasitoid density. Therefore, both host community structure and host susceptibility will determine infectivity in the field.

INTRODUCTION

Historically, the role of parasitic protists in marine planktonic ecosystems has been largely neglected. New molecular tools have revealed that parasitism is a widespread interaction in aquatic microbial communities with a high diversity of unclassified parasites (Lefèvre et al., 2008; de Vargas et al., 2015) even in marine ecosystems not considered previously (Cleary and Durbin, 2016). There is increasing evidence that protist parasites may have a significant effect on plankton at the population, community, and ecosystem levels (Chambouvet et al., 2008; Lepère et al., 2008).

Parasite-mediated effects on their host populations are strongly dependent on parasitic specificity, i.e. the strength of the interactions between them (Anderson and May, 1981). Host species differ

in their susceptibility to a certain parasite; therefore parasite transmission between species is often asymmetrical, where one host species might be highly infected resulting in a higher parasite load to the system (Woolhouse et al., 2001). In some host-parasite systems, generalist parasites infecting multiple host species possess traits to discriminate amongst host species (Krasnov et al., 2002; Goubault et al., 2004; Wang and Messing, 2004; Sears et al., 2012). Host abundance, species identity or host susceptibility are characteristics suggested to influence parasite preferences for choosing a certain host to infect in these systems, since these preferences are supposed to be adaptive strategies that maximize parasite fitness. Given that hosts can vary in their susceptibility to a certain parasite, and that host relative abundance in natural communities shift, parasite selection amongst host species is a very relevant question that has not yet been explored in great detail in parasitoid-phytoplankton systems.

Dinoflagellates are a dominant group of eukaryotic phytoplankton and an important component in marine ecosystem functioning, playing a key role in primary production and the marine food web (Margalef, 1978; Reynolds, 2006). Many dinoflagellate species can cause blooms and some of them produce potent toxins that cause negative impacts for human health, aquaculture and marine ecosystems (Zingone and Enevoldsen, 2000). Currently, three groups of zoosporic parasitoids with different phylogenetic origin are known to infect dinoflagellates, '*Amoebophrya ceratii*' complex (Syndiniales), *Parvilucifera* (Perkinsids) and *Dinomyces* (Chytrid) , moreover, environmental molecular surveys have unveiled a high hidden diversity amongst these groups (Guillou et al., 2008; Chambouvet et al., 2014). The characteristics of these parasitoids are to kill their host, to have short generation times and to produce a huge amount of offspring following host infection (Coats and Park, 2002; Garcés et al., 2013a; Lepelletier et al., 2014a),thereby reducing the abundance of their hosts, potentially altering host population processes, such as competition, which in turn influence community composition.

Several studies have evaluated the range and specificity in host-parasitoid systems. In the case of the '*Amoebophrya ceratii*' complex, some clades are specialists (Chambouvet et al., 2008), whereas others have a broader host range (Coats and Park, 2002; Kim, 2006). However, in some generalist strains, after infecting a host, the offspring are unable to produce a second generation (Coats and Park, 2002). *Dinomyces* and *Parvilucifera* species (with the exception of *P. prorocentri*) have been described as generalist parasitoids, able to infect a wide range of hosts within dinoflagellates, including toxic species (Garcés et al., 2013a; Lepelletier et al., 2014a; Lepelletier et al., 2014b). In the case of *Parvilucifera* parasitoids, although a generalist in terms of the number of species they are able to infect, intra and inter-species variability still exists at the level of host susceptibility or infectivity (Figueroa et al., 2008; Råberg et al., 2014; Turon et al., 2015). The extent to which *Parvilucifera* parasitoids show preferences for certain hosts has not been fully investigated. Further research is required in order to understand the potential effects this parasitoid may have in marine microbial communities.

A system comprised of *Parvilucifera sinerae* and their dinoflagellate hosts provides a good model to address whether generalists *Parvilucifera* parasitoids exhibit preferences for the most susceptible hosts available, given that, (i) the reproductive success of the parasitoid depends on its ability to infect a host, (ii) it can infect a wide range of hosts from among dinoflagellates, and (iii) it uses chemical cues, such as dimethylsulphide, to detect host presence (Garcés et al., 2013b). As such, the objectives of the present work were to determine if *P. sinerae* shows preferences among possible dinoflagellate hosts, and evaluate whether the host susceptibility or the host dominance (in terms of abundance), are decisive factors when the parasitoid infects a host.

MATERIAL AND METHODS

Host and parasitoid cultures

Experiments were conducted with host strains of several dinoflagellate taxa obtained from the culture collection of the Centro Oceanográfico (CCVIEO) in Vigo, Spain. Specifically, we used two strains belonging to Gonyaulacales: *Alexandrium minutum* (AMP4), and *Protoceratium reticulatum* (GC1AM); two strains belonging to Gymnodiniales: *Gymnodinium catenatum* (GC11V), and *Amphidinium carterae* (ACRN03); and two strains belonging to Peridiniales: *Scrippsiella trochoidea* (S3V), and *Heterocapsa niei* (VGO 623).

Cultures were maintained in 50 mL polystyrene tissue culture flasks filled with 20 mL of L1 medium (Guillard, 1995) without silica. The medium was prepared with filtered (0.2 µm), autoclaved seawater, adjusting the salinity to 31 by the addition of sterile MilliQ water. Cultures were grown at 20 ± 1 ºC with a photoperiod of 12:12 h (light:dark) cycle. Illumination was provided by fluorescent tubes with a photon irradiance of about 90 µmol photons m^{-2} s^{-1}.

Stock parasitoid culture of *Parvilucifera sinerae* (ICMB852) was propagated by transferring a 1 mL aliquot of mature sporangium every six to seven days into an uninfected host stock culture of exponentially growing *A. minutum* strain AMP4 in sterile polystyrene six well-plates, each well with a growth area of 9.6 cm^2 and a volume of 15.5 mL (BD Biosciences). These cultures were maintained under the same culture conditions mentioned above.

All experiments were conducted in triplicate using host cultures growing exponentially and recently formed sporangium of *P. sinerae* culture (strain ICMB 852). To obtain recently formed sporangia, four days after infection of an *A. minutum* (AMP4) culture, sporangia produced from the subsequent parasite generation were harvested for the inoculation of the experiments. Sporangia concentration was estimated by counting at least 300 mature sporangia (late sporocyte) using a Sedgewick-rafter chamber. Zoospore concentration was estimated by multiplying the number of zoospores contained in a single sporangium (250 in the case of *A. minutum*; Garcés et al. 2013) by the sporangia concentration. For the experiments, the volume added from the parasitoid mother culture was adjusted to obtain the final zoospore concentration required in each of the experiments.

Parasitoid generation time and transmission in the different host populations

For each host species, triplicate 30 mL cultures at initial density of $1x10^4$ mL^{-1} were inoculated with recently formed sporangia at zoospore: host ratio of 1:60. We used this low parasitoid ratio to mimic the initial phase of an epidemic, avoiding killing the entire host population in the first generation, and then obtain two to three parasitoid generations in the same host population.

Infected cultures were performed in 50 mL-polystyrene tissue culture flasks, and incubated under growth conditions (described above) for 14 to 16 days. This incubation time was required to observe at least two parasitoid generations depending on the host species that was infected. We took a 1 mL aliquot daily, preserved it with formaldehyde (1% final concentration) and the mature sporangia abundance were counted by inverted light microscopy (Leica–Leitz DMIRB) using a

Sedgewick-rafter chamber by scoring at least 300 sporangia, with the exception of the first generation, where the infections where very low. Mature sporangia (late sporocyte) of each host species can be seen in figure 1.

Generation time was estimated by following the evolution of infected cells over time, which showed clear peaks associated with each parasitoid generation. We decomposed the evolution for each species into a sum of Gaussian peak shapes using an unconstrained non-linear optimization algorithm based on an iterative least-squares method, where the fraction of infected cells is the division of each individual Gaussian peak shape by the total number of infected cell at each time step. This fraction data allowed us to calculate the parasitoid generation time, following an adaptation of the methodology of Carpenter and Chang (1988) for the quantification of parasitoid generation time, by knowing the fraction of infected cells for each generation.

Host selection experiment

Selection chambers were used to determine whether *Parvilucifera* zoospores demonstrated a putative behavioral attraction among three dinoflagellate species: the high-susceptible host *Alexandrium minutum*, the low-susceptible host *Heterocapsa niei*, and the non-susceptible host *Amphidinium carterae*. Since *A. carterae* is a dinoflagellate but not a potential host (*Parvilucifera* is not able to infect *Amphidinium*; see table 2 of the study of Garcés et al. (2013)), we used this resistant species to know whether *Parvilucifera* zoospores are attracted to dinoflagellates in general, both those that can be infected (susceptible) as well as those not in their host range (resistant or non-susceptible). We also tested the attractiveness of two infochemicals, dimethylsulphide (DMS) and dimethylsulfoniopropionate (DMSP), which are related to dinoflagellate metabolism and were previously identified as chemical signals that activated the release of the zoospores from the dormant sporangium (Garcés et al., 2013b). Each selection chamber consisted of four 5 mL-syringes placed vertically, separated by 1 cm, into a 17-mL well volume (6-deep well plates, BioCoat™) containing 15 mL of L1 medium (n=9). In each of the nine wells, three of the syringes contained 1.5 mL of exudates from *A. minutum*, *H. niei* and *A. carterae*, while the fourth syringe contained L1 medium (control). Exudates were prepared by filtering 5 mL of the host culture at 10^4 cells mL^{-1} through 0.22 µm pore size Swinnex filters (Millipore) right before the experiment. Then, we added 1 mL of swimming zoospores at a concentration of $5x10^4$ in the center of the well and syringes remained dipped for 30 min. After this period, syringes were removed and the whole content inside the syringe was fixed with formaldehyde (1 % final concentration). The number of zoospores that entered inside the syringe was estimated by counting at least 400 cells using a Sedgewick-Rafter chamber under light microscopy. To test whether the zoospores were attracted to specific chemicals cues, triplicate syringes containing lab-prepared DMS and DMSP at a concentration of 300 nM were placed inside a well filled with L1 medium and $5x10^4$ swimming zoospores. After 30 min, syringes were removed and zoospores were counted as above.

Parasitoid preferences for host species

Parasitoid preferences for infecting certain host species in an artificial mixed community of *Alexandrium minutum*, *Scrippsiella trochoidea*, *Protoceratium reticulatum*, *Heterocapsa niei* and *Gymnodinium catenatum* was tested in triplicate. The initial host concentration of each species was normalized by host cell biovolume in order to obtain a zoospore:host ratio of 1:1 taking into account the biovolume of $1.5x10^3$ *Gymnodinium catenatum* cells mL^{-1} which is the largest host.

As the sizes of the host species used in this experiment vary, normalization by host cell biovolume avoids having different encounter probability rates between the parasitoid and the host. Infected cells of each species were counted during the first three days after parasitoid addition. We counted at least 300 cells as either infected or uninfected, identifying the infected ones of the whole artificial community by optical microscopy using a Sedgewick-rafter chamber. Clear identification of the infected species was obtained, as infection is easily recognizable in the host species (Figure 1 column 2: early trophocyte).

Susceptibility of host species

Parasitoid prevalence in the five host species used in the preference experiment was determined as a function of inoculum size. For each experiment, sets of triplicates 50 mL-polystyrene tissue

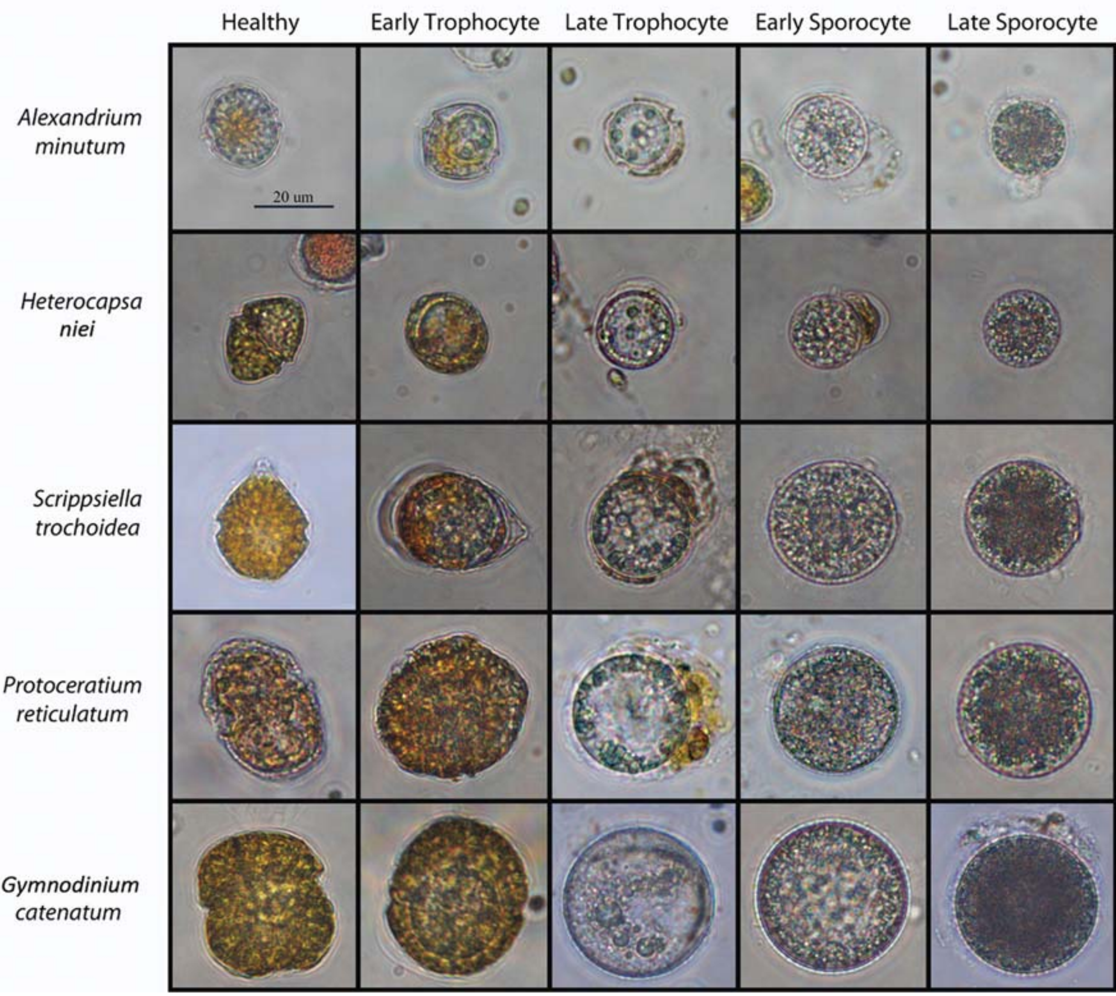

Figure 1. Optical micrographs of the different life-cycle stages of *Parvilucifera sinerae* infecting five dinoflagellate hosts. Scale bar = 20 µm.

culture flasks containing 20 mL of host cells at initial density of 5 x 10^3 mL^{-1} were inoculated with recently formed sporangia and incubated for three to four days under the same growth conditions as described above. Inoculum size of parasitoid for each set of triplicate vials was adjusted to give zoospores: host ratios of 1:1, 2:1, 5:1, 10:1, 20:1, 40:1, and 80:1. In two host species (*H. niei* and *G. catenatum*) the prevalence curve was not stabilized at ratio of 80:1, so we also inoculated both species with an inoculum size of 120:1 ad hoc. The time required to detect easily if the cell was infected or not was 3-5 days of incubation and that time was shorter than the time needed for the parasitoid to initiate a second round of infection (a second generation) according to the results obtained in the generation time experiment. After incubation, samples were preserved with formaldehyde (1% final concentration) and examined by inverted light microscopy (Leica–Leitz DMIRB) to estimate parasitoid prevalence. Parasitoid prevalence was calculated as a percentage of infected cells and was determined by scoring at least 300 cells per sample as infected (taking into account any of the infection stages) or uninfected (healthy) in a Sedgwick-Rafter chamber.

Data for each host species were fitted to a single two parameter exponential rise to maximum following the method of Coats and Park (2002). The equation for the curve fit was $y = a(1 - e^{-bx})$, where a is the maximum infection level (I_{max}) and b is α/I_{max}. Alpha (α) represents the slope of the initial linear portion of the fitted curve and reflects the potential of zoospores to infect host cells. Alpha was estimated as I_{max}*b.

Host abundance experiment

The effect of host abundance on parasitoid preferences was assessed in two systems; *System A* a mixed culture comprised of two species that were equally preferred in the preference experiment, *A. minutum* and *S. trochoidea,* and *System B*, a mixed culture containing a preferred host, *A. minutum* and a less preferred host, *Heterocapsa niei*. For each system, we establish a set of triplicates in 50 mL-culture flasks of varying dominance with i) the two hosts at the same initial host cell concentration (10^3cells mL^{-1}); ii) a mixed culture with *A. minutum* and *S. trochoidea* at 10^3cells mL^{-1} and at 10^4cells mL^{-1} initial cell concentration, respectively; iii) a mixed culture with *A. minutum* and *S. trochoidea* at 10^4cells mL^{-1} and at 10^3cells mL^{-1} initial cell concentration, respectively. The same set up was established for *System B;* the *A. minutum*/*H. niei* system. We inoculated 20 sporangia mL^{-1} of *P. sinerae* to each culture in order to obtain a 5:1 zoospore:host ratio matched to the less abundant host (10^3cells mL^{-1}). By matching the zoospore ratio to the lowest density host we were able to minimize obscuring host preferences, as a higher number of zoospores could result in over-infection of both host populations, masking the true preference of the parasitoid. Prevalence in each host was determined during the first 4 days after parasitoid addition in *System A* and *System B*, by scoring at least 300 infected cells and identifying the species that was infected using a Sedgewick-rafter chamber under light microscopy. All the infection stages were considered as infected when samples were counted, as shown in figure 1, from the second to the last column (from early trophocyte to late sporocyte).

Statistical analyses

For the host selection experiment, to analyze whether *P. sinerae* zoospores were attracted by specific chemical cues (DMS and DMSP) and, if the parasitoid select among three different host species that differ in their susceptibility, we conducted a one-way analysis of similarity (ANOSIM).

The analysis was performed on the number of zoospores that choose each treatment or each of the host species. ANOSIM is a multivariate non-parametric permutation test, analogue to a one-way ANOVA (Clarke and Warwick, 1994). Prior to ANOSIM, similarity matrices were calculated by using the Bray-Curtis similarity coefficient. We used an alpha = 0.01 to test significance. In the case of significance, we conducted a post-hoc test by multiple Pairwise Comparisons.

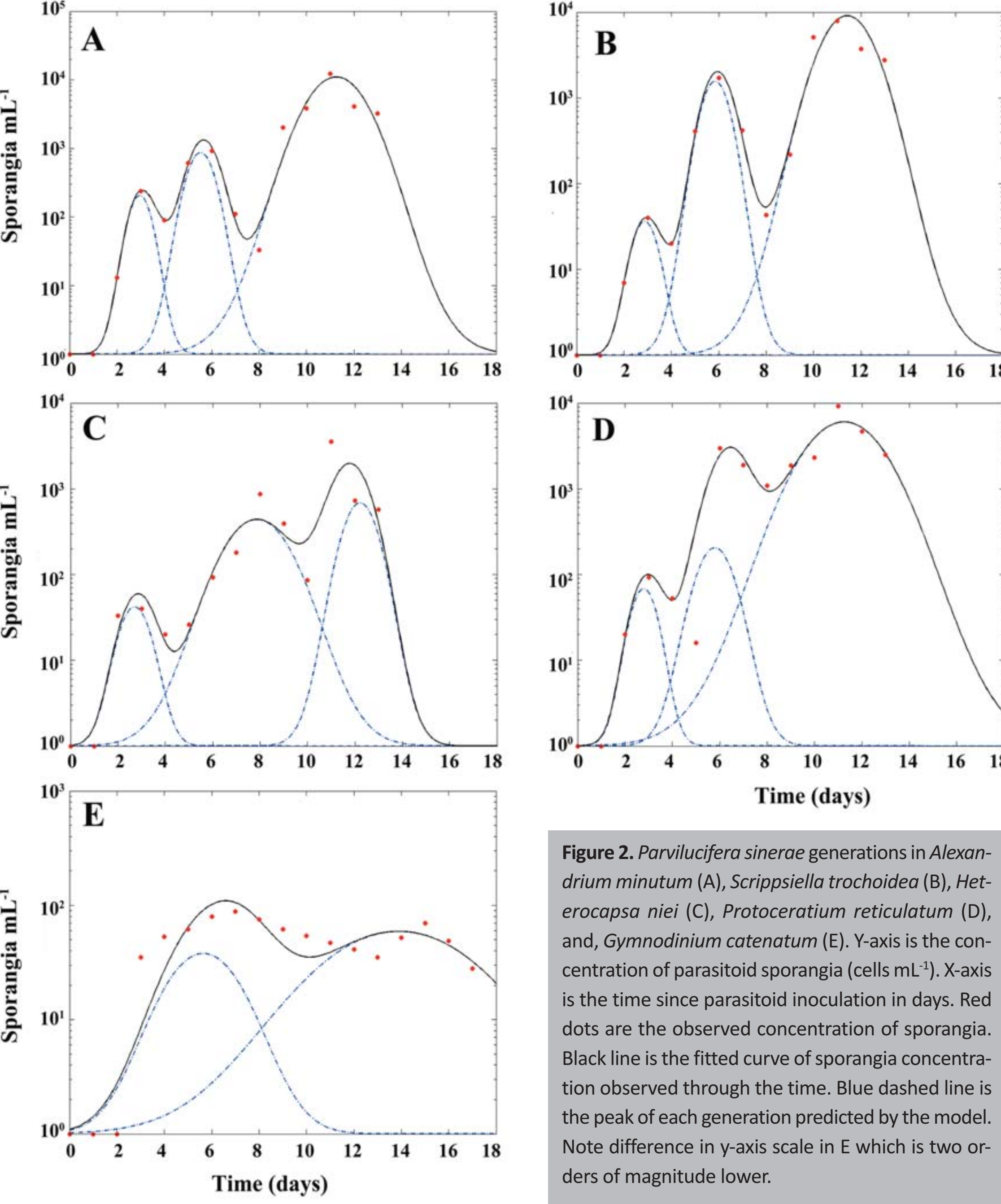

Figure 2. *Parvilucifera sinerae* generations in *Alexandrium minutum* (A), *Scrippsiella trochoidea* (B), *Heterocapsa niei* (C), *Protoceratium reticulatum* (D), and, *Gymnodinium catenatum* (E). Y-axis is the concentration of parasitoid sporangia (cells mL^{-1}). X-axis is the time since parasitoid inoculation in days. Red dots are the observed concentration of sporangia. Black line is the fitted curve of sporangia concentration observed through the time. Blue dashed line is the peak of each generation predicted by the model. Note difference in y-axis scale in E which is two orders of magnitude lower.

For the host preference experiment, to test if there were significant differences between species in the artificial community, we conducted the same statistical analyses as above, on the percentage of infected cells of each species at day three in the artificial community.

To test for significant differences in host susceptibility to the parasitic infection by *P. sinerae* we used two variables, the maximum infection level (I_{max}) and the alpha value (α), which is the slope of the linear portion of the fitted curve. Prior to analysis data were transformed as log(X+1), because the two variables presented values that differed by one order of magnitude. Then, the same statistical analysis and post-hoc test as above were performed. All the analyses were performed by using the statistical software PRIMER 6.1.2 (Clarke and Gorley, 2006).

RESULTS

Parasitoid generation time in the different host species

Inoculation of *A. minutum*, *H. niei*, *S. trochoidea* and *P. reticulatum* with zoospores at 1:60 ratio at a high initial host concentration of 10^4cells mL^{-1}, produced an increased number of mature sporangia over the 16 days, showing three peaks corresponding to three generations of parasitoid life-cycle (Figure 2A-D). The same inoculation of *G. catenatum* resulted in a more gradual increase of the mature sporangia, showing only two peaks during the same time period (Figure 2E).

The estimated time for the first generation of *Parvilucifera sinerae* was 62 and 137 hours for the second generation in *Alexandrium minutum* (r^2 = 0.98), being the species with the shortest generation time (Figure 2A). In the case of parasitoid infection in *Scrippsiella trochoidea* (r^2 = 0.99) (Figure 2B) and *Protoceratium reticulatum* (r^2 = 0.92) (Figure 2D) the averaged generation time was the same for both species, being 72 and 132 hours for the first and the second generations, respectively. Infecting *H. niei* (r^2 = 0.93) (Figure 2C), the parasitoid showed a generation time of 108 and 154 hours for the first and second generations, respectively. Finally, for *P. sinerae* infecting *G. catenatum* (r^2 = 0.88) (Figure 2E) we were only able to estimate the time for the first generation, because we observed two peaks, around 192 hours. In all the species studied, the increase

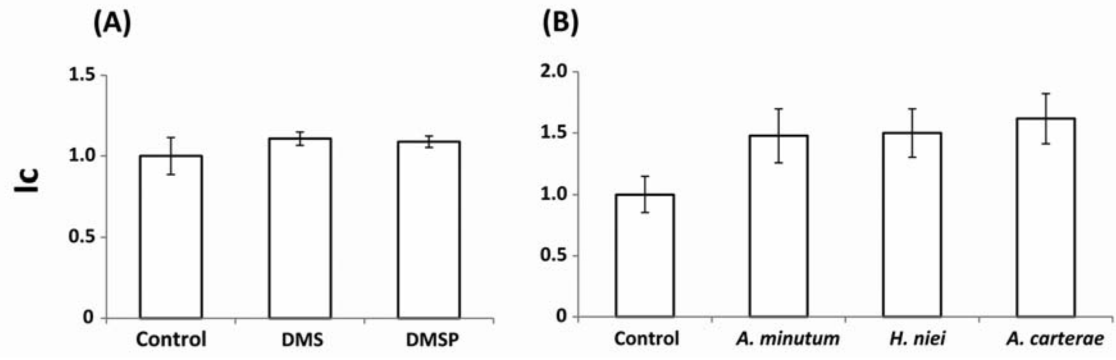

Figure 3. Parasitoid zoospore chemotaxis for two chemical cues (A) and three dinoflagellate species (B). Ic is the chemotaxis index, defined as the proportion of zoospores that enter the syringe relative to the control (L1 medium). Data are expressed as mean±s.d.

in the sporangia concentration through the different parasitoid generations was more than one order of magnitude between the successive peaks, with the exception of *G. catenatum,* where only low levels of infection were achieved in both generations.

Host selection

The response of the zoospores to the info-chemicals DMS and DMSP was not different from that of the control (p=0.23) (Figure 3A). DMS, despite being involved in activating dormant zoospores inside the sporangium and acting as a chemical cue for high host abundance, did not play any role in host location. However, the response of zoospores to a signal from the three dinoflagellates species tested (Figure 3B) differed significantly from that of the control (L1 medium) (p=0.0001), suggesting the presence of a substance that is released by the living dinoflagellates which acts as a chemoattractant to the free-living parasitoids. Concerning host attractiveness through chemotaxis experiments, the pairwise comparisons between the different hosts, confirmed that zoospores did not present significant differences between host species (Figure 3B), indicating that the infective stage of *P. sinerae* does not select amongst its dinoflagellate hosts.

Parasitoid preference for host species

Inoculation of *Parvilucifera sinerae* in a mixed artificial dinoflagellate community revealed that the parasitoid preference for hosts significantly differed between host species (p=0.0007) (Figure 4). The parasitoid showed a gradient in the prevalence in the different hosts, showing the strongest preference for *A. minutum* and *S. trochoidea* species, reaching approximately 60% infection in both populations 3 days after parasitoid addition. The parasitoid showed no significant preference between these two species. The next most preferred species by *P. sinerae* was *P. reticulatum,* with 38% of its population infected, followed by *H. niei* (17%), and finally *G. catenatum*, which was hardly infected, showing infection prevalence in less than 3% of the population.

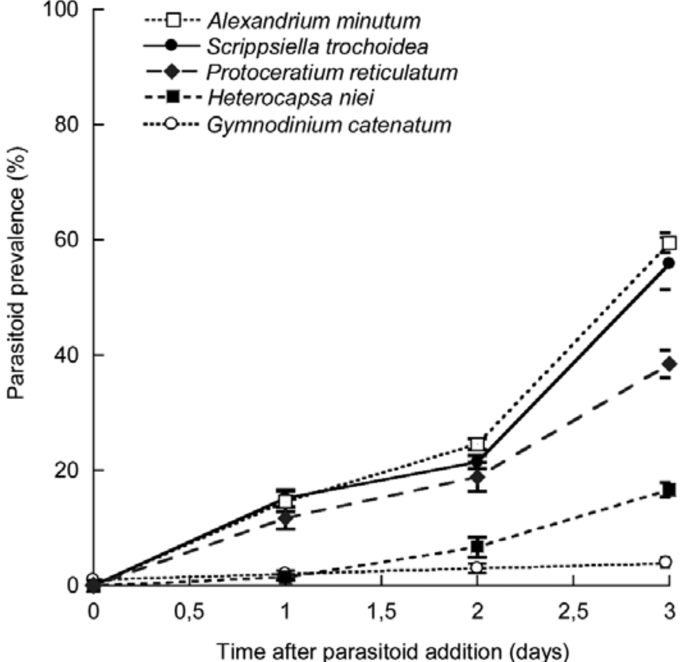

Figure 4. Parasitoid prevalence (%) in each of the five host species mixed in an artificial community during the three days after parasitoid inoculation. Data are expressed as mean±s.d.

Susceptibility of host species

Parasitoid prevalence showed an exponential increase to a maximum relative to inoculum size in all five species tested (Figure 5). Estimates for maximum infection levels (I_{max}) and initial slope of the fitted curves

(α) were I_{max}= 98.2±2.2; α = 27.4±0.04 (r^2=0.98) for *A. minutum*, I_{max}= 100.9±1.91; α = 27.9±0.04 (r^2=0.99) for *S. trochoidea*, I_{max}=100±3.5; α= 28±0.21 (r^2=0.94) for *P. reticulatum*, I_{max} = 81 ± 3.5; α = 3.5 ± 0.01 (r^2=0.94) for *H. niei*, and I_{max} = 58 ± 8.8; α = 0.98 ± 0.3 (r^2=0.90) for *G. catenatum*.

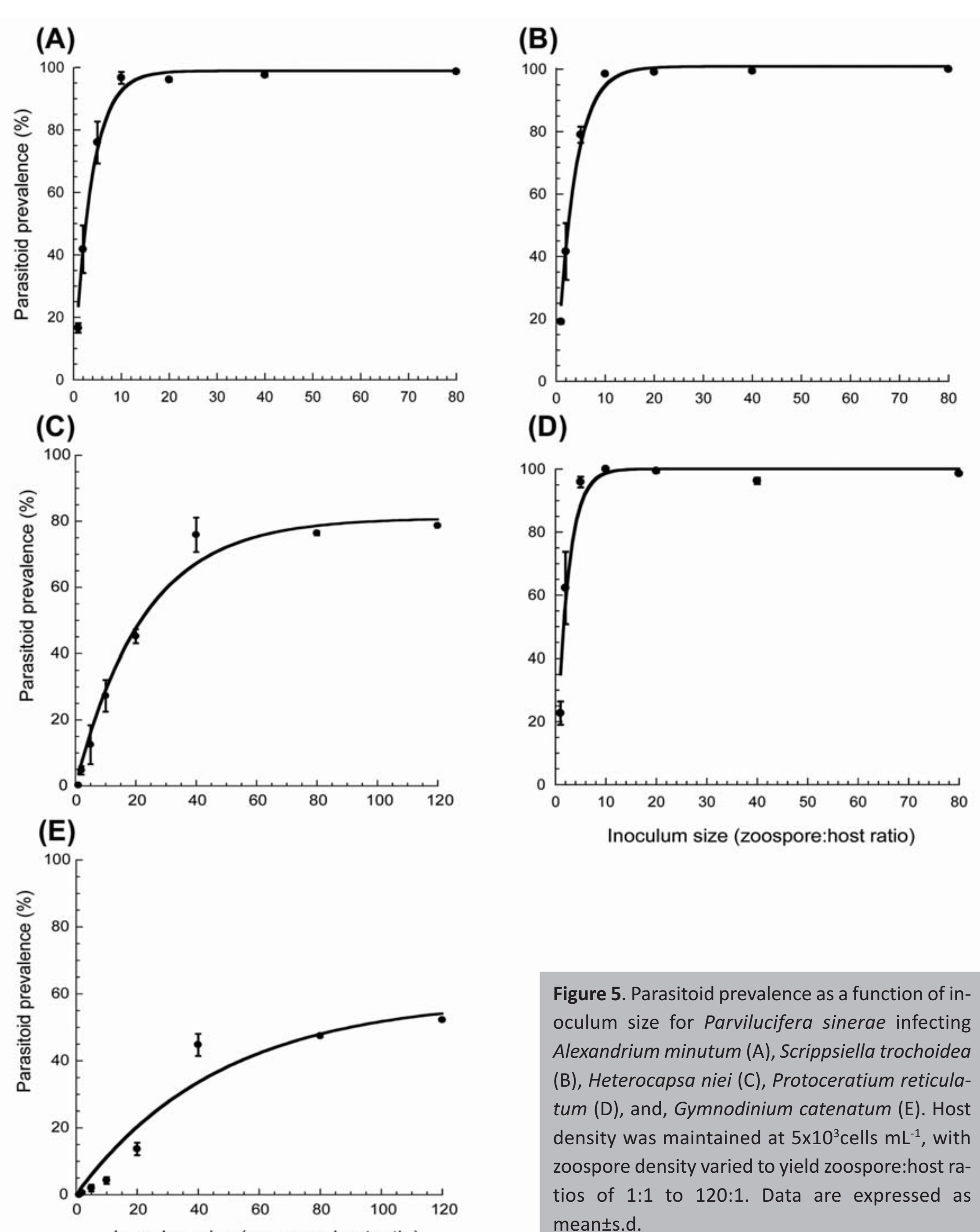

Figure 5. Parasitoid prevalence as a function of inoculum size for *Parvilucifera sinerae* infecting *Alexandrium minutum* (A), *Scrippsiella trochoidea* (B), *Heterocapsa niei* (C), *Protoceratium reticulatum* (D), and, *Gymnodinium catenatum* (E). Host density was maintained at $5x10^3$cells mL^{-1}, with zoospore density varied to yield zoospore:host ratios of 1:1 to 120:1. Data are expressed as mean±s.d.

Host species varied significantly in their susceptibility to infection (p=0.001) showing a gradient, with *A. minutum*, *S. trochoidea*, and *P. reticulatum* being the most susceptible (Figure 5A, B and D, respectively), followed by *H. niei*, and *G. catenatum* (Figure 5C and E, respectively). In the most susceptible species (*A. minutum*, *S. trochoidea* and *P. reticulatum*) the maximum infection level was reached at 10:1 zoospore: host ratio where the whole dinoflagellate population was completely exterminated. In contrast, in the less susceptible species (*H. niei* and *G. catenatum*) the prevalence showed a more gradual increase to saturation (40:1 ratio) and failed to reach 100% infection levels, even at higher ratios (120:1).

Effect of host abundance in host infection

The effect of host abundance in the choice of *P. sinerae* infection is highly dependent on host susceptibility (Figure 6). In the system comprised of equal host densities of two highly susceptible species, *A. minutum* and *S. trochoidea* (*System A*; Figure 6A), both species were infected without distinction. However, when the density of one of these species was higher than the other (Figure 6B and C), *P. sinerae* chose to infect the most abundant species in both experiments. In contrast, when the system was composed of one high-susceptible species (*A. minutum*) and one low-susceptible species (*H. niei*) (*System B*), the parasitoid always reached higher infection in the one that is more susceptible, i.e. *A. minutum* (Figure 6D, E, and F), independently of the initial density of the low-susceptible species. Nevertheless, an interesting effect was observed after the first generation took place in *System B* (Figure 6E), where after the parasitoid completed its first generation (day three) killing the whole *A. minutum* population, the rapid increase in the parasitoid population allowed for high infection of the low-susceptible species *H. niei* (Figure 6E, day four).

DISCUSSION

Parasitism is made up of many different strategies for infection, each one representing unique ecological interactions (Skovgaard, 2014). Understanding the relationship between parasitoids and hosts is crucial to know the role played by parasitoids, the impact that they can exert on a community and to quantify these processes for the modelling of natural phytoplankton communities.

Parvilucifera's strategy of seeking out a host to infect

In screening experiments, *Parvilucifera sinerae* and the other species within the genera have been described as generalist parasitoids of dinoflagellates (Norén et al., 1999; Garcés et al., 2013a; Lepelletier et al., 2014b), however, the strategy of infection has never been studied. All *Parvilucifera* species complete their life-cycle in one individual host, which dies at the end of the infection. After reproducing, it produces many offspring inside a sporangium that remains dormant until the adequate signal. Garcés et al. (2013b) identified DMS as a density-dependent chemical cue for *P. sinerae* activation, where high concentrations of DMS communicate the presence of a high number of potential hosts in the marine environment. Upon activation, the zoospores abandon the sporangium in order to infect a new host. DMS is produced by several phytoplankton species, however *Parvilucifera* are generalists so it follows that they may be activated by a general chemical cue. In this study, the chemotaxis experiment demonstrated that once outside the sporangium, the motile zoospores do not use the DMS/DMSP to locate a suitable host, but some other signal from living cells, which seems to be involved in host location (Figure 3). In a previous study involving an *Amoe-*

bophrya parasite and the toxic *Karlodinium veneficum*, the authors found that high-toxin-producers were more infected than non-toxic strains (Bai et al., 2007). We did not measure the toxicity of the species tested; however, whether the parasitoid locates the host by a specific substance is an in-

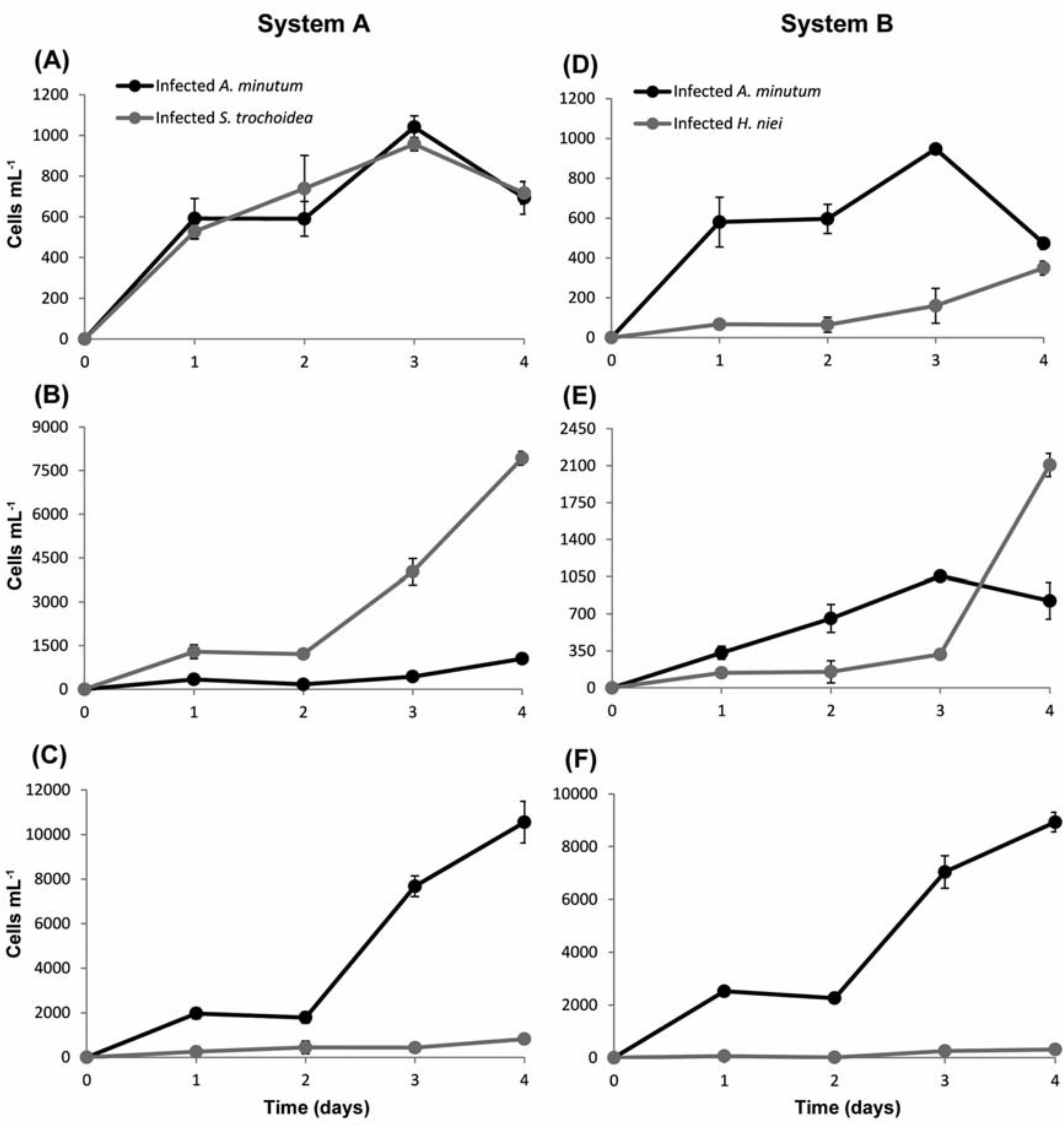

Figure 6. Effect of host abundance in host infection in System A: a mixed culture of *Alexandrium minutum* and *Scrippsiella trochoidea* (A-C), and in System B: a mixed culture of *A. minutum* and *Heterocapsa niei* (D-F). (A, D) Initial host density of both host was the same 10^3cells mL^{-1}; (B, E) *S. trochoidea* and *H. niei* were at 10^4cells mL^{-1} and *A. minutum* was at 10^3cells mL^{-1}. (C, F) *A. minutum* was at initial density of 10^4cells mL^{-1}, and *S. trochoidea* and *H. niei* at 10^3cells mL^{-1}. Data are expressed as mean±s.d.

teresting question worthy of further investigation. Our results show that *Parvilucifera* does not select amongst potential dinoflagellate hosts tested in this study, instead, the parasitoid attacks all hosts encountered, regardless of species. In fact, the zoospores exhibit the same level of attraction to a high-susceptible, low-susceptible and a non-susceptible host. These data suggest that the infection strategy of *Parvilucifera* is more like a game of Russian roulette, where the zoospores seek out and contact a host at random, and it is only once the zoospores have encountered their host, that their fate is determined. Instead of choosing a host that will allow them to proliferate, successful infection is simply a game of chance and it is the hosts' susceptibility that determines whether or not the parasitoid can attach and penetrate into the host cell to develop the infection.

Parasitoid preferences and specificity

In the artificial mixed community, where the probability of encounter was the same for all dinoflagellate hosts used, we determined a preference for *Parvilucifera* to infect a certain species (Figure 4). A plausible hypothesis to test was that the parasitoid preferred to infect the largest host, as a strategy to increase parasitoid reproduction rate, since zoosporic parasitoids produce an amount of offspring proportional to host size, where the bigger the host biovolume, the more zoospores are produced (Garcés et al., 2013a). Certainly, the size of the host is significant, but, in terms of parasitoid transmission, parasitoid generation time in the different hosts and the number of hosts infected is also relevant. For instance, in this study the largest sporangium was obtained through infecting *G. catenatum*, but the total number of sporangia in two consecutive generations was orders of magnitude lower than in the other host species. Add to that the generation time, which was much longer, and the maximum population size of *P. sinerae* was much lower than the other more susceptible species. Moreover, in the preference experiment the greatest infection occurrence was reached in two species of different sizes, *A. minutum* and *S. trochoidea,* with a mean biovolume (n=30 cells) of $1.6 \times 10^3 \mu m^3$ and $4 \times 10^3 \mu m^3$, respectively. As such, in the case of *Parvilucifera* parasitoids, the size of the host is not a determinant of host preference.

The *P. sinerae* strain used in this study was isolated from an *A. minutum* bloom, which often appears with *S. trochoidea* in the natural environment (Figueroa et al., 2008). Therefore, we cannot rule out the possibility that *Parvilucifera* shows an innate preference for a particular host species due to the result of historical sympatry., This would suggest that *P. sinerae* preferences are a result of host phylogeny, whereby the parasitoid easily infects more closely related dinoflagellates (Figueroa et al., 2008). The results of this study, where *P. sinerae* heavily infected *A. minutum* and *S. trochoidea* but not *G. catenatum* in the same extent (Figure 4 and 5), give weight to this idea of historical sympatry and are consistent with a study by Llaveria et al. (2010) on a natural population, in which *P. sinerae* heavily infected *A. minutum* and *S. trochoidea*, but not the more distantly related *Prorocentrum*. Similarly, results by Garcés et al. (2013a) support this idea, where *P. sinerae* was able to infect many species belonging to Gonyaulacales and Peridiniales, being less successful infecting Gymnodiniales and not able to infect any species belonging to Prorocentrales. Congruent results were obtained from *P. rostrata* and *P. infectans* (Lepelletier et al., 2014b), however, in the case of *P. prorocentri*, which is the most morphologically and phylogenetically distanced of the four *Parvilucifera* species described to date, it is the only *Parvilucifera* known to infect Prorocentrales (Leander and Hoppenrath, 2008).

We observed that *Parvilucifera sinerae* prefers to infect *A. minutum* and *S. trochoidea* in a mixed community (Figure 4), which at the same time were the most susceptible species (Figure 5)

showing i) a high prevalence in the host populations, ii) the zoospores being highly infective in these species (high α values), iii) presenting shorter generation times, and iv) producing denser parasitoid populations with each generation. So, *P. sinerae* is well adapted to its primary hosts maximizing parasitoid transmission, which could be a result of antagonistic coevolution. This refers to reciprocal evolution of host defense and parasitoid infectivity, which plays an important role in determining the outcome of infection. The study of Råberg et al. (2014) demonstrated that host susceptibility and parasitoid virulence in *P. sinerae*-*A. minutum* systems depends strongly on the combination of host and parasitoid genotypes involved. Also, these evolutionary processes could lead to intra-species phenotypic variability of several *P. sinerae* traits, such as host invasion and parasitoid transmission (zoospores success, infection rate and sporangia viability) (Turon et al., 2015). Interestingly, *H. niei* and *G. catenatum* presented a higher resistance to parasitic infection, supporting higher zoospore load, which we had to increase to reach maximal levels of prevalence. Studies on parasite-induced defense reactions in dinoflagellate hosts to avoid infection are still scarce. Some hosts have evolved defenses by their capacity to produce cysts. Parasitoids alter or shift the community from planktonic life-forms to benthic, producing resistant cysts that avoid infection development (Toth et al., 2004; Chambouvet et al., 2011). Figueroa et al. (2010) found that parasitoid presence induced sexual recombination, where some phases of the life-cycle became infected but others did not, and promoting new host genotypes by genetic recombination that might be resistant to parasitic infection.

Our density-dependent experiments have shown that host abundance together with susceptibility, play an important role in parasitic infection (Figure 6), as *Parvilucifera* presents a frequency-dependent transmission. This is supported by the study of Johansson et al. (2006), which suggested that *P. infectans* distribution in the coast of Sweden is not only governed by the total dinoflagellate population but also the community dominance, which can significantly affect infectivity in the field. As our data show, in a situation of coexistence of two preferred competent species (those that propagate the parasitoid well, enabling its maintenance and spread), the host abundance is the determinant in the infection. The parasitoid will infect the most abundant species (Figure 6B and C), because the probability of an encounter with the dominant species is higher. In contrast, in a community dominated by two species with a different degree of susceptibility, for instance, *A. minutum* and *H. niei*, the key to parasitic infection is host susceptibility, where the parasitoid preferentially infects the most susceptible species rather than the most abundant one (Figure 6D-F). However, once the most susceptible host population has been infected during the first generation (Figure 6E), this newly increased parasitoid population allows *P. sinerae* to reach higher prevalence in the less susceptible host species during the second generation (see Figure 6E day four), as the level of infection depends on the parasitoid population size (Figure 5C).

Potential effects in the community

The characteristics of zoosporic parasitoids are to kill their host, to have short generation times, to produce many progeny, and to exert top-down controls by reducing the size of their host populations, which in turn influence phytoplankton dynamics (Coats et al., 1996; Chambouvet et al., 2008; Velo-Suárez et al., 2013). Several authors have modelled the impact these parasites exert under a mono-specific dinoflagellate bloom situation (Montagnes et al., 2008), or in a three-host-species model (Salomon and Stolte, 2010) and the results obtained were similar to field studies. However, mono-specific dinoflagellate blooms happen only under very specific conditions, so most of the time phytoplankton communities are composed of a mixture of different species.

Therefore, understanding the impact that generalist parasitoids infecting multiple dinoflagellate species could have on natural communities (i.e. *Parvilucifera* parasitoids) to incorporate in models is important, as it has the potential to completely change system's dynamics (Dobson, 2004).

The potential effects that a generalist parasite could have in the community are diverse, moreover, if it exhibits host preferences, the effects are potentially even more asymmetrical. *Parvilucifera,* as a generalist parasitoid, has a direct negative effect on the original host that they are infecting (*A. minutum*), which in turn may have an indirect effect, both positive and negative, on additional host populations and in those of non-host species. Our results suggest that, when competent hosts are present enabling a dense parasitoid population and good transmission, *Parvilucifera* plays an important role in shaping the structure of the community (Figure 7; Hatcher et al. (2012)). In the first case (Figure 7A), *Parvilucifera* mediates coexistence of two competent species, *A. minutum* and *S. trochoidea*. The population of the most abundant species, or in other words the superior competitor, is regulated by parasitic infection, enabling the other, less-harmed species to persist. In this way *Parvilucifera* can enhance the coexistence of both species by reducing competitive advantage through preferential infection of the superior competitor. In an alternative

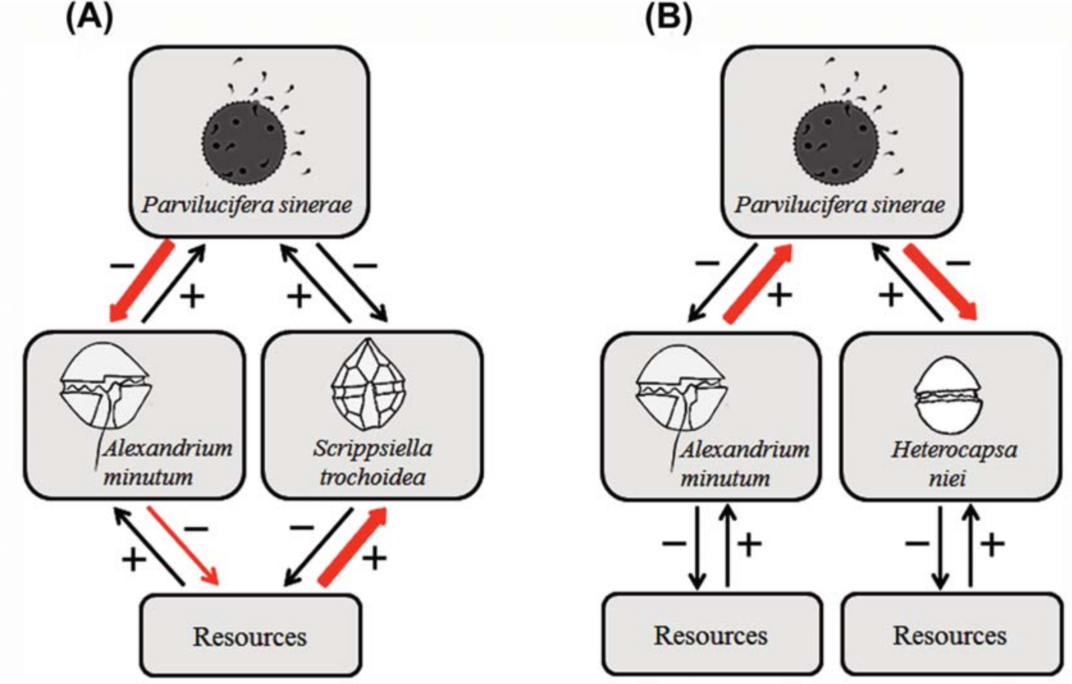

Figure 7. Potential effects of *Parvilucifera sinerae* (adapted from Figure 1 of Hatcher et al. (2012). Arrows depict positive (+) and negative (–) direct effects (numerical effects) on population density resulting from the impact of a consumer or the resources; arrow thickness indicates strength of interaction; red arrows indicate key interactions, leading to the following patterns: A) Parasitoid-mediated coexistence: regulation of a superior competitor by the parasitoid, i.e. *A. minutum*, enables *S. trochoidea*, less harmed by the parasitoid, to persist. B) Apparent competition: higher densities of *A. minutum* host result in higher parasitoid population densities, which have a detrimental effect on *H. niei* host: thus, *A. minutum* acts as a reservoir of infection to *H. niei*.

situation where *Parvilucifera* is shared by two species with different susceptibility (Figure 7B), the most susceptible, *A. minutum*, acts as a reservoir of infection to *H. niei*. First, *Parvilucifera* infects *A. minutum*, its preferred host, where parasitoid transmission is highest, allowing the increase of parasitoid load. This in turn facilitates the infection of the less susceptible, but abundant *H. niei*, reaching higher levels of infection than would be attainable without the presence of the original host (Figure 6E). This situation can cause apparent competition, leading to species exclusion, as one host enhances parasitic infection in the other. In contrast to competitive and reservoir species, hosts that are inefficient propagators of *Parvilucifera*, like *G. catenatum*, can create a dilution effect, thereby lowering infection prevalence and reducing parasitoid population, but maintaining it in low concentrations until preferred hosts become dominant. In agreement to Lapchin (2002), in an unpredictable and changing environment, such as marine phytoplankton communities, *P. sinerae* biology and their infection plan makes for a successful strategy in the evolution of this species. *Parvilucifera sinerae* is able to infect different species successfully, while having a higher fitness in a few of the hosts. This partial specialization allows the parasitoid to survive or maintain a small population when the most susceptible host becomes rare in the community.

Our results highlight the importance of understanding the mechanisms underlying specificity, which are presumably unique in each host-parasite system. The degree of specificity is very important when incorporating parasites into ecosystem models, especially for understanding how parasite prevalence and persistence impacts the marine microbial interactions, from the level of the community to the entire ecosystem.

ACKNOWLEDGEMENTS

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Chapter 5

"*In situ* occurrence, prevalence and dynamics of *Parvilucifera* parasitoids during recurrent blooms of the toxic dinoflagellate *Alexandrium minutum*"

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In situ occurrence, prevalence and dynamics of *Parvilucifera* parasitoids during recurrent blooms of the toxic dinoflagellate *Alexandrium minutum*

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ABSTRACT

Dinoflagellate blooms are natural phenomena that often occur in coastal areas, which in addition to their large number of nutrient-rich sites are characterized by highly restricted hydrodynamics within bays, marinas, enclosed beaches, and harbors. In these areas, the massive cell proliferations have harmful effects on humans and the ecosystem. However, the very high cell density and low diversity of blooms make them vulnerable to parasitic infections. In nature, *Parvilucifera* parasitoids infect the toxic dinoflagellate *Alexandrium minutum* during bloom conditions. Under laboratory conditions parasitoids are able to exterminate an entire host population, but the prevalence and impact of *Parvilucifera* parasitoids in the field remains unexplored. In this study, we evaluated the in situ occurrence, prevalence, and dynamics of *Parvilucifera* parasitoids during recurrent blooms of the toxic dinoflagellate *A. minutum* in a confined site in the NW Mediterranean Sea as well as the contribution of parasitism to bloom termination. *Parvilucifera* parasitoids were recurrently detected from 2009 to 2013, during seasonal outbreaks of *A. minutum*. Parasitic infections in surface waters occurred after the abundance of *A. minutum* reached 10^5 cells L^{-1}, suggesting a density threshold beyond which *Parvilucifera* infection becomes established and transmitted. Moreover, host and parasitoid abundances were not in phase. Instead, there was a lag between maximum *A. minutum* and *Parvilucifera* densities, indicative of a delayed density-dependent response of the parasitoid to host abundances, similar to the temporal dynamics of predator-prey interactions. The highest parasitoid prevalence was reached after a peak in host abundance, coinciding with the decay phase of the bloom, when a maximum of 38% of the *A. minutum* population was infected. According to our estimates, *Parvilucifera* infections accounted for 5–18% of the total *A. minutum* mortality, which suggested that the contribution of parasitism to bloom termination is similar to that of other biological factors, such as encystment and grazing.

INTRODUCTION

In the last decades, toxic and harmful phytoplankton species have been the focus of attention due to their environmental, economic, and public health impacts in coastal areas, which are of major importance for food production (Zingone and Enevoldsen, 2000). Around 200 species belonging to diverse groups of marine microalgae, including dinoflagellates, diatoms, pelagophytes, raphydophytes, and prymnesiophytes, have been identified as potentially harmful. Of these, about 90 species, mainly those of dinoflagellates, are known to be potentially toxic (Zingone and Enevoldsen, 2000; Hallegraeff et al., 2003).

In the Mediterranean Sea, harmful algal blooms (HABs) commonly occur in areas with restricted hydrodynamics, such as bays, lagoons, harbors, beaches, and estuaries. These coastal prolifera-

tions are an emerging problem whose frequency has increased in response to intensive urbanization and recreational use of the Mediterranean shoreline, which has resulted in nutrient-rich (semi-) confined areas with low turbulence levels. These areas constitute a unique environment that favors HAB formation by several phytoplankton species (Garcés and Camp, 2012). For example, the worldwide distributed *Alexandrium minutum* is responsible for outbreaks of paralytic shellfish poisoning in humans and for the high mortality of wild and cultured fauna (Anderson et al., 2012). It also forms recurrent blooms along the Catalan coast (NW Mediterranean Sea) (Vila et al., 2001; Vila et al., 2005; Bravo et al., 2008), with its large number of harbors and huge nutrient inputs from inland sources (Vila et al., 2001; Garcés et al., 2003; Bravo et al., 2008). However, many physical, chemical and biological factors are involved in the development, persistence, and termination of a bloom (Garcés and Camp, 2012). While most studies on HAB dynamics have focused on bottom-up factors, recent investigations have demonstrated a role for the top-down control exerted by biotic factors, such as parasitism and grazing (Coats et al., 1996; Johansson and Coats, 2002; Calbet et al., 2003; Chambouvet et al., 2008; Montagnes et al., 2008).

Parasitism on marine dinoflagellates by eukaryotic parasitoids is mainly due to members of the globally distributed genera *Parvilucifera* and *Amoebophrya* (Alveolata) (Park et al., 2004). In the marine ecosystem, *Amoebophryidae* species are abundant in the water column (Guillou et al., 2008; de Vargas et al., 2015; Massana et al., 2015), while those of *Parvilucifera* predominate in the marine sediment (Chambouvet et al., 2014). Both groups of parasitoids infect and kill several genera of dinoflagellates, among them noxious species, with potentially very strong virulence and high prevalences in both laboratory experiments and in the field (Coats and Park, 2002; Chambouvet et al., 2008). Thus, some authors have proposed the use of parasitoids as biological control agents for bloom mitigation (Norén et al., 1999; Erard-Le Denn et al., 2000). However, little is known about the specificity of these parasites or about the potential unintended side-effects on other dinoflagellate populations (Anderson, 2009). The mechanisms underlying specificity are also not well understood, as intra- and inter-species variability may depend on the host phylogeny (Chambouvet et al., 2008) and/or the specific genetic features of the host and parasite. Both of these factors will determine the outcome of infection (Råberg et al., 2014; Turon et al., 2015; Alacid et al., 2016).

A few studies have addressed the interaction between parasites and their dinoflagellate hosts in the marine environment in order to assess the impact of these organisms in natural communities. Some have shown that, under certain conditions, parasitism has a greater impact than grazing with respect to dinoflagellate population dynamics (Montagnes et al., 2008; Salomon and Stolte, 2010; Jordi et al., 2015). In the field, the prevalence of *Amoebophyra* parasites in dinoflagellate blooms was moderate to high (Coats et al., 1996; Chambouvet et al., 2008; Alves-de-Souza et al., 2012; Velo-Suárez et al., 2013) and in some cases was the main cause of dinoflagellate mortality. Although a high *Parvilucifera* abundance has been correlated with a reduction in the relative abundance of *A. minutum* in short-lasting blooms (Blanquart et al., 2016), field studies on the prevalence impact of *Parvilucifera* infections on their natural host populations as well as the contribution of infection to bloom termination are lacking.

The main goals of this study were: (i) to determine the timing of *Parvilucifera* parasitoid occurrence in Arenys de Mar harbor, a confined area in the NW Mediterranean Sea; (ii) to assess host-parasitoid dynamics during a bloom of the toxic dinoflagellate *A. minutum*; and (iii) to quantify the impact and contribution of *Parvilucifera* spp. parasitism to bloom termination. The present work constitutes the first record of the impact of *Parvilucifera* parasitoids in the field.

MATERIAL AND METHODS

Study area

Arenys de Mar harbor (41° 34.30'N and 2° 32.40'E) is located on the coast of Catalonia (NE Spain) in the NW Mediterranean Sea (Fig. 1). Fishing and leisure are the main human activities in the harbor. The harbor measures 0.4 km^2, has a depth ranging from 1 m at confined sites to 6 m at the entrance, and receives large freshwater inputs rich in nutrients. Intense and recurrent *Alexandrium minutum* blooms between December and August have been recorded every year since 1999 (Vila et al., 2001; Garcés et al., 2004; Van Lenning et al., 2007; Bravo et al., 2008; Anglès et al., 2012), as part of the extensive study of *A. minutum* ecology and bloom dynamics in this confined system (Garcés et al., 2004; Van Lenning et al., 2007; Anglès et al., 2010; Anglès et al., 2012). In this study, we assessed parasitic occurrence and infection during *A. minutum* blooms at two sampling sites (A and B, Fig. 1) where maximum abundances of vegetative cells and resting cysts were documented (Anglès et al., 2010; Garcés et al., 2004).

Phytoplankton sampling and determination of the dinoflagellate community

From 2009 to 2012, phytoplankton surface samples were collected from location A once a week between January and September, and once a fortnight from October to December. From January

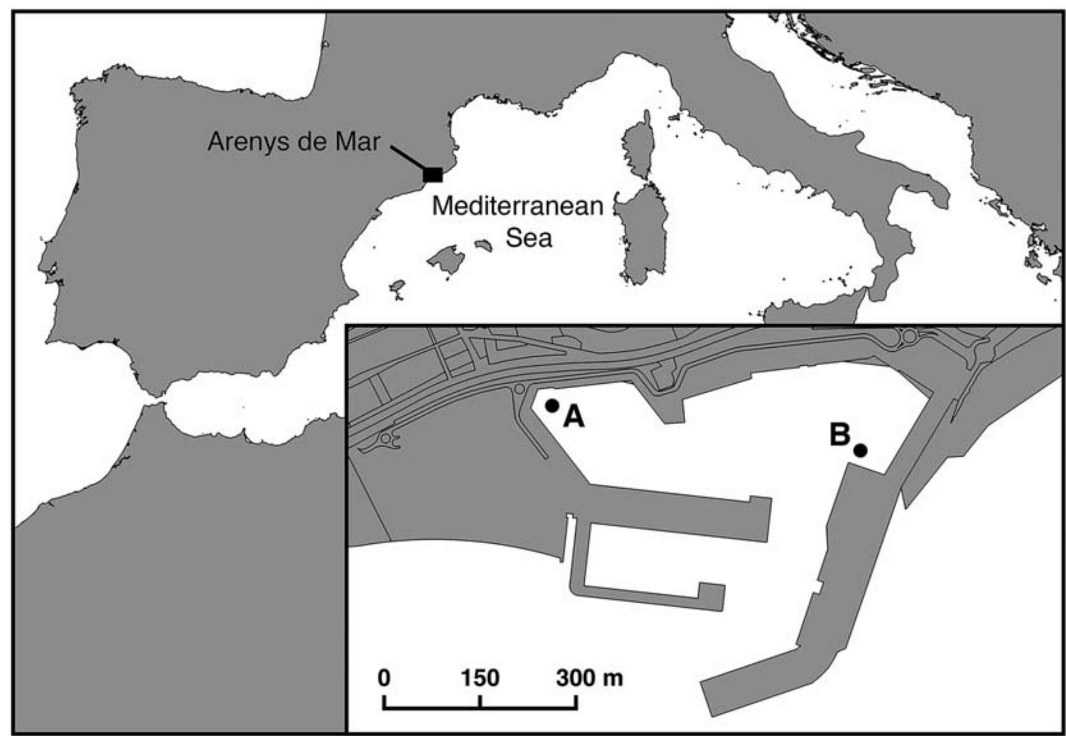

Figure 1. Location of Arenys de Mar harbor in the northwest Mediterranean Sea. The two sampling stations (A and B) are shown in the inset.

to April 2013, samples were collected at 12:00 GMT at locations A and B (Fig. 1) every 5-7 days for the whole *A. minutum* bloom period. To quantify the main dinoflagellate species abundances and standing stock of dinoflagellate hosts, 10- or 50-mL subsamples were fixed in Lugol (2%) and allowed to sediment in settling chambers for 24 h. A Leica–Leitz DMIRB inverted microscope fitted with epifluorescence filters was then used to count the cells in an appropriate (depending on cell abundances) area on phytoplankton settling chambers at 20× magnification (Andersen and Throndsen, 2003). *A. minutum* cells were identified by staining the thecal plates with Calcofluor white solution (Fritz and Triemer, 1985).

Occurrence of *Parvilucifera* parasitoids

Parvilucifera parasitoid occurrence was assessed in 6-L surface-water samples from January to May in 2009 and from January to July in 2010 to 2012. The seawater was pre-filtered through a mesh with a 60-µm pore size to discard possible predators and then incubated for 4–5 days at room temperature (20ºC) with natural light. Four L of the sample were concentrated by inverse filtration (10-µm pore size) and the presence of infected cells, defined as the detection of sporangia, was determined under light microscopy. Less than 10 sporangia per concentrated sample was considered as a low presence of *Parvilucifera* spp., and more than 10 as a high presence. Samples with no sporangia were considered as non-infected.

Identification and quantification of *Parvilucifera* infections

To quantify infections caused by *Parvilucifera* parasitoids during the *A. minutum* bloom in 2013 and because the dinoflagellate host sinks after infection (Alacid et al., 2015; Turon et al., 2015), we deployed two sediment traps to estimate the flux of infected cells at locations A and B (Fig. 1). Each trap consisted of a cylindrical collection vessel (height 33 cm, diameter 10 cm) moored 0.5 m from the bottom (the depth at both stations was 2 m). These traps were similar to those previously employed by Anglès et al. (2012) to quantify the encystment flux of a natural population of *A. minutum*. The traps were collected and replaced every 5–7 days (the same sampling frequency as that of the surface-water samples) throughout the bloom period (from January to April) in 2013. All settled material was fixed with formaldehyde (1% final concentration) and stored for 1 h in the dark at 4ºC. Subsamples (100 mL) were filtered through a mesh of 10-µm, rinsed with 250 mL of autoclaved seawater to remove small sediment particles, and then concentrated in 10 mL of autoclaved seawater in 15-mL Falcon tubes (BDFalcon). From the latter, 1–5mL were filtered onto 0.8-µm polycarbonate filters (25-mm diameter) using a vacuum pump at 150 mbar at room temperature. Cellulose-acetate support filters were used during filtration to promote the homogeneous distribution of the cells. The filters were cut into pieces with a razor blade and, to avoid cell loss, dipped in low-gelling-point agarose (0.1%), then they were dried face down on Parafilm. The filter sections were then mounted on a microscope slide, placed in a mixture consisting of four parts Citifluor and one part Vecta Shield containing 4′-6′-diamidino-2-phelylindole (DAPI) (final concentration 1 µg mL^{-1}), and stored at 4ºC in the dark until they were observed at 400× using an Olympus BX61 epifluorescence microscope. Ultraviolet excitation allowed detection of the DAPI signal of the host nuclei as well as the parasitoid nuclei in the sporangium stage. Blue light excitation was used to detect the green autofluorescence of *Parvilucifera* parasitoids in the sporangium stage vs. the red autofluorescence of host chlorophyll. *Parvilucifera* sporangia were classified into morphotypes based on the size and disposition of the nuclei inside the sporangia and on sporangial morphology. Micrographs were taken using an Olympus DP72 camera (Olympus

America Inc.) attached to the microscope. *Parvilucifera* sporangia were counted in 3-4 transects (~11 × 0.5 mm^2 each) across the filter piece to analyze a representative area of the whole filter. The detection limit under these conditions was ~75 cells L^{-1}.

Parasitoid infection flux and prevalence

The abundance of total parasitoid infections in the sediment traps was used to determine the daily infection fluxes (infected cells/m^2 day^{-1}) during the bloom. Infection fluxes were obtained by multiplying the abundance of infected cells (infected cells L^{-1}) by the trap volume (L^{-1}) and dividing first by the trap aperture (cm^2) and then by the corresponding interval of time (days) between the deployment and removal of the traps. Parasitoid prevalence on *A. minutum* was calculated as the percentage of infected cells of the total *A. minutum* population according to:

$$\text{Prevalence} = \frac{\text{infected cells}}{\textit{A. minutum}\text{ standing stock} + \text{infected cells}} \times 100$$

It was also determined with respect to total dinoflagellates as the percentage of infected cells of the total dinoflagellate community:

$$\text{Prevalence} = \frac{\text{infected cells}}{\text{Total Dinoflagellates standing stock} + \text{infected cells}} \times 100$$

This was done to compare the effect of these parasitoids on the blooming host population and on the total dinoflagellate community, since under laboratory conditions *Parvilucifera* species infect a wide range of dinoflagellate species, reflecting their large number of potential hosts in the field.

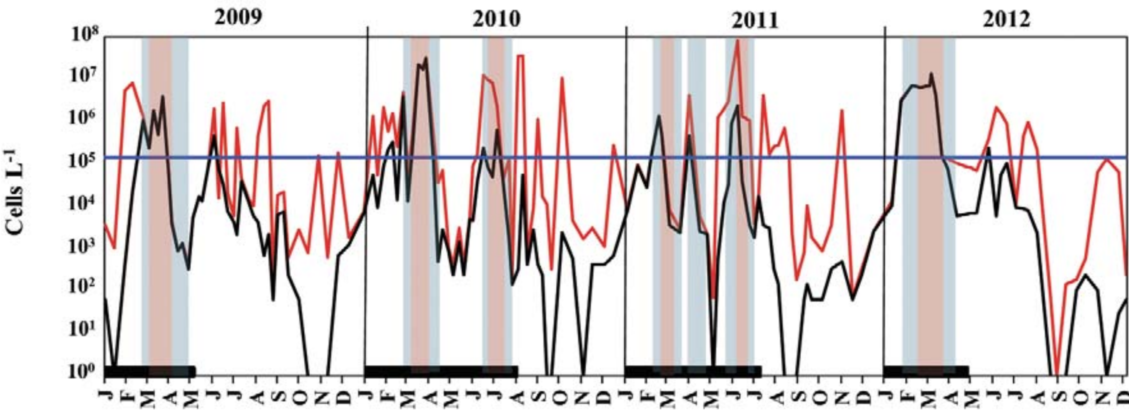

Figure 2. Seasonal patterns of total dinoflagellates (in red), and *Alexandrium minutum* abundance (in black) and parasitoid occurrence during a 4-year period (2009–2012) in Arenys de Mar harbor. The thick black line along the bottom indicates the sampling period for *Parvilucifera* parasitoids occurrence. The blue horizontal line is the concentration threshold (10^5 cells L^{-1}) of *A. minutum* needed to trigger *Parvilucifera* spp. infection. Blue and red shading indicates the low and high presence of *Parvilucifera* spp., respectively.

Host mortality due to parasitism

Host mortality induced by *Parvilucifera* parasitoids, i.e., the percentage of hosts killed per day, was estimated as described by Coats and Bockstahler (1994):

$$\text{Host mortality} = \frac{\frac{\text{Prevalence}}{\text{gt}}}{100}$$

where gt is the generation time from the sporangium stage until zoospore release, estimated to be 1.6 days. The gt was temperature corrected by applying the Q_{10} temperature coefficient of 2 units, considering the average ambient temperature (14ºC) recorded during the *A. minutum* bloom in 2013 and the 1 day needed for sporangia to release their zoospores at 20ºC (Turon et al., 2015). The Q_{10} is a measure of the rate of change of a biological system as a consequence of an increase in temperature of 10°C. It was calculated using the gt determined for *Parvilucifera sinerae* at 20ºC by Alacid et al. (2015) and at 15ºC by Råberg et al. (2014) under culture conditions (3.5 and 5 days, respectively). These gt values were then converted to per day rates of 0.2–0.28 day^{-1}, respectively. Finally, these rates were applied to the Q_{10} equation:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)}$$

To estimate the contribution of parasitoid-induced mortality to bloom termination, the *in situ* net mortality rate of *A. minutum* was calculated as the decrease in host cell abundance during the decaying phase, in this case from March 26th to April 23rd, following the method of Guillard (1973):

$$\mu = \frac{1}{(t_2 - t_1)} \ln \frac{N_2}{N_1}$$

where μ is the mortality rate in days^{-1} and N_2 and N_1 are the cell abundances at t_2 and t_1, respectively. The contribution of parasitoid infection to bloom termination was estimated based on the mean percentage of *A. minutum* and the mortality caused by *Parvilucifera* spp. during the decaying phase of the bloom.

Figure 3. Host-parasitoid dynamics during the winter bloom (from February to May 2013) in Arenys de Mar harbor at locations A (A–C) and B (D–F). (A, D) Major species composition and contribution to the total dinoflagellate abundance. Note the log scale. (B, E) Infection dynamics and prevalence during the bloom. The left-*y* axis is the infection flux (infected cells m^{-2} day^{-1}) and host cell abundance (cells L^{-1}) of the standing stock. The right-*y* axis is the prevalence (% of infected cells of the population). (C, F) The left-*y* axis is the contribution of the different *Parvilucifera* spp. sporangia morphotypes to the total parasitoid density (cells m^{-2}), and the right-*y* axis host mortality (day^{-1}) caused by the parasitic infection. Asterisks indicate the bloom phase: (*) initial phase, (**) exponential growth, and (***) bloom decrease.

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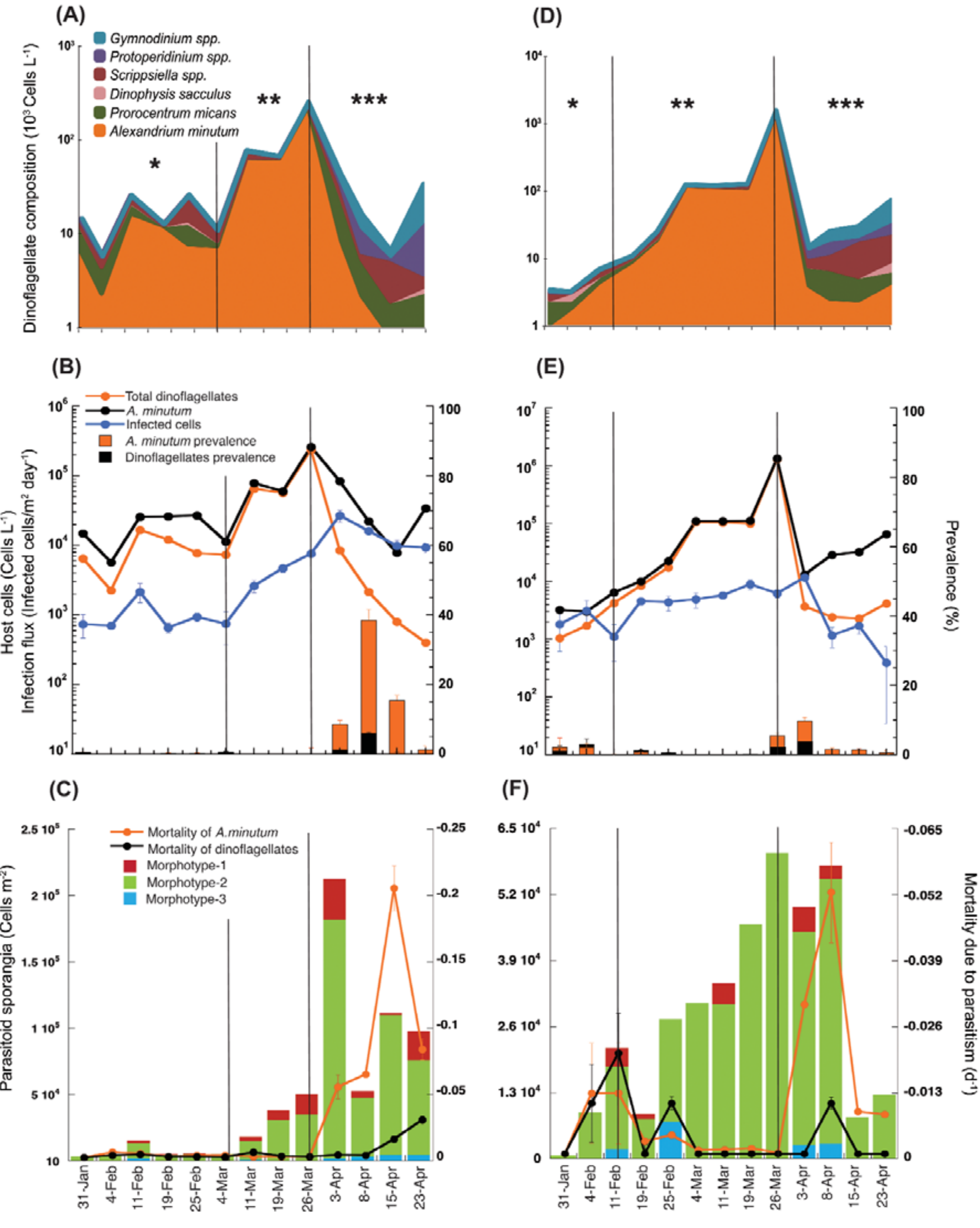
(A)
Gymnodinium spp.
Protoperidinium spp.
Scrippsiella spp.
Dinophysis sacculus
Prorocentrum micans
Alexandrium minutum
Dinoflagellate composition (10³ Cells L⁻¹)
(D)
(B)
Total dinoflagellates
A. minutum
Infected cells
A. minutum prevalence
Dinoflagellates prevalence
Host cells (Cells L⁻¹)
Infection flux (Infected cells/m² day⁻¹)
(E)
Prevalence (%)
(C)
Mortality of A.minutum
Mortality of dinoflagellates
Morphotype-1
Morphotype-2
Morphotype-3
Parasitoid sporangia (Cells m⁻²)
(F)
Mortality due to parasitism (d⁻¹)
31-Jan
4-Feb
11-Feb
19-Feb
25-Feb
4-Mar
11-Mar
19-Mar
26-Mar
3-Apr
8-Apr
15-Apr
23-Apr

RESULTS

Seasonal patterns of *Alexandrium minutum* and its parasitoids during 4 consecutive years

Dinoflagellates caused recurrent high-biomass blooms at location A every year from 2009 to 2012, where concentrations of up to 10^7 cells L^{-1} were recorded between June 2010 and 2011 (Figure 2). A recurrent peak in *A. minutum* abundance was consistently detected in winter (late February-beginning of March) and caused high-biomass blooms. Other peaks in the abundance of this species occurred in June and July, although the abundances were lower than those reached in winter.

During the sampling period, *Parvilucifera* parasitoids were detected every year, from 2009 to 2012, when the *A. minutum* reached bloom abundances as high as 10^4–10^5 cells L^{-1} (Figure 2). The parasitic infection in the surface waters was first detected when *A. minutum* abundances were > 10^5 cells L^{-1} (blue line in Fig. 2). After these peaks in host abundance, parasite occurrence continued, even when *A. minutum* abundance declined to 10^2 cells L^{-1}. In general, total *Parvilucifera* spp. occurrences lasted 1–2 months, depending on the duration of the specific bloom. In the first weeks of the bloom, the parasitoids had a low-level presence. However, when high host abundances were maintained over 2–3 weeks, a high presence of parasitoids was observed, including during the initial phase of the bloom decrease. Finally, at an advanced phase of bloom termination, the parasitoids again reached low abundances and continued to decline until they were no longer detected in the incubated surface samples.

Dinoflagellate abundance and composition, infection flux, and parasitoid prevalence during the 2013 winter bloom

During the sampling period, from February to April 2013 and at both sampling sites, the total dinoflagellate abundance increased two-fold from January to late March, reaching a peak on March 26th of up to 10^6 cells L^{-1} and decreasing to as low as 10^3 cells L^{-1} in early April (Figure 3A and D). Total dinoflagellate abundance coincided with the fluctuations of *A. minutum*, which was the dominant species during almost the whole sampling period. The bloom of this species lasted 2 months, from early February to early April. During this period, the dinoflagellate species composition changed depending on the bloom phase. Thus, initially, the dinoflagellate community was highly diverse, but composed principally of *A. minutum*, *Prorocentrum micans*, and *Scrippsiella* spp. Thereafter, *A. minutum* grew exponentially, with a several-fold increase in its abundance until it dominated the dinoflagellate community, representing up to 90% of the species contribution. Exponential growth stopped when the *A. minutum* reached a peak abundance of up to 10^5 cells L^{-1} at location A and 10^6 cells L^{-1} at location B. Abundances of10^5 cells L^{-1} were sustained for 10 days at location A and for 15 days at location B, and in the latter even increased to 10^6 cells L^{-1} during a 1-week period. After the peak of the bloom, during the decline in *A. minutum*, the dinoflagellate community again became more diverse, with an increased dominance of *Scrippsiella* spp. and *P. micans*, whereas *A. minutum* abundances reached their lowest values.

Parvilucifera spp. infections were restricted to dinoflagellates, but from a total of 17 recorded dinoflagellate taxa (data not shown) samples of only three of them were infected: *A. minutum*, *Scrippsiella* spp. and *P. micans*. However, while infected cells of *A. minutum* were observed throughout the bloom at both locations, a few infected cells of *Scrippsiella* spp. were observed

at location A on only two sampling dates, February 19th and March 4th, and only a single infection on *P. micans* on March 4th, also at location A. The detection of infected *Scrippsiella* sp. cells coincided with the maximum abundance of this dinoflagellate (10^4 cells L^{-1}) and with a decrease of *A. minutum*.

The infection flux caused by *Parvilucifera* spp. parasites followed dynamics similar to those of their hosts but with a one-period phase lag between *A. minutum* and *Parvilucifera* densities (Figure 3 B and E). The number of infected cells increased gradually at both locations albeit with a delay and pointed to a density-dependent response to the increase in host cells during the bloom period. At location A (Figure 3B), during the initial phase of the bloom, host cell abundance in the water column was relatively stable, with values of ~10^4 cells L^{-1}, until March 11th. During this period, the infection flux was also stable, with *Parvilucifera* spp. parasitoids infecting ~10^3 cells/m^2 day^{-1}. After March 11th, corresponding to the exponential growth phase of the bloom, the infection flux increased one-fold, reaching a maximum on April 3rd of up to 10^4 infected cells/m^2 day^{-1}. This

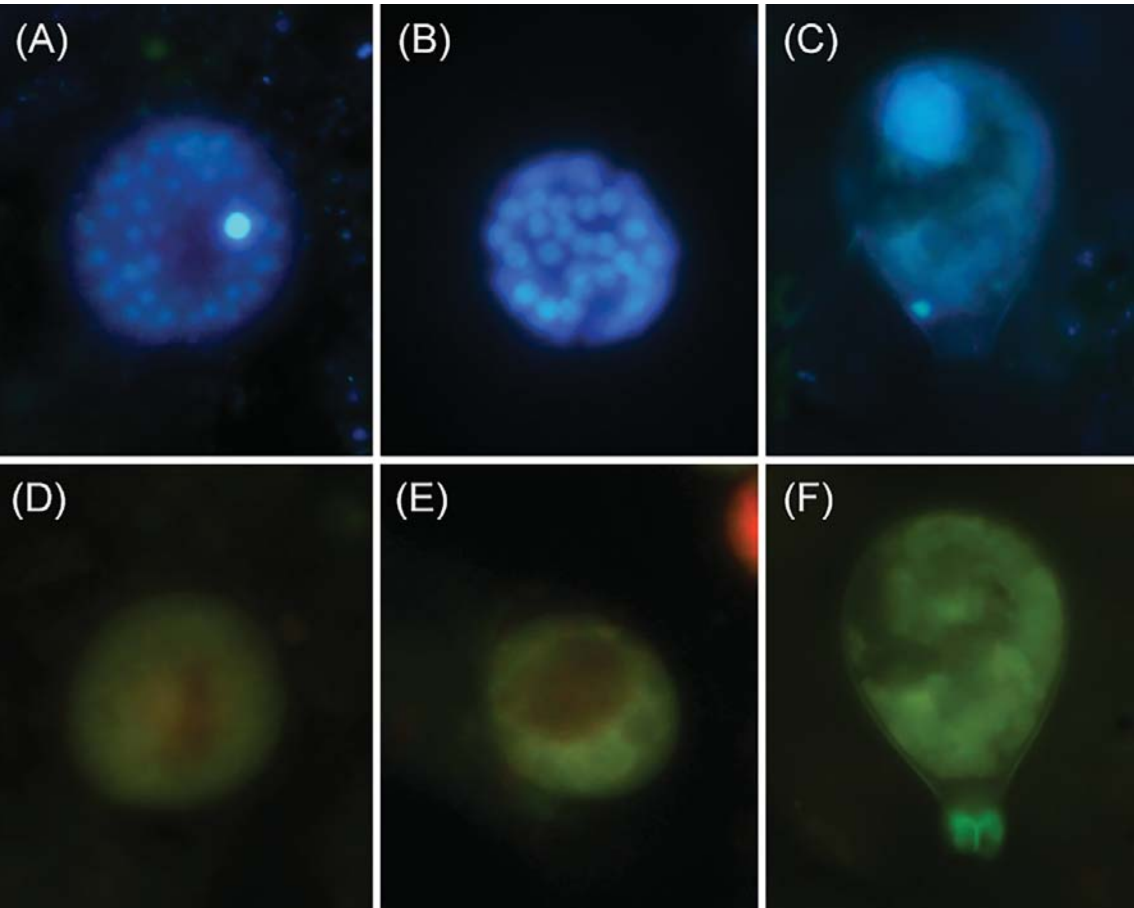

Figure 4. *Parvilucifera* spp. morphotypes identified in the sediment traps during the *A. minutum* winter bloom of 2013. (A–C) Sporangia and their DAPI-stained nucleus/nuclei. (D–F) The green autofluorescence of the sporangia under blue light excitation. (A, D) Morphotype-1; (B, E) morphotype-2; (C, F) morphotype-3.

maximum occurred immediately after the peak in the *A. minutum* concentration (10^5 cells L^{-1}) on March 26th. After the peak, the abundance of A. *minutum* gradually decreased, to 10^3 cells L^{-1}, although the total dinoflagellate concentration was 10^4 cells L^{-1}. The slight decrease in the infection flux coincided with the decrease in *A. minutum* abundance. At location B (Figure 3E), the initial phase of the bloom was much shorter, with the exponential phase starting on February 11th. The infection flux during this initial period was about 10^3 cells/m^2 day^{-1}, which slightly increased until a maximum of 10^4 cells/m^2 day^{-1} was reached on April 3rd. The *A. minutum* standing stock of vegetative cells at location B was one order of magnitude higher than that at location A, although the maximum infection flux (10^4 cells/m^2 day^{-1}), achieved immediately after the peak of the bloom, was the same at the two sites. Following the dramatic decline of the *A. minutum* population, by more than two orders of magnitude, the infection flux declined by one order of magnitude.

The parasitoid prevalences at locations A and B followed a similar pattern (Figure 3B and E), with very low percentages (0–5%) of the host population infected before peak bloom development during the initial phase and the exponential growth phase. The percentage of infected hosts increased after the bloom peak, with a mean of 18% at location A and 6% at location B and coinciding with the rapid decrease in *A. minutum*. Maximum prevalence values, reached between April 3rd and 15th, were much higher at location A than at location B, evidenced by maximum values of 38% and 12% of the *A. minutum* population, respectively. The impact of the parasite prevalence on the total dinoflagellate community was very low at both locations over the entire course of the bloom, with a maximum of 7% at location A and 4% at location B.

Parasitoid sporangial abundance and dinoflagellate mortality due to parasitic infection

Total sporangial abundance followed the same dynamics as the *A. minutum* density, with a higher abundance occurring close to the *A. minutum* (host) peak and then diminishing as the bloom declined (Figure 3C and F). At the initial phase of the bloom, sporangial abundance was slightly lower than during the exponential growth phase, ranging from 10^2 to 10^4 cells m^{-2} in the sediment traps. The mean sporangial abundance was $2.5{\cdot}10^3$ cells m^{-2} at location A and $4.5{\cdot}10^3$ cells m^{-2} at location B. After the maximum of *A. minutum* abundance, the sporangial density at location A (Figure 3C) increased by one order of magnitude, reaching concentrations of $2{\cdot}10^5$ cells m^{-2}. The concentration of sporangia decreased only marginally thereafter and was thus maintained at $\sim 5{\cdot}10^4$–10^5 cells m^{-2} during the decay phase of the bloom. At location B (Figure 3F), the maximum sporangial density in the traps was $6{\cdot}10^4$ cells m^{-2}, coinciding with the peak in *A. minutum* abundance but continuing for 2 weeks. This density was one order of magnitude lower than that in location A, although the host cell concentration was higher. Sporangial abundance underwent a sharp decrease when *A. minutum* abundance declined to $< 10^3$ cells L^{-1}.

Three different *Parvilucifera* sporangial morphologies were identified in the sediment traps placed during the *A. minutum* bloom. These three morphotypes could be distinguished based on nuclear distribution and sporangial morphology (Fig. 4). While we were unable to attribute morphotype-1 to any of the five existent *Parvilucifera* species described to date, morphotype-2 was linked to *P. sinerae*, based on the morphological similarities of the sporangia, determined under optical and epifluorescence microscopy, to those described in Alacid et al. (2015). Morphotype-3 was likely related to *Parvilucifera prorocentri*, due to the pear-shaped sporangial morphology (Leander and Hoppenrath, 2008). Morphotype-1 was the second most abundant parasitoid, with a density ranging from 10^2 to 10^4 cells m^{-2}. This morphotype was present in almost all samples obtained during

the whole bloom. Morphotype-2 was the most abundant in the sediment traps and was the cause of most of the infections. In addition, it was dominant during the whole bloom and at both locations (green bar, Figure 3C and F). At location A (Figure 3C), the density of morphotype-2 ranged from 10^3 to 10^5 cells m^{-2}. Morphotype-3 was only detected sporadically, before the peak of the bloom, and at low concentrations (10^2 cells m^{-2}). It was recurrently present at the late phase of the bloom, from April 3rd to 23rd and at a higher concentration (~10^3 cells m^{-2}). At location B (Figure 3F), the density of morphotype-2 was stable throughout the bloom period at ~10^4 cells m^{-2}, with the lowest abundances at the very early phase and again at the late phase. The other two morphotypes were intermittently detected over the course of the bloom at abundances of ~10^3 cells m^{-2}.

The highest *A. minutum* mortalities due to *Parvilucifera* parasitism occurred after the maximum parasite density (Figure 3C and F) and after the peak in the host concentration, coinciding with the bloom decay phase at both locations. Before the peak of host and parasite abundance, *Parvilucifera* spp. killed an average of 0.2% (i.e. –0.002 day^{-1}) and 0.7% (i.e. –0.007day^{-1}) of the host population per day at locations A and B, respectively. Host mortality increased after the peak of *Parvilucifera* abundance, with the parasites killing, on average, 10% (–0.1 day^{-1}) of the *A. minutum* population every day. The estimated mortality rate during the termination phase of the bloom was lower at location B than at location A, with 2.3% (–0.023 day^{-1}) of the host population killed. Maximum host mortalities of –0.21 day^{-1} and –0.053 day^{-1} at locations A and B respectively, were reached immediately after the maximum in *Parvilucifera* abundances. On average, at location A, *Parvilucifera* parasites killed 3.4% of the *A. minutum* population (–0.034 day^{-1}) each day. Considering a decrease of –0.2 day^{-1} in *A. minutum* abundance at the end of the bloom, parasitism due to *Parvilucifera* was estimated to account for 18% of the total *A. minutum* mortality at location A between March 26th and April 23rd, and for 5% at location B during the same period.

DISCUSSION

Coupled host-parasitoid occurrence and dynamics in the field

Alexandrium minutum is a potentially toxic dinoflagellate and a common species in the Mediterranean. In the NW Mediterranean, it is present at low abundances throughout the year in the studied location, but once or twice per year, mainly in winter, it proliferates to form high-biomass blooms. As demonstrated in this study, *Parvilucifera* infections accompany these outbreaks, becoming prominent when the dinoflagellate community is almost mono-specific (> 90%) for *A. minutum*. Algal blooms, with their low diversity and very high abundances, are temporary states of the dinoflagellate community. According to the diversity-disease hypothesis of Elton (1958), these communities are vulnerable to parasitic infection and transmission. Elton observed that infectious disease outbreaks due to parasitism most often involve dense, human-simplified communities, such as cultivated land, or, using the example of more recent cultivation systems, marine farmed species (Lafferty et al., 2015). Dinoflagellate blooms, with their lack of diversity and high cell densities, resemble these systems and thus also support parasitic occurrences. The high host densities reached during dinoflagellate blooms increase the rates of contact between the parasitoids and their hosts, thereby increasing infection transmission and parasitoid load to the system. By contrast, in the absence of a bloom, the low host densities reduce the probability of encounter with susceptible hosts and the infection accordingly subsides.

Infections by *Parvilucifera* parasites at the surface waters occurred only after a peak in *A. minutum* abundance, suggesting the existence of a density threshold that allows the establishment and transmission of *Parvilucifera* infections. As determined in this study, the minimum surface-water *A. minutum* concentration is ~10^5 host cells L^{-1}. Blanquart et al. (2016) followed *A. minutum* and *Parvilucifera* dynamics in two estuaries in France. Whereas qPCR failed to detect the parasite in the water column at the beginning of the blooms, *Parvilucifera* parasitoids were detected when the density of *A. minutum* reached 10^5 cells L^{-1} and the latter was the major contributor to the dinoflagellate community. The need for a minimum host density for infection establishment demonstrates a direct host density dependence, in agreement with the other density-dependent response described by Garcés et al. (2013b) for the same host-parasitoid system: sporangial activation from a dormant stage. In that process, higher host densities release high concentrations of dimethylsulfide (DMS), which through chemical signaling activates a higher proportion of sporangia containing dormant infective zoospores. DMS "informs" the parasitoid of the presence of a high host abundance in the environment and thus facilitates infection and transmission within the dense host population.

Moreover, as the bloom advances, the increasing host density provokes an increase in the number of infections, thereby enlarging the *Parvilucifera* population. Interestingly, parasitoid abundance was not in phase with the abundance of *A. minutum*, as *Parvilucifera* spp. followed a time-delay response to the temporal fluctuations of its blooming hosts, similar to the temporal dynamics of predator-prey interactions. We observed that the flux of infected cells is characterized by a lag with host abundance such that a peak was reached after the host cells achieved their maximum density. Then, the parasitoid population in the water column decreased as the bloom declined. This finding is consistent with a direct negative density dependence such as also occurs for predator-prey systems, and sometimes, but not as usual for parasite-host dynamics (Begon et al., 2005). Comparable dynamics were also observed in the study by Blanquart et al. (2016) described above and for parasitoids belonging to the *Amoebophryidae* during blooms of several dinoflagellate species in different locations (Coats and Park, 2002; Chambouvet et al., 2008; Alves-de-Souza et al., 2012). Both *Amoebophryidae* and *Parvilucifera* grow inside the host, killing it as an obligate part of their life-cycles. Therefore, the ecology and population dynamics of parasitoids lie somewhere in between those of predators and true infectious parasites, such as bacteria and protozoa (Hassell, 2000).

The parasitoid community during the bloom could be classified into three *Parvilucifera* morphotypes. The dominance and coupled dynamics of morphotype-2 (*P. sinerae*), during the *A. minutum* bloom agreed with the results reported by Turon et al. (2015), who, based on the 18S rDNA gene, determined that all *Parvilucifera* isolates from *A. minutum* blooms from the Atlantic and Mediterranean coast (most of them in Arenys de Mar harbor) were *P. sinerae*. Although in host-range laboratory experiments using monospecific cultures *Parvilucifera* species were shown to be generalist pathogens of dinoflagellates (Norén et al., 2001; Garcés et al., 2013; Lepelletier et al., 2014), the persistent occurrence and dominance of *P. sinerae* during *A. minutum* natural blooms indicates a greater specialization of *P. sinerae*. This field specificity agrees with the preference of *P. sinerae* for *A. minutum*, as demonstrated in an artificial mixed community (Alacid et al., 2016). A strong in situ specialization is known for *Amoebophrya* parasitoids. Chambouvet et al. (2008) reported the coexistence of several *Amoebophrya* clades, with consecutive blooms caused by a different dinoflagellate species and followed by an increase in the abundance of a specific parasitoid clade. Whether *Parvilucifera* species are characterized by the same dynamics as *Amoe-*

bophrya requires further study on the *in situ* specificity of these parasitoids, which would reveal details of their species- and community-level dynamics as well as their co-evolution with their hosts.

The host density threshold required to trigger a *Parvilucifera* infection and the delayed density-dependent response of the parasitoid to host abundances have relevant sampling implications. Thus, a specific parasitoid will be significantly abundant in the water column only if its host is also abundant. If at the time of sampling, the host is absent or its density is below the threshold for infection, the parasitoid will not be detected. Moreover, studies of the ecology of *Parvilucifera* must also take into account the meroplanktonic life cycle of these parasitoids, which have a documented benthic stage and thus alternate between the water-column and the sediment. Taken together, our results explain why, in discrete environmental samples, *Parvilucifera* parasitoids were reported to be more abundant and active in the marine sediment (Chambouvet et al., 2014) than in the water column, where they have been scarcely detected (de Vargas et al., 2015; Lepère et al., 2015; Massana et al., 2015).

Parasitism and bloom termination

Historically, studies on the causes of dinoflagellates bloom decline only considered environmental and physico-chemical factors (Margalef, 1978). However, recent studies have identified biological interactions, such as parasitism and grazing, as important factors in bloom dynamics, since the nature of those interactions affect host population densities (Coats et al., 1996; Calbet et al., 2003; Montagnes et al., 2008; Velo-Suárez et al., 2013).

To date, the impact of eukaryotic parasitism in dinoflagellate blooms has been studied in *Amoebophrya* whereas that by the genus *Parvilucifera* in natural populations is unknown, as only data from laboratory experiments are available. The estimated prevalence of *Parvilucifera* during the *A. minutum* natural bloom, as determined in this study, reached a maximum of 38%, which is much lower than demonstrated in laboratory experiments (> 90% for clonal strains) (Alacid et al., 2015; Alacid et al., 2016). These differences can be attributed to the many factors in the field that are absent from laboratory conditions, including factors that cause direct parasitoid losses, such as grazing on the free-living infective zoospores (Johansson and Coats, 2002), and those that reduce infection success, such as dinoflagellate vertical migration (Coats and Bockstahler, 1994), host genetic diversity, and abiotic conditions such as turbulence (Llaveria et al., 2010).

Although locations A and B were in the same harbor, they differed remarkably with respect to parasitoid prevalence and host mortality, which suggests the high spatial heterogeneity and patchiness of parasitism as also reported for dinoflagellate vegetative cells during blooms (Garcés et al., 2004). Since host density appears to be fundamental to infection and transmission, a higher infection level was expected at location B, where *A. minutum* abundance was an order of magnitude higher, than at location A. However, parasitoid prevalence and host mortality were higher at the latter location, which points to the role played by the differences in the physical and biological factors of the two locations. Location B is close to the entrance of the harbor and is therefore more exposed and its waters more turbulent. Turbulence is an important factor that reduces parasite infectivity (Llaveria et al., 2010). Conversely, location A is more protected such that its low hydrodynamics and confinement promote infection and higher prevalences, similar to laboratory conditions. Further studies assessing the role played by abiotic factors on parasitic infection will

allow a better understanding of the variability in parasitic infection observed in natural blooms. As previously suggested for differences in *P. infectans* infectivity in the Swedish coast (Johansson et al., 2006), differences in the community composition of predators may also influence infection rates in the field, as demonstrated for the role played by a community rich in grazers in preventing high infection levels (Johansson and Coats, 2002; Kagami et al., 2004). Although we observed ciliates preying upon *Parvilucifera* zoospores in microscopic observations of living natural samples in the laboratory, nothing is known about the biological interactions of *Parvilucifera* parasitoids with non-host organisms present in the plankton community. Indeed, studies on the relationship of *Parvilucifera* with other trophic levels would be a step forward in the study of energy transfer within marine food webs and in ecological modeling.

In the present study, during the whole bloom period *Parvilucifera* killed, on average, a low percentage (1–3%) of the *A. minutum* planktonic population per day. It was at the end of the bloom, coinciding with the sharp decrease in *A. minutum* abundance, when *Parvilucifera* reached higher prevalences causing maximum host mortalities. Such patterns were also observed in *Amoebophrya* infection dynamics in the field (Alves-de-Souza et al., 2012; Velo-Suárez et al., 2013). In fact, the host mortalities caused by *Amoebophrya* infection may be so extreme that the bloom collapses (Chambouvet et al., 2008; Mazzillo et al., 2011). Based on a 5–18% decrease in the total *A. minutum* population attributable to *Parvilucifera*, it can be concluded that parasitism strongly influences bloom dynamics, as it leads to losses that are of the same order of magnitude as those due to other biological factors, such as encystment (Anglès et al., 2012) and grazing (Calbet et al., 2003). However, it is not the only cause of bloom termination, since density-dependent disease systems were previously shown to be significantly less likely to cause the extinction of a population (Jaffee et al., 1992). In the case of *Parvilucifera* parasitoids and dinoflagellates, since their interaction is host-density dependent, the natural course of the infection will lower the density of the host and thus its contact rate with the parasitoid. Fewer host individuals and lower infection rates will lead to the establishment of a population equilibrium between dinoflagellate cells and their parasitoids.

Our study demonstrates that *Parvilucifera* dynamics are well adapted to those of their blooming hosts and therefore that eukaryotic parasitism is an important factor accounting for biological loss during dinoflagellates massive proliferations. In addition, parasitism exhibits both temporal and spatial heterogeneity during high-biomass blooms. Further investigations of the effects of abiotic and biotic factors on the ecology of these parasitoids are needed to understand parasitoid abundance, host interactions, and the link with other trophic levels of the marine food web.

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General Discussion

General Discussion

Host- parasite interactions have been historically neglected in the study of marine planktonic communities, because the identification of these organisms in nature with traditional methodologies, such as light microscopy and dependent-culture methods was difficult due to i) the divers morphology of their life cycle stages, ii) the small size and scarcity of morphological characters of the free-living stage, and iii) the difficulty to culture them. Recently, the advances in molecular tools combined with advanced microscopy techniques have allowed the ultrastructural and phylogenetic identification and characterization of some species, being some of them available in culture. These studies, together with the era of the "environmental sequencing" have placed parasitism due to zoosporic protists as key components of marine communities (Guillou et al., 2008; Chambouvet et al., 2014; de Vargas et al., 2015). However, fundamental questions regarding their ecology and diversity, such as who are they, who infect whom, which is their life cycle, and their parasitic strategy remains unknown. For these reasons, the present thesis focused the research on host-parasite interactions in marine coastal phytoplankton communities, by studying *Parvilucifera* parasites infecting dinoflagellates as a model organism.

The analysis of this relationship at different scales, from cells to communities, and combining laboratory and field studies, allowed us to understand the dynamics of these parasites in their natural environment, which is discussed below. Moreover, it has also allowed us to detect some methodological problems or limitations in the study of these organisms that are also summarized in this section.

We will finish the discussion recommending the future directions that need to be addressed in the field of research concerning marine protists host-parasite interactions.

Conceptual model of *Parvilucifera sinerae* ecology and population dynamics

Understanding *Parvilucifera sinerae* ecology implies the characterization of its patterns of distribution and prevalence in their natural host populations and the factors responsible for these patterns. For that, it is important to comprehend its life cycle, its mode of transmission (how parasites move from one host to the next) and the factors that may limit transmission in natural populations.

Parvilucifera sinerae present a direct life cycle that causes the host death, with short generation times, and high asexual reproduction rates, producing a huge offspring from a single infection in preferred host populations (**Chapter 1, Chapter 3**). The life cycle is meroplanktonic, alternating between a free-living zoospore that swim in the water column and a benthic stage, the sporangium, that remains in the marine sediment. The parasitoid presents horizontal transmission, where once the parasite infects a host and develop the sporangium, the infective zoospores are released to the environment to infect other hosts (**Chapter 1**).

The coupled ecology and dynamics of *Parvilucifera sinerae* and *Alexandrium minutum* in coastal marine environments show the following paths (Figure 1). In phytoplankton communities, under certain conditions, the growth of a particular dinoflagellate species is enhanced. In that case, when the development of the toxic *A. minutum* is favoured, vegetative cells quickly proliferate

asexually, increasing their abundance. Since one of the mechanisms that activate the parasitoid from their dormant stage is dymetilsulfide (DMS) released by *A. minutum* cells, the increase of host abundance produce a higher release of that compound to the environment which may activate the dormant *Parvilucifera* zoospores (**Chapter 2**).

If *A. minutum* continue growing, its population can reach a high cell abundance, 10^4-10^7 cells L^{-1},

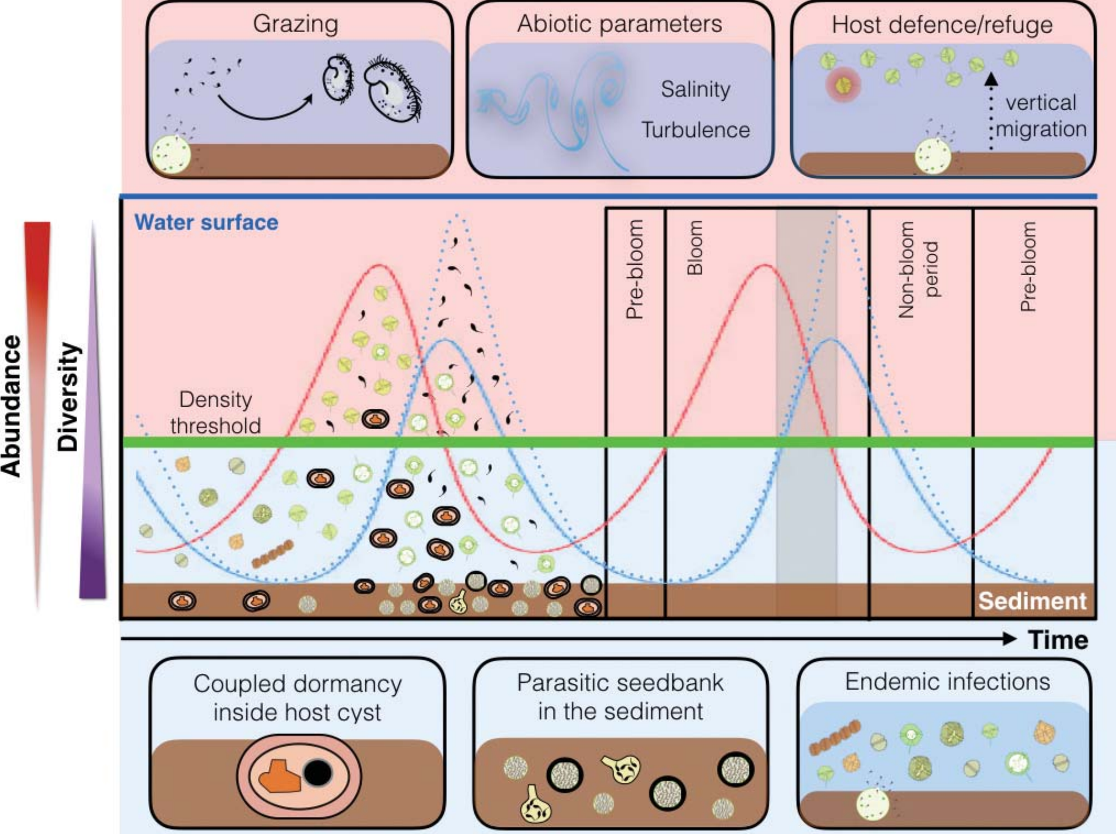


Figure 1. Conceptual model of *Parvilucifera sinerae* ecology and population dynamics, including the water column and the marine sediment. Y-axis represents the changes in abundance and diversity of the dinoflagellate community and parasitoid abundance. X- axis represents the time. Changes in abundance of host and parasitoid stages during time: *Alexandrium minutum* vegetative cells (red line), non-motile stages of *P. sinerae* (light blue line), and *P. sinerae* infective swimming zoospores (light blue dashed line). Abundance threshold beyond which *P. sinerae* infection becomes established and transmitted (horizontal green line). Period of higher prevalence and mortality levels (vertical grey shadow). Periods of high host cell abundance (upper pink) and low host cell abundance (bottom blue). Potential factors controlling the infection levels of *P. sinerae* during periods of high host abundance (insets on the upper pink). Hypotheses of *Parvilucifera* survival strategy during periods of host absence or low abundance (insets on the bottom blue).

referred here as blooms. When *A. minutum* reached a density threshold of 10^5 cells L^{-1}, *Parvilucifera* infection can be sustained in the host population (**Chapter 5**). These dense blooms are transitional states of the dinoflagellate community, where *A. minutum* became the dominant species, therefore decreasing the diversity. Since the mechanisms of these parasites are density-dependent, during *A. minutum* bloom periods, *P. sinerae* infection and transmission is enhanced, due to the increased contact rates between the preferred hosts and the parasitoid (**Chapter 4, Chapter 5**). Such high number of infections, produce a high load of sporangia, that afterwards would imply an increase in the number of *Parvilucifera* zoospores released in the marine environment. Since *P. sinerae* transmission depends on the abundance of its host, their population dynamics present a delayed density-dependent response to host abundances, existing a lag between maximum vegetative *A. minutum* cells, infected cells and *Parvilucifera* zoospores abundance, similar to the temporal dynamics of predator-prey interactions (Salomon and Stolte, 2010).

During the evolving *A. minutum* bloom, when vegetative cell losses are higher than the gain in cell abundance, the bloom decay until it finishes. These cell losses are due to both, biotic factors, such as cell mortality, encystment and negative interspecies interactions, and abiotic mechanisms like dispersion (Steidinger, 1973; Anderson and Lindquist, 1985; Pfiester, 1987; Garcés et al., 1999). It is during the decaying phase of the bloom, when maximum *Parvilucifera* prevalences and maximum mortalities due to parasitism occur (**Chapter 5**). The constant reduction of the number of host cells during the bloom demise, decrease the contact rate between the zoospores and the host, limiting parasitoid transmission and therefore diminishing the number of infections until negligible. In the case of *Parvilucifera*, cell losses due to parasitism are of the same order of magnitude than other biological factors, such as grazing and encystment (Calbet et al., 2003; Anglès et al., 2012).

In the general dynamic patterns of *P. sinerae* during the outbreaks of its host, there is spatial and temporal heterogeneity in terms of the impact these parasitoids cause in their host populations. Intra-bloom variability in terms of prevalence, mortality and therefore parasitoid abundance exists (**Chapter 5**). During periods of high host abundances, local biotic and abiotic factors may limit parasitoid transmission determining the maximum infection levels and mortalities achieved (Figure 1 insets on the upper pink).

Among the biological ones, studies in other zoosporic parasitoid-phytoplankton host systems, demonstrate that grazers feeding on zoospores can heavily reduce parasitic infection (Johansson and Coats, 2002; Kagami et al., 2004). Grazing on *P. sinerae* zoospores observed in laboratory mainly due to ciliates, would directly decrease the number of the infective stage in the marine environment, decreasing the contact rate between the uninfected hosts and the parasitoids, hence reducing the infection and transmission.

Abiotic factors, such as salinity and turbulence have been reported having an effect in infectivity. Changes in salinity can be produced by punctual and localized freshwater discharges in coastal sites, which would produce differences in infectivity since low salinities promote both *P. sinerae* sporangial germination and higher rates of infection (Figueroa et al., 2008). Moreover, more confined or exposed sites, which have different hydrodynamics, would also provoke differences in infectivity since turbulence have been demonstrate to reduce *P. sinerae* infection (Llaveria et al., 2010).

The host itself may have some defence mechanisms to avoid infection, but are still unknown. Ecological refuges may also avoid host being parasitized, such as host vertical migration to surface layers of the water column, where the swimming zoospores may not have access, or other spatial mismatch between host and parasite reducing infection levels (Park et al., 2002).

While the present thesis has contributed to understand *P. sinerae* dynamics during high host abundances, the survival strategy during periods of host absence or low abundance remains to be determined. We will discuss three plausible hypotheses (Figure 1 insets on the bottom blue).

The first hypothesis relay in the study of Chambouvet et al. (2011), where under culture conditions the presence of the parasite *Amoebophrya* promoted dinoflagellate cyst production and its zoospores encysted within their host. During the *A. minutum* bloom, it also forms resting cysts, being the encystment flux higher when the abundance of vegetative cells in the water column is greater (Garcés et al., 2004; Anglès et al., 2012). The coincidence of the high host abundance, the resting cyst formation and the maximum infection caused by *P. sinerae*, make us to propose that zoospore encystment can occur within their host. Both, the parasite and the host, would enter in a coupled dormancy period until their germination months later, re-establishing the host its asexual phase, and the parasitoid the infection.

A second hypothesis is the existence of long-term viable parasitic seedbank that would play the same role than resting cysts in dinoflagellates life cycle. Sporangia would remain dormant in the sediments under adverse conditions (absence or low host abundances) until the next host proliferation. This idea relies on: i) the short life-time of zoospores outside the host but longer life span as sporangium; ii) the high number of sporangia produced at the end of the bloom that sink to the marine sediment; and iii) the density-dependent response of *P. sinerae* biological processes, i.e. the activation response of sporangia (**Chapter 2**) and the density threshold needed for a well-established infection (**Chapter 5**). In the absence or low abundance of *A. minutum*, the parasitic seedbank would contain dormant *P. sinerae* sporangia and *A. minutum* resting cysts. When conditions would be appropriate, resting cysts of dinoflagellate would germinate, re-establishing the vegetative planktonic phase, which by asexual reproduction proliferate causing a bloom. *P. sinerae* sporangia would be activated under these favourable conditions, those ones during *A. minutum* blooms. The high reproduction and transmission of *P. sinerae* during such conditions, would lead to an increase of new sporangia nourishing the parasitic seedbank.

The third hypothesis is that *P. sinerae* subsist causing endemic infections at low prevalences in other non-type hosts, until *A. minutum* became the dominant species and cause a bloom again. In that case, when *A. minutum* bloom decease and the host community diversity increase, *P. sinerae* population could suffer a dilution effect, being undetectable in the water column due to low abundances. This hypothesis is based on the laboratory results that define *P. sinerae* as a generalist parasitoid (**Chapter 3**), which although presenting a preference for certain host species, especially for its primary host *A. minutum* (**Chapter 4**) it can infect many different dinoflagellate species.

Methodological problems and limitations in the study of *Parvilucifera* parasitoids

The study of ecology of *Parvilucifera* parasites of phytoplankton and their interactions is challenging due to several reasons.

Early stages of *Parvilucifera* infection are very difficult to recognise in nature with traditional microscope techniques such as optical microscopy. Furthermore, despite being able to recognise such stages, it is not possible to identify the *Parvilucifera* species causing the infection. The parasitoid life-cycle stage that can be easily recognized is the sporangium, which is a distinctive morphological feature in the taxonomy of *Parvilucifera* species. However, when the sporangium is developed, the host has been completely destroyed being unidentifiable and therefore challenges the correct identification of the host. Consequently, the study of specificity in the field (who infects whom), as well as the quantification of parasitoid impact (prevalence) on dinoflagellates communities is challenging.

The detection of early stages of infection and the identification of both partners of the parasitic association can be solved by using a combination of molecular methods using specific probes of the target organisms, i.e. fluorescent in situ hybridization (FISH) techniques combined with other fluorescent stains. For instance, the use of a tyramide signal amplification-fluorescence *in situ* hybridization (TSA- FISH) approach to detect specific *Amoebophrya* clades, combined with Calcofluor-white stain, which binds to cellulose of the thecate dinoflagellate hosts, has been used to study the specificity of Syndiniales group II (Chambouvet et al., 2008). Another study, in order to quantify the role of *Amoebophrya* parasites in the termination of *Alexandrium fundyense* blooms, used whole cell FISH conjugated to a Cy3$^{\%}$fluorochrome to identify *Alexandrium* cells, combined with TSA-FISH to detect *Amoeboprhya* life-cycle stages (Velo-Suárez et al., 2013).

In the case of *Parvilucifera* parasitoids, the existent probe designed by Johansson et al. (2006) for *P. infectans*/*P. sinerae* was successfully used to describe the life cycle of *P. sinerae* (**Chapter 1)** combining TSA-FISH with confocal microscopy under culture conditions. Although the technique succeeded to mark all the stages of *P. sinerae* life cycle, it failed to mark all the infected cells. This hindered the use of this technique in **Chapter 5** to quantify *in situ* the occurrence and impact of *Parvilucifera* infections during recurrent *Alexandrium* blooms. Since we find several *Parvilucifera* species coexisting, together with the fact that the probe failed in marking all the infected cells, we would have not been able to unequivocally identify and quantify *P. sinerae* in the parasitic pool and the use of these technique was discarded. The reasons why the probe fails have not been addressed, but the optimization of this technique for the study of *Parvilucifera* and other Perkinsozoa parasitoids is urgently needed because of their potential.

Although FISH can solve the difficulty to identify cell-cell interactions at early stages of the infection, the problem of assigning late stages of infection to a certain host remain unresolved. This is especially challenging when studying *Parvilucifera* parasites, which have been described as generalists, presenting a wide host range tested under laboratory conditions. In that case, is necessary to further study their field specificity, to distinguish between the potential host-range, which includes all the species that is capable of utilising as a host, and the field host range which is what actually happens. The field host-range is frequently a subset of the potential host-range, because the parasite and the potential host may never coincide in space and time (Van Klinken, 1999). Reducing the list of host species used in field by *Parvilucifera* parasitoids may help to infer from which host was infected in the field when it is partially or totally destroyed and cannot be identified.

Although the use of molecular probes is very promising, we should be cautious when using it to study the ecology and distribution of *Parvilucifera* species, since the environmental studies are

reporting a growing number of divers sequences belonging to unknown Perkinsozoa organisms and new species of *Parvilucifera* are lately being described (Bråte et al., 2010; Chambouvet et al., 2014; Lepelletier et al., 2014b; Reñé et al., 2016). The probes thought to be general for *Parvilucifera* at the moment of their design, designed with the molecular information available at that time, may not be such general as previously thought.

Future directions

With the knowledge acquired throughout the development of the present thesis, we give some recommendations about the future work needed to advance in the knowledge of Perkinsozoa parasites, and specifically, in the field of *Parvilucifera* parasitism in marine phytoplankton.

- A great effort should be placed in the identification and culturing of Perkinsozoa representatives from different systems, given the huge diversity of unclassified Perkinsozoa sequences revealed by environmental studies in aquatic environments worldwide (Lepère et al., 2008; Bråte et al., 2010; Mangot et al., 2011; Chambouvet et al., 2014; Chambouvet et al., 2015; Reñé et al., 2016). Since these organisms also infect many different type and distantly-related hosts, from fish, frogs, bivalves and phytoplankton (Bower et al., 1994; Park et al., 2004; Chambouvet et al., 2015; Freeman et al., 2017), probably we are in front of one of the successful aquatic parasitic branches of the tree of life, like the Apicomplexans, being its study crucial to understand the origins of parasitism.

- Environmental molecular studies have unveiled that most eukaryotic plankton biodiversity belonged to heterotrophic protistan groups, particularly those known to be parasites or symbiotic hosts (de Vargas et al., 2015). Moreover, they are key players in planktonic food web linkages across taxa (Lima-Mendez et al., 2015) and therefore are crucial in marine biogeochemical cycles (Worden et al., 2015). For that, further research is needed to confirm and quantify organism associations indicative of parasitism, by using novel approaches such as the combination of targeted flow cytometry cell sorting and dual-label fluorescence in situ hybridization (FISH) (Lepère et al., 2015). For instance, zoosporic fungi (chytrids) play an important role in aquatic food webs of freshwater ecosystems, directly feeding zooplankton or facilitating the trophic interaction between microalgae and grazers (Kagami et al., 2014). *Parvilucifera* parasites are likely to play a similar role in coastal marine environments, still nothing is known about their biotic interactions with organisms from other trophic levels. Revealing *Parvilucifera* associations with other organisms belonging to higher and lower trophic levels, will be an advance in the ecology and dynamics of these parasites as well as, to contribute to the knowledge of complex food webs.

- Several studies on parasitism of dinoflagellates during blooms, reported the coexistence of several parasitic clades or species at the same place (**Chapter 5**; Chambouvet et al., (2008); Lepelletier et al., (2014a); Lepelletier et al., (2014b); Blanquart et al., (2016)). Although in coastal areas, Perkinsozoa has been reported more abundant in the marine sediment and Syndiniales in the water column (Chambouvet et al., 2014; Massana et al., 2015), further study about the spatial segregation of these parasites, their infectious temporal window, and the inter-species relations within them, will contribute to the understanding of the structure and dynamics of such parasitic communities in coastal planktonic systems. Moreover, how these parasites survive in nature during the absence of their hosts, still re-

mains unknown. Answering these fundamental questions would help to advance in understanding the life-cycle and ecology of these parasites.

- The host range can be a useful tool for identifying certain parasite and host species in nature, since some of these intimate associations are highly specific and both partners always co-occur in the field. Host range of *Parvilucifera* species tested under laboratory conditions, supported that they are generalist parasitoids of dinoflagellate hosts (Chapter 3; (Norén et al., 1999; Norén et al., 2001; Lepelletier et al., 2014b). However, the noticed preferences of some of these parasites for *Alexandrium* species (**Chapter 4**, Lepelletier et al., (2014b)), together with their persistence occurrence in field blooms of *A. minutum* (**Chapter 5**; Turon et al., (2015); Blanquart et al., (2016)) highlight that their specificity is not yet well-understood. Future research in this topic should focus in the molecular mechanisms underlying specificity in these parasites, and to constraint their field host range. Furthermore, to study whether parasite distribution is related to that of their hosts is needed.

- Since new *Parvilucifera* species have been cultured, new sequences of 18S DNA ribosomal genes regions are now available (Lepelletier et al., 2014b; Turon et al., 2015; Reñé et al., 2016). The development of molecular probes and primers and the optimization of molecular techniques, such as TSA-FISH and qPCR, would be a step forward in: i) the identification and quantification of such host-parasite interactions; ii) to advance in the knowledge of *Parvilucifera* specificity in nature; and iii) to better comprehend the dynamics of the different life-cycle stages in the field.

- To study the interactions between *Parvilucifera* and their dinoflagellate hosts at molecular level, thorough cutting-edge methodologies such as single cell 'omics' methods, and specifically single cell transcriptomics (Kolisko et al., 2014; Liu et al., 2017) will allow to investigate the genes that encode functions that underpin these interactions in natural ecosystems, and infer their ecology and evolution.

Conclusions

Conclusions

1. *Parvilucifera sinerae* are endoparasitoids of dinoflagellates, with a direct life cycle, presenting short generation times (3-4 days), and a high asexual reproduction rate, producing a huge offspring from a single infection. The life cycle is meroplanktonic, alternating between free-living zoospores that swim in the water-column and a benthic stage, the sporangium, that remains in the marine sediment.

2. *P. sinerae* life-cycle stages present ultrastructural features shared with the other species belonging to *Parvilucifera* genus, with Perkinsus and with some apicomplexan parasites. The trophocyte or feeding stage, develops within the host, inside a parasitophorous vacuole. Trophocyte nucleus divide by schizogony, forming a multinucleate sporocyte stage. The formation of the new zoospores inside the sporangium occurs in a process referred as "budding". These characteristics have been previously reported in some Apicomplexa, such as *Plasmodium*, the causative agent of Malaria in humans. Whereas *Perkinsus* species also develops inside the parasitophorous vacuole, differ in the type of nuclei division and zoospore formation.

3. *P. sinerae* infection is a denso-dependent process that depends on the contact rate between the infective zoospores and the host.

4. The algal metabolite dimethylsulphide (DMS) released by dinoflagellates have been identified as one of the mechanisms that activate *P. sinerae* sporangium stage from dormancy, inducing the release of the infective zoospores. The response rate was proportional to the DMS concentration down to a threshold close to 30 nM. DMS may act as a generic density-dependent cue of the presence of potential hosts in the marine environment.

5. *P. sinerae* zoospores are attracted by dinoflagellate cells, but does not select among the potential hosts to infect. Instead, the parasitoid attacks all hosts encountered regardless of the species susceptibility. Thus, the infection strategy of *Parvilucifera* is a game of chance, where the zoospores seek out and contact the potential host at random. A successful infection will occur if the contacted host is susceptible to the parasite.

6. *P. sinerae* reproduction rate in dinoflagellate host populations is a balance between: i) the number of zoospores produced by sporangium, ii) the generation time, and iii) the number of sporangia produced in a certain host population, which depends on host susceptibility.

7. *P. sinerae* is a host-generalist parasitoid. It is able to infect at least 15 different genera, including thecate and athecate species. Potential hosts include toxin producers and harmful species. Some dinoflagellate genera are more susceptible to infection than others. Moreover, intra-strain variability was observed as well. Nevertheless, *P. sinerae* is unable to infect some dinoflagellate genera, neither haptophytes, diatoms, and chlorophytes.

8. *P. sinerae* shows preferences for *Alexandrium* and *Scrippsiella* species, which maximize parasitoid transmission. Such preferences could be the result of historical sympatry of *P. sinerae* and its primary host *A. minutum*. The degree of host specificity may be determined by host phylogeny, whereby the parasitoid easily infects more closely related dinoflagellates.

9. *P. sinerae* infection strategy, being able to infect different species successfully, while having a higher fitness in a few of the hosts, could be a good strategy in unpredictable and changing phytoplankton communities. Such strategy, could allow the parasitoid to survive or maintain a small population when preferred hosts become rare in the community. *P. sinerae* infectivity in the field will be determined by both, host community structure and susceptibility.

10. Since *P. sinerae* is a generalist parasite that exhibits host preferences, it may differentially impact dinoflagellate populations, driving changes in community composition and microalgal successions in natural communities.

11. *Parvilucifera* occur together with dinoflagellate outbreaks in the field. This transcient community characterized by low diversity and high abundances support parasitic infection. The high host densities reached during blooms, increase the rates of contact between the parasitoids and their hosts, thereby increasing infection transmission and parasitoid load to the system.

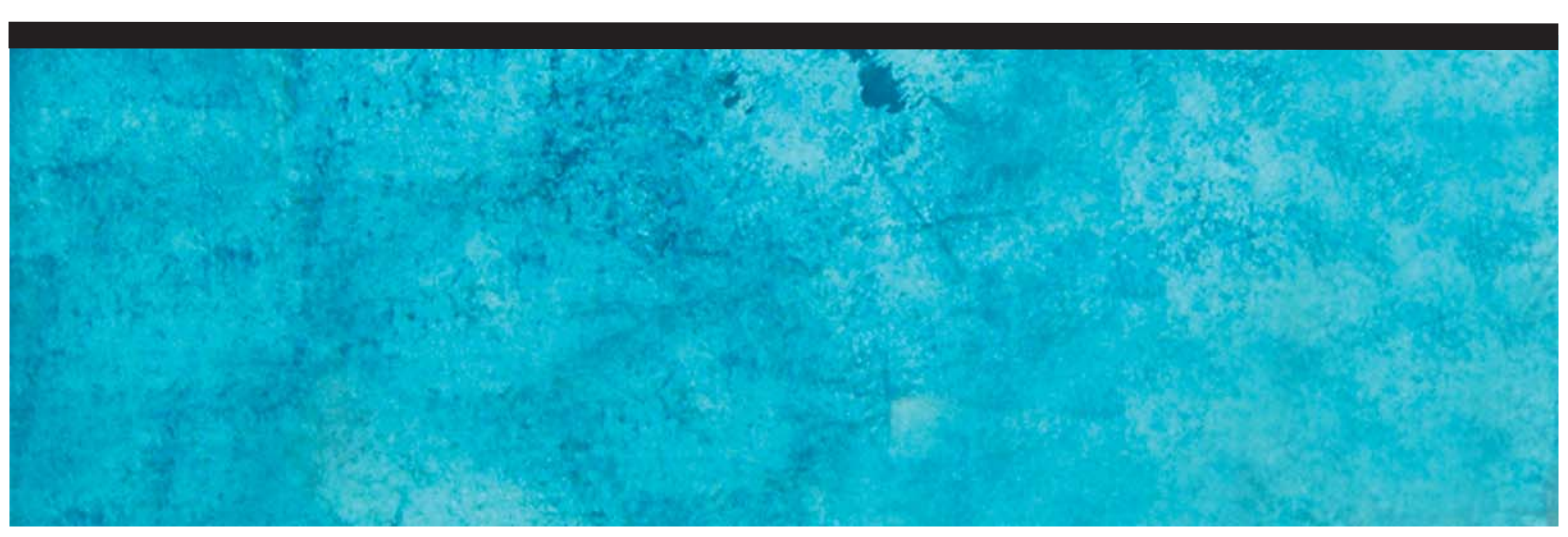
12. *Parvilucifera* abundance followed a time-delay response to the temporal fluctuations of its blooming hosts. This temporal dynamic is similar to that of predator-prey interactions. Since *Parvilucifera* is a parasitoid that kills their hosts, their ecology and population dynamics lie somewhere in between those of predators and true infectious parasites, such as protozoa.

13. *Parvilucifera* parasitism influences bloom dynamics, contributing to bloom termination causing losses that are of the same order of magnitude as those due to other biological factors.

14. *P. sinerae* parasitism exhibits both temporal and spatial heterogeneity during high-biomass blooms. Prevalence and host mortalities differ during the different phases of the bloom, being very low during the pre-bloom and the exponential growth phase, and coinciding the highest values with the bloom decrease. The spatial heterogeneity of these parameters could be attributed to local differences in the physical and biological factors.

15. Prevalence of *Parvilucifera* in the field, is much lower than demonstrated in laboratory experiments. These differences can be attributed to the many factors in nature, including those that cause direct parasitoid losses and those that reduce infection success.

References of General Introduction & Discussion



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