

**Interplay between the parasite *Amoebophrya* sp. (Alveolata) and the cyst formation of the red tide dinoflagellate *Scrippsiella trochoidea***

**Aurélie Chambouvet<sup>a,2</sup>, Catharina Alves-de-Souza<sup>a,b</sup>, Valérie Cueff<sup>a,3</sup>, Dominique Marie<sup>a</sup>, Sergey Karpov<sup>a,4</sup>, Laure Guillou<sup>a,1</sup>**

<sup>a</sup> Université de Pierre et Marie Curie (Paris 06) and CNRS, UMR 7144, Station Biologique de Roscoff, Place Georges Teissier, 29680 Roscoff, France

<sup>b</sup> Instituto de Biología Marina, Universidad Austral de Chile, Campus Isla Teja, P.O. Box 567, Valdivia, Chile.

<sup>1</sup> Corresponding author; fax 33 2 98 29 23 24, e-mail [lguillou@sb-roscoff.fr](mailto:lguillou@sb-roscoff.fr)

<sup>2</sup> Present address: Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK

<sup>3</sup> Present address: Laboratoire de microbiologie des environnements extrêmes, UMR 6197, CNRS and IFREMER, 29280 Plouzané, France

<sup>4</sup> Present address: Zoological Institute RAS, Universitetskaya emb. 1, St. Petersburg, 199034, Russia

## **Abstract**

**Syndiniales (Alveolata) are marine parasites of a wide range of hosts, from unicellular organisms to Metazoa. Many Syndiniales obligatorily kill their hosts to accomplish their life cycle. This is the case for *Amoebophrya* spp. infecting dinoflagellates. However, several dinoflagellate species known to be infected by these parasites produce diploid resting cysts as part of their life history. These resting cysts may survive several seasons in the sediment before germinating. How these parasites survive during the dormancy of their host remained an open question. We successfully established infections by *Amoebophrya* sp. in the red tide dinoflagellate *Scrippsiella trochoidea*. This host strain was homothallic and able to continuously produce typical calcified cysts covered by calcareous spines. Presence of the parasite significantly speeded up the host cyst production, and cysts produced were the only cells to resist infections. However, some of them were clearly infected, probably earlier in their formation. After 10 months, cysts produced in presence of the parasite were able to germinate and new infective cycles of the parasite were rapidly observed. Thus, a very novel relationship for protists is demonstrated, one in which parasite and host simultaneously enter dormancy, emerging months later to propagate both species.**

**Keywords: *Amoebophrya*, Dinoflagellate, Parasite, Resting cyst, Sexual reproduction.**

## Introduction

Recent molecular studies using culture-independent methods for direct sequencing of genes, generally the small subunit of the ribosome (SSU rDNA gene or 18S), have demonstrated the widespread occurrence of enigmatic novel eukaryotic lineages within the smallest size fraction (< 3-5  $\mu\text{m}$ ) of marine plankton (López-García et al. 2001; Moon-van der Staay et al. 2001). By phylogeny, a large part of these environmental sequences belongs to Alveolata and are sister groups to the dinoflagellates (Guillou et al. 2008; Massana et al. 2008). These environmental lineages, usually known as marine alveolates (MALV), have been retrieved in all marine ecosystems studied so far, from coastal waters to deep hydrothermal vents. They usually represent 10 to 50 % of the total environmental sequences from genetic libraries based upon the SSU rDNA (Guillou et al. 2008). Recently, several formally described species were placed within these marine alveolate lineages (Harada et al. 2007; Moon-van der Staay et al. 2001; Skovgaard et al. 2005; Skovgaard and Daugbjerg 2008). All of them belong to Syndiniales, a group exclusively composed of obligated marine parasites. These pathogens infect a wide range of hosts, from unicellular microalgae and micro-predators (such as ciliates), to metazoan including Crustacea (copepods, crabs, langoustines), fish eggs, and *Appendicularia*. Indeed, the widespread occurrence of MALV sequences in environmental clone libraries and the potential host range of the Syndiniales suggest that these parasites are ecologically important in marine waters. Thus far, syndinians have been shown to severely damage commercial stocks of several species, such as crabs, Norway lobsters and fish eggs (Mori et al. 2007; Stentiford and Shields 2005; Stratoudakis et al. 2000; Yuasa et al. 2007). The marine alveolate Group II (MALV II) undoubtedly represents the most widespread and genetically diversified group within the Syndiniales. Up to 44 different genetic clades have been recorded within the MALV II, most known only by environmental sequences (Guillou et al. 2008). *Amoebophrya*, with seven species described so far, is the only known genus

belonging to MALV II. It infects a wide range of organisms, including dinoflagellates, ciliates, and radiolaria (Cachon 1964; Cachon and Cachon 1987). The species complex '*Amoebophrya ceratii*' was recently reported to cause the rapid decline of several bloom-forming dinoflagellates (Chambouvet et al. 2008; Coats et al. 1996). These parasites are very virulent. They prevent reproduction of host cells and, in a few days, kill their host and release small free-living cells, called dinospores. These dinospores rapidly lose their capacity to initiate new infections and most of them disappeared after 2 to 10 days in cultures (Coats and Park 2002). Propagation of the parasite, however, is ensured by the number of dinospores produced, as one infected dinoflagellate cell potentially produces between 60 to 400 dinospores (Coats and Park 2002). Indeed, both field observations and model predictions indicate that these parasites have the capacity to control their host populations (Cachon 1964; Chambouvet et al. 2008; Coats et al. 1996; Montagnes et al. 2008). A study conducted in the Penzé estuary (Brittany, France) during early summer of three consecutive years, showed chronic infection of dominant dinoflagellates species, including the toxic species *Alexandrium minutum*, with prevalences up to 40 % (Chambouvet et al. 2008). Using fluorescent oligonucleotide probes, these parasites were proved to be highly specific, the same parasite distinguishable at the genetic level infecting the same dinoflagellate host year after year (Chambouvet et al. 2008). From an ecological and an evolutionary perspectives, however, such pathogens pose a paradox, as by controlling proliferation of its host, a parasite reduces the probability of its own survival.

Many dinoflagellates produce over-wintering resting cysts as part of their sexual cycle. In such case, haploid vegetative cells transform into gametes that fuse to produce motile diploid planozygotes, that after a period of growth form thick-walled resting cysts (also called hypnozygotes) that can remain viable in the sediments for long periods (Dale 2001). These cysts form seed banks that ensure the survival of dinoflagellates from year to year. However,

how do syndinian parasites that infect dinoflagellates survive while their hosts reside in the sediment as resting cysts is still an open question. Do syndinian parasites form cysts? Do they lie dormant within the cyst of their host? Or, do they infect other host taxa during the winter? We recently isolated a strain of *Amoebophrya* able to infect the red tide dinoflagellate *Scrippsiella trochoidea*. This homothallic dinoflagellate continuously undergoes sexual reproduction in monoclonal cultures and produces typical calcified cysts covered by calcareous spines. This host-parasite system offered a unique opportunity to follow the interplay between infection by *Amoebophrya* and host encystment. Here we used cultures of *Scrippsiella trochoidea* and its complementary strain of *Amoebophrya* to test the hypothesis that the parasite survives in the absence of motile host cells by remaining dormant inside the host resting cysts.

## Results

### Characteristics of strains

The clonal strain of *Scrippsiella trochoidea* used in this study belongs to the clade STR1 (Gottschling et al. 2005; Gu et al. 2008), based on analysis of the ITS1-5.8S-ITS2 sequence (access number: HQ658160, not show). Vegetative cells averaged 23  $\mu\text{m}$  long and 18  $\mu\text{m}$  wide. This strain is homothallic, continuously producing two types of resting cysts in culture. One cyst type had a thick wall, was covered by numerous calcareous spines, and had a mean length of 38.4  $\mu\text{m}$  and mean width of 28.9  $\mu\text{m}$ . The second type was characterized by an absence of calcification, and was smaller, with mean dimensions of 32.4  $\mu\text{m}$  long and 25.0  $\mu\text{m}$  wide. Both cyst types had distinctive double wall and contained at least one bright red accumulation body.

SSU rDNA gene sequence (access number: HQ658161), placed the parasite in the Amoeboophryidae (Syndiniales), basal to the dinoflagellate lineage, and in the MALV II, clade 2 (classification published in Guillou et al. 2008). The parasite exhibited the typical life cycle of *Amoeboophrya ceratii* as described by Cachon in 1964 (Fig. 1). Infections were initiated by the entrance of dinospores (3.5  $\mu\text{m}$  in mean length) into the host cell. Infections developed in the nucleus of the host and the mature multinucleated trophont had a typical beehive organization. After 2-3 days, the parasite emerged from the host cell as a motile vermiform stage. When observed under the microscope, dinospores were rapidly produced by the dissociation of the vermiform stage. Like other strains of *Amoeboophrya* (Coats and Bockstahler 1994), all life-history stages of the parasite, including dinospores and vermiforms, emitted *in vivo* a bright green autofluorescence under blue-violet excitation (Fig. 1).

### Dynamic of infection and cyst production

Infections occurred in all flasks inoculated with dinospores, vegetative host cells completely disappearing after 10 days (data not shown). The host growth showed an initial lag period of one day observed in almost all incubations (Fig. 2). Net growth rates were maximal in all incubations between days 1 and 2, with mean values of  $1.07 \text{ d}^{-1}$  in uninfected host and of 0.89, 1.30, and  $1.65 \text{ d}^{-1}$  in infected cultures inoculated with 2:1, 10:1, and 100:1 host:dinospore ratio, respectively (Fig. 2). Flasks inoculated with the highest concentration of parasite (1 parasite for 2 hosts) showed a positive net growth rate until the second day, followed by rapid decline of the host population (Fig. 2). The timing of the decline in the host population was directly linked to the initial host:dinospore ratio, as disappearance of host cells was progressively delayed in inoculations using 1 dinospore for 10 and 100 hosts, respectively (Fig. 2). At their initial concentration, dinospores (less than  $1.4 \times 10^3 \text{ cells mL}^{-1}$ ) were barely distinguishable using flow cytometry because they were in low numbers. However, dinospores were clearly detected by the flow cytometer after 3 days of inoculation in the 2:1 host:dinospore ratio treatment, and after 4 to 5 days in the other two treatments (Fig. 2). At this time, the free-living stage of the parasite became eight times more abundant ( $6.5 \times 10^4 \text{ cells mL}^{-1}$ ) than the remaining hosts ( $8 \times 10^3 \text{ cells mL}^{-1}$ ) in the first condition. Dinospore production reached highest density ( $8.1 \times 10^5 \text{ cells mL}^{-1}$ ) in the 2:1 inoculation (Fig. 2). Heterotrophic bacteria rapidly increased from  $2.9 \times 10^7 \text{ cells mL}^{-1}$  to  $9.7 \times 10^7 \text{ cells mL}^{-1}$  concomitantly with the decline of host populations in the 2:1 inoculation (Fig. 2). Similar trends were observed in the two other conditions.

Although both types of cysts of *S. trochoidea* were formed in all incubations, the majority of them were calcified (Fig. 2). Calcified cysts were occasionally produced after 7 days in controls and in 100:1 incubations, with zero or one cyst observed per well, corresponding to less than  $0.3 \text{ cysts mL}^{-1}$ . Total number of cysts clearly increased in the presence of the parasite with the initial ratio of 2:1 and 10:1 reaching 42 and 4 mean cysts  $\text{mL}^{-1}$  after 7 days,

respectively (Fig. 2). However, cyst abundance was still very low compared to the host density. These cysts were similar in size, averaging about 24.7  $\mu\text{m}$  long and 20.1  $\mu\text{m}$  wide. After day 4, the number of calcified cyst remained constant for months in all conditions. Production of non-calcified cysts was 10 times less important in all conditions excepted for the 2:1 ratio incubations, where non-calcified cysts reached 6  $\text{mL}^{-1}$  on day 7 (Fig. 2). However, the number of non-calcified cysts was decreasing over time, completely disappearing after one month (data not shown).

### **Influence of the initial host cell density and water-bone signal on the cyst production.**

Cyst production was followed for different initial host concentrations ranging from 1,000 to 20,000 hosts  $\text{mL}^{-1}$ , but for a constant host:dinospore ratio (2:1, Fig. 3). After 6 days, cyst abundance was similar in controls without parasites (initial host concentration of 20,000 cells  $\text{mL}^{-1}$ , data not show) and the lowest host concentration inoculated with parasites (1,000 cells  $\text{mL}^{-1}$ ). However, cyst abundance increased almost linearly with the initial host concentration, until a plateau that was reached at a concentration  $\geq 10^4$  cells  $\text{mL}^{-1}$ . At this plateau, mean cyst abundance was 60 cysts  $\text{mL}^{-1}$  after 6 days. Most cysts (93 %) were produced during the first 3 days in all conditions.

We then tested for the produce and release of a water-bone signal triggering host encystment during infections. Cyst production observed in controls (without parasite) was similar to that observed in host culture inoculated with a  $< 0.22 \mu\text{m}$  filtrate of a well infected culture (Fig. 4).

### **Presence of the parasite inside the host resting cysts**

After two weeks, we carefully observed by epifluorescence microscopy resting cysts produced during the first experiment. Intriguingly, several fresh host cysts produced in the presence of the pathogen (2:1 host:dinospore incubations) exhibit the typical bright green autofluorescence of the parasite in part of their cellular content (Fig. 5). This green fluorescence was observed in both types of cysts (calcified and non-calcified). This

fluorescence rapidly faded during microscopic examination and over time as cysts aged, and completely disappeared after one month. After 10 months, between 10 and 20 cysts were collected and mixed with exponentially growing hosts. One week later, new infection cycles were observed, with the concomitant presence of well infected host cells and dinospores (data not shown).

Green autofluorescent calcified cysts were observed by TEM in thin sections (Fig. 6). They had a resistant, thick, multi-layered cell wall, storage products, and reduced plastid material, indicative of lowered metabolism. The presence of the viable parasite inside these cysts was observed (Fig. 6). The *Amoebophrya* trophont lied inside the nucleus of the host, occupying most of the main nuclear volume. The parasite had no special parasitophorous vacuole; its plasma membrane was in direct contact with the nucleoplasm. The parasite produced several cytoplasmic protrusions in the host nucleus similar to pseudopodia of amoebae (Fig. 6). The cytoplasm of the parasite was rather dense, with a lot of ribosomes, some small vesicles, and lipid droplets. There were dictyosomes of the Golgi apparatus and rare membranes of cytoplasmic reticulum. Some small vesicles filled the pseudopodial protrusions of the parasite. No mitochondria were found in this stage of the parasite. In some sections we found two, or even three nuclei in the parasite (not shown).

## Discussion

### Characteristics of strains used in this study

Both the host and the parasite were isolated from the same geographic area (the Penzé estuary, France). This parasite belongs to the MALV Group II clade 2, which is in fact the genetic clade that was observed to chronically infect *S. trochoidea* during three consecutive years from the same location (Chambouvet et al. 2008). These observations strongly suggest that *S. trochoidea* is the primary host for this parasitic strain. This *Amoebophrya* strain is the seventh obtained in culture and described so far in the literature (Coats et al. 1996; Coats and Park 2002; Kim et al. 2004; Place et al. 2009). However, this is the first report of a parasitic strain infecting a homothallic dinoflagellate species, meaning that the host is able to continuously have sex in clonal culture.

The host strain produces two types of cysts, which are either calcified or not. Calcified cysts are widely considered as diploid assuming that their formation requires prior sexual reproduction (Kim and Han 2000; Kremp and Heiskanen 1999). This is in agreement with their long-term encystment observed during our experiments (more than 10 months). By contrast, the non-calcified cysts disappeared after few weeks in presence of the parasite. These cysts have typical shape of true resting cysts (or hypnozygotes) by having distinctive double wall and containing red accumulation body. They might represent an aborted stock of immature cysts, too weak to transform durably in more typical calcified cysts, and perhaps sensitive to the parasite infections. However, as the state of non-calcified cysts among *Scrippsiella* genus is still controversial (Kremp and Parrow 2006), we cannot even exclude that these cysts have a different nature (haploid?) and/or a shorter dormancy period than the calcified cysts. Overall, the calcified cysts were the only ones to resist infections by *Amoebophrya* on a long term.

### **Did the parasite promote the host sexuality?**

During experiments, the production of sexual and resistant cysts were promoted in presence of parasite. This process is undoubtedly important processes for host survival. This phenotypic trait may be comparable to the fecundity compensation (more reproduction early in life) widely observed for metazoans exposed to parasites (Hochberg et al. 1992; Minchella 1985). However, having sex for unicellular organisms has a more direct advantage, as deep morphological modifications, providing new resistances, may be eventually induced by the change of ploidy level. Diachronous ploidy modifications, with radical phenotypical changes, may be a widespread strategy to resist to pathogens for unicellular organisms having a diplobiontic life cycle. This mechanism, called the Cheshire cat strategy (Frada et al. 2008), was recently described for the widespread diploid stage of *Emiliana huxleyi* (Haptophyta) that is sensitive to specific giant phycodnavirus EhVs (Frada et al. 2008). This species may survive infections by producing a discreet, but resistant, haploid stage. This ‘Cheshire cat’ escape strategy may actively participate in the maintenance of sexuality in most of unicellular eukaryotic lineages.

However, there is no evidence that host sexuality was directly promoted by the parasite addition. During long-term experiments, diploid *E. huxleyi* cultures were impacted by viral infections in only few days. By contrast, an actively growing haploid population was visible only after three weeks of incubation. These haploid and diploid stages are distinct phases that can proliferate independently by clonal divisions in the same water masse or culture (this is a true haplo-diplontic life cycle). Indeed, it is not clear if the diploid host changed into the haploid form due to contact with the viruses, or if the haploid stage, potentially initially present at low concentration in cultures, were promoted by the decline of the diploid stage. Similarly, these doubts also persist for *S. trochoidea* infected by *Amoebophrya*. First, the

encystment process involves certain definite steps that take time in dinoflagellates (Fig. 5). Such steps include the formation of gametes from haploid vegetative cells (Fig. 5, H1 to H2), their fusion to form diploid biflagellate cells (called the planozygote, H3), and the final encystment process leading to the formation of the hypnozygote or resting cyst (H4). Gametes were observed to form by two successive and rapid divisions (in less than 24 hours) and fusing of gametes appears to immediately succeed gametogenesis (Coats et al. 1984). Then, the planozygote resulting from this fusion accumulate storage compounds required for the long-term survival of resting cysts, such as lipidic granules, glucids, and carbohydrates. Accordingly, the diploid planozygote generally requires 3 to 20 days in cultures before encystment (Dale 2001; Figueroa et al. 2006; Pfiester and Anderson 1984). More specifically, *Scrippsiella cf. lachrymosa* and *S. hangoi* planozygotes have been described to require a few days to one week before complete encystment (Heiskanen 1993; Kremp and Heiskanen 1999; Olli and Anderson 2002). Considering the time required for all of these processes, cysts formed during these first days likely resulted from the pool of diploid planozygotes ready to encyst at the beginning of our experiments. Another argument supporting this hypothesis is the relatively low number of cysts produced compared to the total number of vegetative cells at beginning of the experiment (*i.e.* 0.8 %), although this relative low cyst production might partially be explained by the use of full strength medium rather than reduced nutrient level media generally used to assess dinoflagellate cyst production (Binder and Anderson 1987). In addition, they were relatively small compared to cysts produced from cultures without parasites, a characteristic likely induced by a shorter life-time of planozygotes. Thus, addition of the parasite probably does not induce sexuality itself, but only promotes the maturation of diploid planozygotes present in the culture at the beginning of the experiment.

**How explaining this host cyst production during infection?**

Toth et al (2004) reported the production of *Alexandrium ostenfeldii* pellicle cysts by water-borne signals in presence of the parasite *Parvilucifera infectans*. However, water-borne signals did not appear to be involved in the *S. trochoidea* calcified cyst production in presence of *Amoebophrya* sp., as suggested by the experiment where the medium from dinospore suspensions were added to *S. trochoidea* cultures. However, we must admit that this experiment was done by a single addition of the filtrates from the contaminated cultures, which assumed the stability of the potential allelochemical activity during the first three days of incubations.

In other hand, the ratio of dinospores:hosts was a relevant factor affecting cyst formation. This fact suggests that the encystment process requires physical contact with the parasite. The importance of cell contacts in the encystment process of *S. trochoidea* was highlighted by Uchida in 2001. In fact, all planozygotes did not necessary transform into resting cysts (Fig. 7). When individually isolated in fresh medium, planozygotes directly divide to produce haploid vegetative cells without undergoing cysts (Uchida 2001). Thus, encystment was suspected to require cell contact among congeners (Uchida 2001). This is the probable mechanism favouring the formation of calcified cyst of *S. trochoidea* in cultures mixed with the parasite that naturally increased external contact signals.

Double capacity for planozygotes to form resting cysts and undergoing direct meiosis is probably a key parameter to resist pathogens. Indeed, it was recently demonstrated that crosses between two mating strains, one resistant and one sensitive, of the heterothallic dinoflagellate *Alexandrium minutum* produced planozygotes that preferentially divided instead forming resting cysts in presence of the parasite *Parvilucifera sinerae* (Figueroa et al. 2010). This strategy may favour the rapid integration of resistance in recombinant progeny, additionally escaping the mandatory dormancy period (Figueroa et al. 2010). However, we cannot exclude in these experiments that the meiosis route was preferred because these

planozygotes were too young to encyst. Likely, resting cysts production resulted in all cases in the equilibrium between the pool of planozygotes present nearly at the beginning of the experiment, their degree of maturity, and the relative concentration of parasites.

### **Physiology of the parasite inside the cyst of its host.**

In the case of *Amoebophrya* infecting *Scrippsiella trochoidea*, calcified cysts were resistant to the infection. Thus, host cysts bearing parasites were likely infected prior to encystment process. The parasite appeared to be active in pre-cysts, penetrating the host nucleoplasm with cell projections. An absence of active mitochondrion inside the parasite at this stage clearly demonstrated its dependency on host activity. Absence of mitochondrion in early stages of infection of vegetative cells can also be noticed in TEM micrographs published by Fritz and Nass (1992). Accumulation of host mitochondrion close to the trophont was also observed in *Amoebophrya acanthometrae* (Cachon and Cachon 1969). These well developed host mitochondria around the parasite is reminiscent of the situation with Microsporidia, a group of obligatory intracellular parasites that infect a broad variety of metazoan hosts, as well as a small number of protists, and which use ATP directly from the mitochondria of their host (Williams et al. 2002).

As the green autofluorescence of the parasite disappears as soon as the parasite is fixed or dies out, this property might be linked to the metabolic activity of the parasite. This autofluorescence turns off also with the dormancy of the host. However, the *Amoebophrya* strain infecting *Scrippsiella trochoidea* survived inside its host and was still infective after months. Thus, a very novel relationship for protists is demonstrated, one in which parasite and host simultaneously enter dormancy, emerging months later to propagate both species. Considering that the first target of this parasite is the host nucleus, production of a new host generation from these infected cysts is unlikely. Thus, transmission of infections is in fact

always horizontal, a characteristic supposed to promote the evolution of harmful symbionts (Sachs and Wilcox 2006).

### **Ecological significance of this process.**

From an ecological point of view, the capacity of the parasite to lie dormant inside the cyst of its host nicely explains how this parasite chronically infects the same host species year after year, as it was demonstrated in the Penzé estuary during three consecutive years (Chambouvet et al. 2008). This physical link, in time and space, is undoubtedly favourable for the co-evolution of both species at local scales. Given the importance of cyst formation as a factor regulating the initiation and decline of dinoflagellate blooms (Dale 2001), this host-parasite interaction could have additional implications for the understanding of dinoflagellate blooms dynamics. Although the studies about dinoflagellate population dynamics are usually focussed on the role of abiotic variables such as nutrients, light, and temperature, there is growing evidence of the importance of biotic factors, mainly grazing and parasitism, in the demise of blooms (Coats et al. 1996; Montagnes et al. 2008; Watras et al. 1985). However, recent studies pointed out that biotic factors, like pathogens, can also indirectly contribute for the removal of vegetative populations from the plankton by promoting the encystment or affecting the excystment processes (Figuerola et al. 2010; Fistarol et al. 2004; Rengefors et al. 1998; Toth et al. 2004). In this context, this study brings new evidences that the formation of dinoflagellate resting cyst, produced throughout the sexuality, could also be regulated by antagonist interactions. Results from laboratory studies suggest that dinoflagellate resting cysts are formed in response to adverse conditions related to nutrient concentrations, light and temperature (Anderson et al. 1984; Sgroso et al. 2001). However, under field conditions, encystment is not always linked to obvious environmental cues and seems to occur under apparent optimal conditions (Anderson et al. 1983; Estrada et al. 2010; Kremp and Heiskanen

1999). Thus, our results point out the importance of assessing the potential role of Amoebophryidae parasites in studies about dinoflagellate encystment under field conditions.

## Methods

**Cultures:** A culture of the non-toxic dinoflagellate *Scrippsiella trochoidea* was previously established from the germination of a single cyst collected in 2005 from the Penzé estuary sediment (North-West of France, English Channel, 48°37'N; 3°56'W). A monoclonal strain was subsequently obtained after the re-isolation of a single vegetative cell (deposited at the Roscoff Culture Collection, RCC 1627, <http://www.sb-roscoff.fr/Phyto/RCC/>).

From natural samples, *S. trochoidea* cells were detected from the Penzé estuary during summer 2007, with a maximal abundance observed the 10 of July ( $3.2 \times 10^5$  cells L<sup>-1</sup>).

*Amoebophrya*-like parasites infecting *S. trochoidea* were detected by their natural bright green autofluorescence using an epifluorescence microscope (BX51, Olympus) equipped with the U-MWB2 cube (450- to 480-nm excitation, 500-nm emission, Coats and Bockstahler 1994, Kim et al. 2004). The *Amoebophrya* strain was established from samples collected the 23 of June 2007, using a glass micropipette to transfer a single infected cell in late-stage of infection to the monoclonal *S. trochoidea* culture (RCC 1627) at exponential phase. The *Amoebophrya* strain obtained was subsequently re-isolated twice using the same procedure (deposited at the Roscoff Culture Collection, RCC 1626).

Infected and uninfected host cultures were maintained in a F/2 medium (Marine Water Enrichment Solution, Sigma), using autoclaved natural seawater from the Penzé estuary collected in June the year before (27 of salinity) and stored at dark. After preparation, the water salinity was adjusted to 27 with distilled water and complemented with 5% (v/v) soil extract (Starr and Zeikus 1993). A final filtration using a 0.22 µm pore size filter was processed under sterile conditions. Stock cultures of the parasite were propagated using 10

mL culture flasks, by transferring 300  $\mu$ L of infected host culture into 3 mL of exponentially growing uninfected *S. trochoidea* stock every 2-3 days. All stock cultures and experiments were maintained at 19 °C and on a L:D cycle of 12:12 h at 80  $\mu$ Einstein  $m^2 s^{-1}$ .

**Genotypic characterization of strains:** Genomic DNA of the parasite and the uninfected host were extracted from 10 mL of cultures. For the parasite, dinospores were separated from the remaining host cells by filtration through 5- $\mu$ m cellulose acetate filter (Minisart, SARTORIUS, France). Cells were then harvested by centrifugation. DNA was extracted by a standard phenol/chloroform protocol. PCR amplifications were performed using the GoTaq DNA polymerase from Promega. The Internal Transcribed Spacer 1 (ITS 1), the 5.8S rDNA, and the ITS 2 genes of both the host and the parasitic strains were amplified by PCR using the forward primer ITS-Cer-F 5'-GTCGCTCCTACCGATTGAGT-3' and the reverse primer D1R-Cer-R 5'-TATGCTTAAATTCAGCRGGT-3' (modified from Scholin et al, 1994). The SSU rRNA gene of the parasite was amplified using the eukaryotic primers 328-F 5'-ACCTGGTTGATCCTGCCAG-3' and 329-R 5'-TGATCCTTCYGCAGGTTAC-3' described by Moon-van der Staay et al. (2001).

The PCR program included a denaturation step at 95 °C for 5 minutes, followed by 35 cycles of denaturation for 1 min at 95 °C, hybridization for 45 seconds at 55 °C, and elongation for 1 min 15 seconds at 72 °C. The final elongation step lasted for 7 minutes at 72 °C. PCR products were visualised on a 1 % agarose gel, and then purified before being sequenced. PCR products were purified using the ExoSAP-IT procedure (USB, Cleveland, Ohio) following the manufacturer recommendations and directly sequenced on an ABI Prism 3100 automatic sequencer (Applied Biosystems).

**Dynamic of infections:** The experiment was conducted using recently formed dinospores, harvested after 2 days of infection. For that, stock infected cultures of *S.*

*trochoidea* were filtered by gravity throughout 10 µm pore-size filter (polycarbonate membrane, Whatman) to remove uninfected and infected hosts. Just before incubations, aliquots of 1.5 mL of harvested dinospores were fixed with glutaraldehyde (0.25 % final concentration) for at least 15 minutes and the dinospore concentration was determined by flow cytometry (FACS Aria, Becton Dickinson) after DNA staining with SYBR Green-I at a final dilution of 1/50,000, following the protocol described by Marie et al (2000). Dinospores are distinguished from heterotrophic bacteria based upon their DNA content (F11 parameter) and their SSC. To assess the effect of parasites on the host cyst formation, untreated culture 24 well plates (Nalgene) were filled with an exponentially growing *S. trochoidea* culture ( $2,700 \text{ cells mL}^{-1}$ ), with a final volume of 2 mL in each well. Half of the wells were inoculated with dinospores to produce three different host:dinospore ratios (2:1, 10:1 and 100:1). Rest of wells was considered as controls. After inoculation (T0), the experiment was followed during six days (T1-T6). Two replicates of each treatment and their respective controls were daily fixed with glutaraldehyde before being frozen into liquid nitrogen and stored at  $-80 \text{ }^{\circ}\text{C}$  until analysis by flow cytometry. Four replicates of each treatment (the different host:dinospore ratios) were collected during all the experiment and screened every day by inverted microscope (IX71, Olympus) for the quantification of cysts. Flow cytometry (same protocol as mentioned previously) was used to further deduce the host abundances, and when it was possible abundances of dinospores and heterotrophic bacteria, as they may also influence dinoflagellate cyst production (Adachi et al. 1999).

**Influence of the initial host concentration on encystment:** Similar incubations (four replicates per conditions) were initiated using seven different host concentrations ranging from 1,000 to  $18,800 \text{ cells mL}^{-1}$  (final concentration), inoculated with freshly collected dinospores at a 2:1, host:dinospore ratio. Four additional wells were sowed with uninfected

host cultures at 20,000 cells mL<sup>-1</sup> (final concentration). The number of cysts in each well was counted at approximately daily intervals using an inverted microscope.

**Influence of a water-borne signal on the cyst production:** A mixture of host contaminated by the parasite at a final 2:1 host:dinospore ratio was prepared two days before this experiment. At T0, one aliquot of this culture was filtrated by gravity through a 10- $\mu$ m filter (Polycarbonate membrane, Whatman), in order to remove host cells. Fresh dinospores were then collected by filtration through a 0.22- $\mu$ m filter (Polycarbonate membrane, Whatman) and diluted with this filtrate to obtain a final concentration of 2,700 cells mL<sup>-1</sup>. A second aliquot of this culture was directly filtered through a 0.22- $\mu$ m cellulose acetate filter (Minisart, SARTORIUS, France) to collect the filtrate devoid of host and parasite cells. One culture plate was inoculated using an exponentially growing *S. trochoidea* stock cultures (5,400 cells mL<sup>-1</sup>, 1 mL per well). In the first row (4 replicates), 1 mL of F/2 medium complemented with soil extract was added (controls for host only). In the second row, dinospores were added to the host (Vol/Vol, corresponding to a final 2:1 host:dinospore ratio). In the last row, 1 mL of the filtrate only (< 0.22 $\mu$ m) was added (Vol/Vol). Cyst production in all replicates was daily measured by inverted microscopy.

**Electronic Microscopy:** For the EM studies the infected cysts of *Scrippsiella* were isolated from the culture and fixed with 0.25% OsO<sub>4</sub> for 25 min at room temperature. Without rinsing, 2.5% glutaraldehyde on 0.05 M cacodilate buffer with marine medium has been added for 2.5 hours at 4° C. The post fixation with 1% OsO<sub>4</sub> on marine medium was processed overnight at 4° C. The material was then dehydrated using 30 to 100% alcohol series. After a final dehydration with propylene oxide the material was embedded in the Epon. The material was cut using the ultramicrotome Leica ultracut UCT, stained with uranyl acetate and lead citrate, and viewed at the TEM Jeol 1400 at 80 kv, equipped with digital camera Orius SC1000.

**Germination of cysts:** During a first experiment, uninfected hosts (3 mL) were incubated with fresh dinospores (300  $\mu$ L of the stock solution) for one week. These flasks were then stored in dark and covered by parafilm to avoid evaporation. After 10 months, these culture tubes were checked in order to collect cysts, and this solution was mixed with 3 mL of exponentially growing *S. trochoidea* cultures into 6 ml culture plates. Infections were checked daily using natural fluorescence of the parasite (as described above) by inverted epifluorescence microscope (IX71, Olympus).

## **Acknowledgements.**

We thank D. W. Coats for insightful discussion and comment on a draft of this manuscript, Mohamed Laabir and Benjamin Genovesi-Giunti for their help in cyst isolation and germination, Fabien Jouenne for his help with photography and the “Service Mer et Observation” of the Roscoff Biological Station for sampling. This work was financially supported by the French ANR Aquaparadox and Paralex, and by the CNRS (SK grant as associated professor).

## **References**

- Adachi M, Kanno T, Matsubara T, Nishijima T, Itakura S and Yamaguchi M** (1999) Promotion of cyst formation in the toxic dinoflagellate *Alexandrium* (Dinophyceae) by natural bacterial assemblages from Hiroshima Bay, Japan. *Mar Ecol Prog Ser* **191**: 175-185
- Anderson DM, Chisholm SW and Watras CJ** (1983) Importance of life cycle events in the population dynamics of *Gonyaulax tamarensis*. *Mar Biol* **76**: 179-189
- Anderson DM, Kulis DM and Binder BJ** (1984) Sexuality and cyst formation in the dinoflagellate *Gonyaulax tamarensis*: cyst yield in batch cultures. *J Phycol* **20**: 418-425
- Binder BJ and Anderson DM** (1987) Physiological and environmental control of germination in *Scrippsiella trochoidea* (Dinophyceae) resting cysts. *J Phycol* **23**: 99-107
- Cachon J** (1964) Contribution à l'étude des péridiniens parasites. *Cytologie, cycles évolutifs*. *Ann Sc Nat Zool Paris* **VI**: 1-158
- Cachon J and Cachon M** (1969) Ultrastructure des Amoebophyidae (péridiniens Duboscquidinida) I.- Manifestations des rapports entre l'hôte et le parasite. *Protistologica* **Tome V**: 535-547
- Cachon J and Cachon M** (1987) Parasitic dinoflagellates. In Taylor FJR (ed) *The biology of dinoflagellates*. Oxford, Blackwell scientific publications. 21: 571-611

- Chambouvet A, Morin P, Marie D and Guillou L** (2008) Control of toxic marine dinoflagellate blooms by serial parasitic killers. *Science* **322**: 1254-1257
- Coats DW, Tyler MA and Anderson DM** (1984) Sexual processes in the life cycle of *Gyrodinium uncatenatum* (Dinophyceae): a morphogenetic overview. *J Phycol* **20**: 351-361
- Coats DW and Bockstahler KR** (1994) Occurrence of the parasitic dinoflagellate *Amoebophrya ceratii* in Chesapeake Bay populations of *Gymnodinium sanguineum*. *J Eukaryot Microbiol* **41**: 586-593
- Coats DW, Adam EJ, Gallegos CL and Hedrick S** (1996) Parasitism of photosynthetic dinoflagellates in a shallow subestuary of Chesapeake Bay, USA. *Aquatic Microb Ecol* **11**: 1-9
- Coats DW and Park MG** (2002) Parasitism of photosynthetic dinoflagellates by three strains of *Amoebophrya* (Dinophyta): parasite survival, infectivity, generation time, and host specificity. *J Phycol* **38**: 520-528
- Dale B** (2001) The sedimentary record of dinoflagellate cysts: looking back into the future of phytoplankton blooms. *Scientia Marina* **65**: 257-272
- Estrada M, Solé J, Anglès S and Garcés E** (2010) The role of resting cysts in *Alexandrium minutum* population dynamics. *Deep-Sea Res Part II* **57**: 308-321
- Figuerola RI, Bravo I and Garcés E** (2006) Multiple routes of sexuality in *Alexandrium taylori* (Dinophyceae) in culture. *J Phycol* **42**: 1028-1039
- Figuerola RI, Garcés E and Camp J** (2010) Reproductive plasticity and local adaptation in the host-parasite system formed by the toxic *Alexandrium minutum* and the dinoflagellate parasite *Parvilucifera sinerae*. *Harmful Algae* doi:10.1016/j.hal.2010.06.002
- Fistarol GO, Legrand C, Rengefors K and Granéli E** (2004) Temporary cyst formation in phytoplankton: a response to allelopathic competitors? *Environ Microbiol* **6**: 791-798

- Frada M, Probert I, Allen MJ, Wilson WH and de Vargas C** (2008) The "Cheshire Cat" escape strategy of the coccolithophore *Emiliania huxleyi* in response to viral infection. Proc Natl Acad Sci USA **105**: 15944-15949
- Fritz L and Nass M** (1992) Development of two endoparasitic dinoflagellate *Ameobophrya ceratii* within host dinoflagellate species. J Phycol **28**: 312-320
- Gottschling M, Knop R, Plötner J, Kirsch M, Willems H and Keupp H** (2005) A molecular phylogeny of *Scrippsiella sensu lato* (Calciodinellaceae, Dinophyta) with interpretations on morphology and distribution. Eur J Phycol **40**: 207-220
- Gu H, Sun J, Kooistra WHCF and Zeng R** (2008) Phylogenetic position and morphology of thecae and cysts of *Scrippsiella* (Dinophyceae) species in the East China Sea. J Phycol **44**: 478-494
- Guillou L, Viprey M, Chambouvet A, Welsh RM, Kirkham AR, Massana R, Scanlan DJ and Worden AZ** (2008) Widespread occurrence and genetic diversity of marine parasitoids belonging to Syndiniales (Alveolata). Environ Microbiol **10**: 3349-3365
- Harada A, Ohtsuka S and Horiguchi T** (2007) Species of the parasitic genus *Duboscquella* are members of the enigmatic marine alveolate Group I. Protist **158**: 337-347
- Heiskanen A-S** (1993) Mass encystment and sinking of dinoflagellate during a spring bloom. Mar Biol **116**: 161-167
- Hochberg ME, Michalakis Y and de Meeus T** (1992) Parasitism as a constraint on the rate of life-history evolution. J Evol Biol **5**: 491-503
- Kim S, Park MG, Yih W and Coats DW** (2004) Infection of the bloom-forming thecate dinoflagellates *Alexandrium affine* and *Gonyaulax spinifera* by two strains of *Amoebophrya* (Dinophyta). J Phycol **40**: 815-822

- Kim YO and Han MS** (2000) Seasonal relationships between cyst germination and vegetative population of *Scrippsiella trochoidea* (Dinophyceae). *Mar Ecol Prog Ser* **204**: 111-118
- Kremp A and Heiskanen A-S** (1999) Sexuality and cyst formation of the spring-bloom dinoflagellate *Scrippsiella hangoei* in the coastal northern Baltic Sea. *Mar Biol* **134**: 771-777
- Kremp A and Parrow MW** (2006) Evidence for asexual resting cysts in the life cycle of the marine peridinioid dinoflagellate, *Scrippsiella hangoei*. *J Phycol* **42**: 400-409
- López-García P, Rodríguez-Valera F, Pedrós-Alió C and Moreira D** (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. *Nature* **409**: 603-607
- Marie D, Simon N, Guillou L, Partensky F and Vaulot D** (2000) DNA/RNA analysis of phytoplankton by flow cytometry. In Robinson JP, Darzynkiewicz Z, Dean PN, Orfao A, Rabinovitch PS, Stewart CC, Tanke HJ and Wheelless LL (eds) *Current protocols in cytometry*. New York, John Wiley & Sons, Inc. 11.12.: 1-18
- Massana R, Karniol B, Pommier T, Bodaker I and Béjà O** (2008) Metagenomic retrieval of a ribosomal DNA repeat array from an uncultured marine alveolate. *Environ Microbiol* **10**: 1335-1343
- Minchella DJ** (1985) Host life-history variation in response to parasitism. *Parasitology* **90**: 205-216
- Montagnes DJS, Chambouvet A, Guillou L and Fenton A** (2008) Can microzooplankton and parasite pressure be responsible for the demise of toxic dinoflagellate blooms? *Aquatic Microb Ecol* **53**: 201-210
- Moon-van der Staay SY, Watcher RD and Vaulot D** (2001) Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature* **409**: 607-610
- Mori K-I, Yamamoto K, Teruya K, Shiozama S, Yoseda K, Sugaya T, Shirakashi S, Itoh N and Ogawa K** (2007) Endoparasitic dinoflagellate of the genus *Ichthyodinium* infecting

fertilized eggs and hatched larvae observed in the seed production of Leopard Coral Grouper *Plectropomus leopardus*. Fish Pathology **42**: 49-57

**Olli K and Anderson DM** (2002) High encystment success of the dinoflagellate *Scrippsiella* cf. *lachrymosa* in culture experiments. J Phycol **38**: 145-156

**Pfiester LA and Anderson DM** (1984) Dinoflagellate life cycles and their environmental control. In Taylor FJR (ed) The biology of the Dinoflagellates. Oxford, Blackwell Scientific Publications, Ltd.: 611-648

**Place AR, Bai X, Kim S, Sengco MR and Coats DW** (2009) Dinoflagellate host-parasite sterol profiles dictate karlotoxin sensitivity. J Phycol **45**: 375-385

**Rengefors K, Karlsson I and Hansson L-A** (1998) Algal cyst dormancy: a temporal escape from herbivory. Proceedings of the Royal Society, London B **265**: 1353-1358

**Sachs JL and Wilcox TP** (2006) A shift to parasitism in the jellyfish symbiont *Symbiodinium microadriaticum*. Proc R Soc Ser B **273**: 425-429

**Scholin CA, Herzog M, Sogin M and Anderson DM** (1994) Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. J Phycol **30**: 999-1011

**Sgrosso S, Esposito F and Montresor M** (2001) Temperature and daylength regulate encystment in calcareous cyst-forming dinoflagellates. Mar Ecol Prog Ser **211**: 77-87

**Skovgaard A, Massana R, Balagué V and Saiz E** (2005) Phylogenetic position of the copepod-infesting parasite *Syndinium turbo* (Dinoflagellata, Syndinea). Protist **156**: 413-423

**Skovgaard A and Daugbjerg N** (2008) Identity and systematic position of *Paradinium poucheti* and other *Paradinium*-like parasites of marine copepods based on morphology and nuclear-encoded SSU rDNA. Protist **159**: 401-413

**Starr R and Zeikus J** (1993) UTEX-The culture collection of algae at the University of Texas at Austin. J Phycol **29**: 1-106

- Stentiford GD and Shields JD** (2005) A review of the parasitic dinoflagellates *Hematodinium* species and *Hematodinium*-like infections in marine crustaceans. *Dis Aquat Org* **66**: 47-70
- Stratoudakis Y, Barbosa A and Meneses I** (2000) Infection of sardine eggs by the protistan endoparasite *Ichthyodinium chabelardi* off Portugal. *J Fish Biol* **57**: 476-482
- Toth GB, Norén F, Selander E and Pavia H** (2004) Marine dinoflagellates show induced life-history shifts to escape parasite infection in response to water-borne signals. *Proc R Soc Ser B* **271**: 733-738
- Uchida T** (2001) The role of cell contact in the life cycle of some dinoflagellate species. *J Plankton Res* **23**: 889-891
- Watras CJ, Garcon VC, Olson RJ, Chisholm SW and Anderson DM** (1985) The effect of zooplankton grazing on estuarine blooms of the toxic dinoflagellate *Gonyaulax tamarensis*. *J Plankton Res* **7**: 891-908
- Yuasa K, Kamaishi T, Mori K-I, Hutapea JH, Permana GN and Nakazawa A** (2007) Infection by a protozoan endoparasite of the genus *Ichthyodinium* in embryos and Yolk-sac larvae of yellowfin tuna *Thunnus albacares*. *Fish Pathology* **42**: 59-66

## Figure legends

**Figure 1.** Developmental stages of *Amoebophrya* sp. infecting the red tide dinoflagellate *Scrippsiella trochoidea*. Cells observed under bright field (A to D, phase contrast, upper part) and blue light excitation (E to H, bottom part). **A, B.** free living stage of the parasite (also called dinospores). **C.** Vermiform stage. **D.** Infected vegetative host cell. **E-H.** Green autofluorescence was from the parasite, red from plastids of the host. Scale bars = 20µm.

**Figure 2.** Cyst production during infection cycles. In lines, panel A-C, D-F, and G-I represent three condition treatments monitored during 6 days for initial host:parasite ratio (1:2, 10:1, and 100:1 respectively). First column represents *Scrippsiella trochoidea* abundances, in grey for incubations mixed with the parasite and in dark for healthy cultures. Second column figures out the production of calcified (black) and non-calcified (grey) in the respective proportion of parasites. Third column concerns the dinospore concentrations (when it was possible to assess by flow cytometry) during incubations mixed with parasite (black line), and heterotrophic bacteria concentration compared to controls ( $\Delta H_{bact} = H_{bact_{parasite}} - H_{bact_{control}}$ , in grey). Error bars indicate standard deviation between replicates.

**Figure 3.** Influence of the initial concentration of the host on the production of calcified cysts by *Scrippsiella trochoidea* in presence of *Amoebophrya* sp. after 3 and 6 days. Initial host:parasite ratio fixed at 2:1. Error bars indicate standard deviation between quadruplicates.

**Figure 4.** Calcified cyst production over days under different culture conditions. In plain curve: host alone. In dash lines and rectangular: host culture incubated with < 0.22 µm filtrate

of a two-days-old culture of *Scrippsiella trochoidea* infected with *Amoebophrya* sp. In dash line and triangle: host culture incubated in the presence of the free-living stage of the parasite.

**Figure 5.** *Amoebophrya* sp. infecting *Scrippsiella trochoidea* cysts. **A-B.** Calcified cysts. The resting cyst with green autofluorescence at the left is infected. **C-D.** Infected non-calcified cysts. A and C: Cells observed under white light (phase contrast). B and D. Same cells observed under blue light excitation (parasite revealed by its natural green autofluorescence). Scale bars = 20  $\mu\text{m}$ .

**Figure 6.** Thin section throughout a portion of calcified pre-cyst of *Scrippsiella trochoidea*. Cytoplasmic protrusions of parasite (cp), nucleus of the host (hn), lipid droplet (l), host mitochondrion (m), nucleus of parasite (pn). Scale bar= 1  $\mu\text{m}$ .

**Figure 7.** Interactions between *Scrippsiella trochoidea* and *Amoebophrya* sp. life cycles. Black arrows indicate *S. trochoidea* life cycle with haploid vegetative cells (H1), gametes (H2), diploid planozygote (H3), diploid calcified resting cyst (H4), diploid planomeiocyte (H5) and haploid non-calcified cyst (H6). *Amoebophrya* sp. life cycle (lines in grey) with free-living stage of the parasite (dinospores, P1), able to infect vegetative cells of *S. trochoidea* (P2), mature trophont of *Amoebobophrya* (typical beehive stage, P3), and the vermiform stage (P4). The parasite was additionally detected in non-calcified (P5) and calcified cysts (P6) of its host. Dotted lines illustrated uncertain routes for the parasite. For examples, infected non-calcified (P5) and calcified (P6) cysts eventually give rise to infected vegetative cells (P2) and infected planomeiocyte (P7) respectively or directly to the vermiform stage (P4) and dinospores (P1).

Figure 1

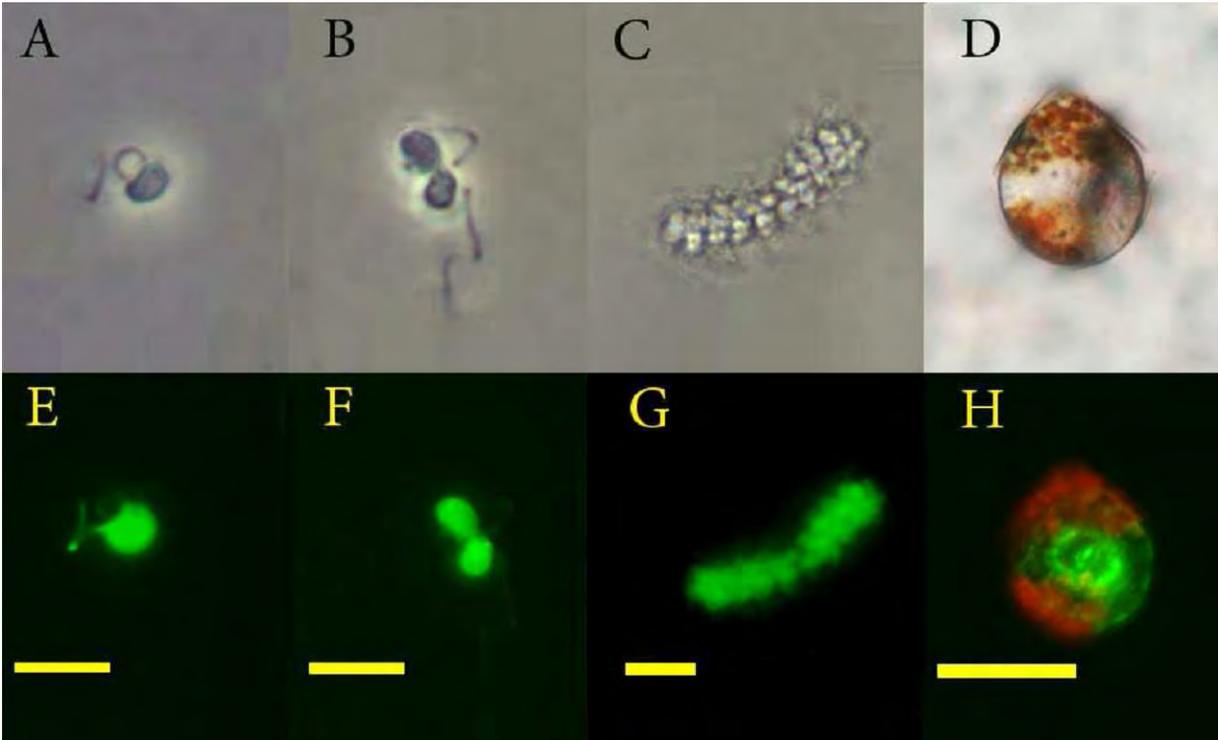


Figure 2

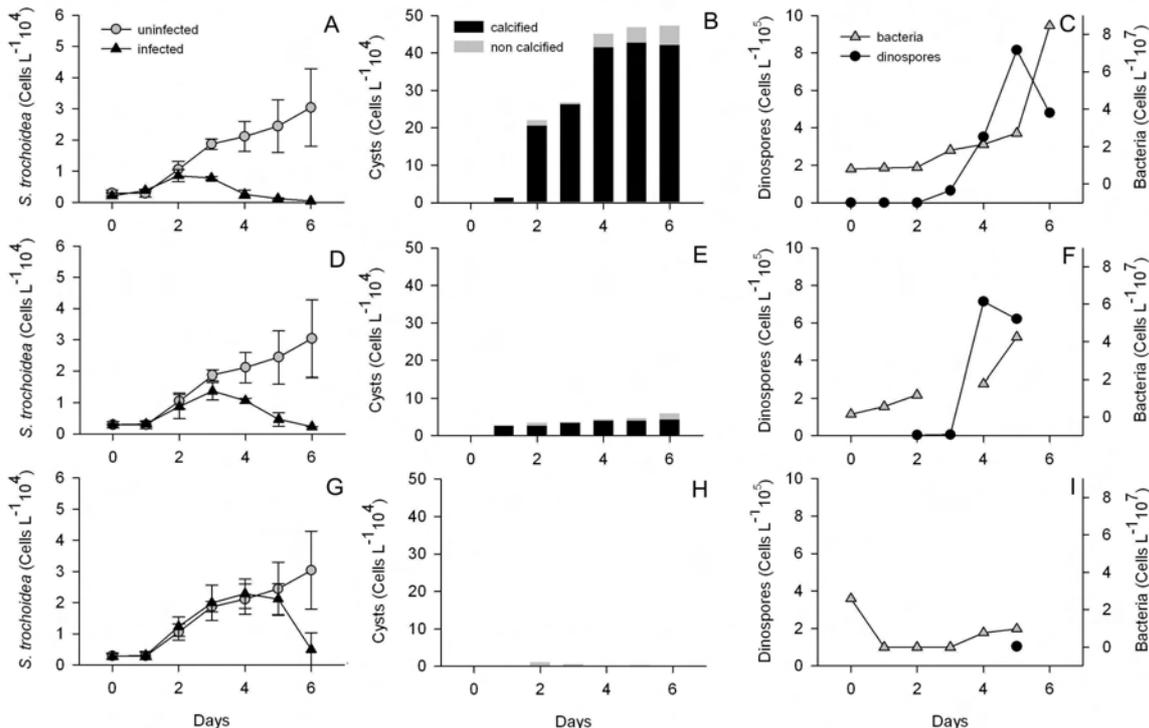


Figure 3

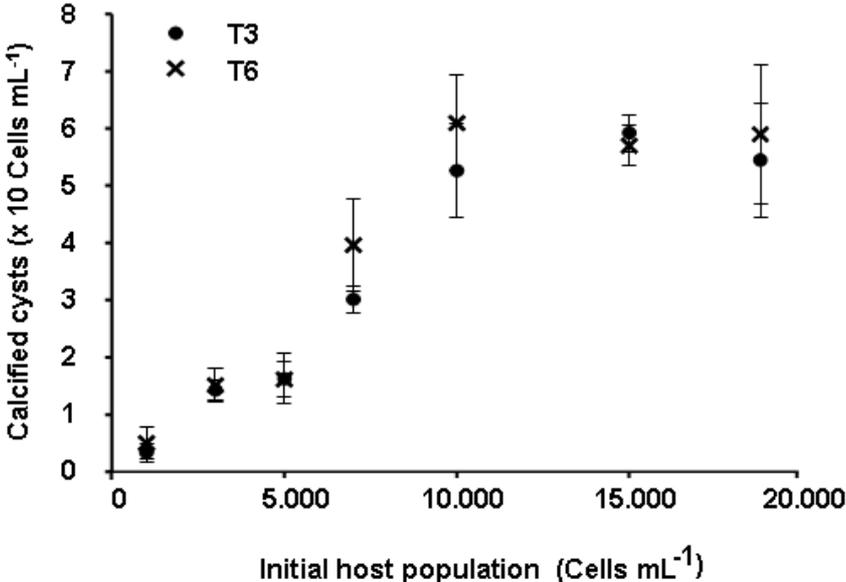


Figure 3

Figure 4

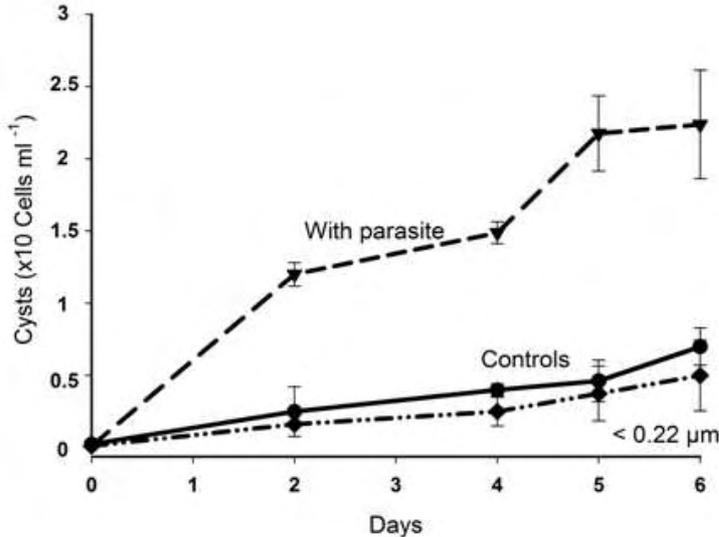


Figure 4

Figure 5

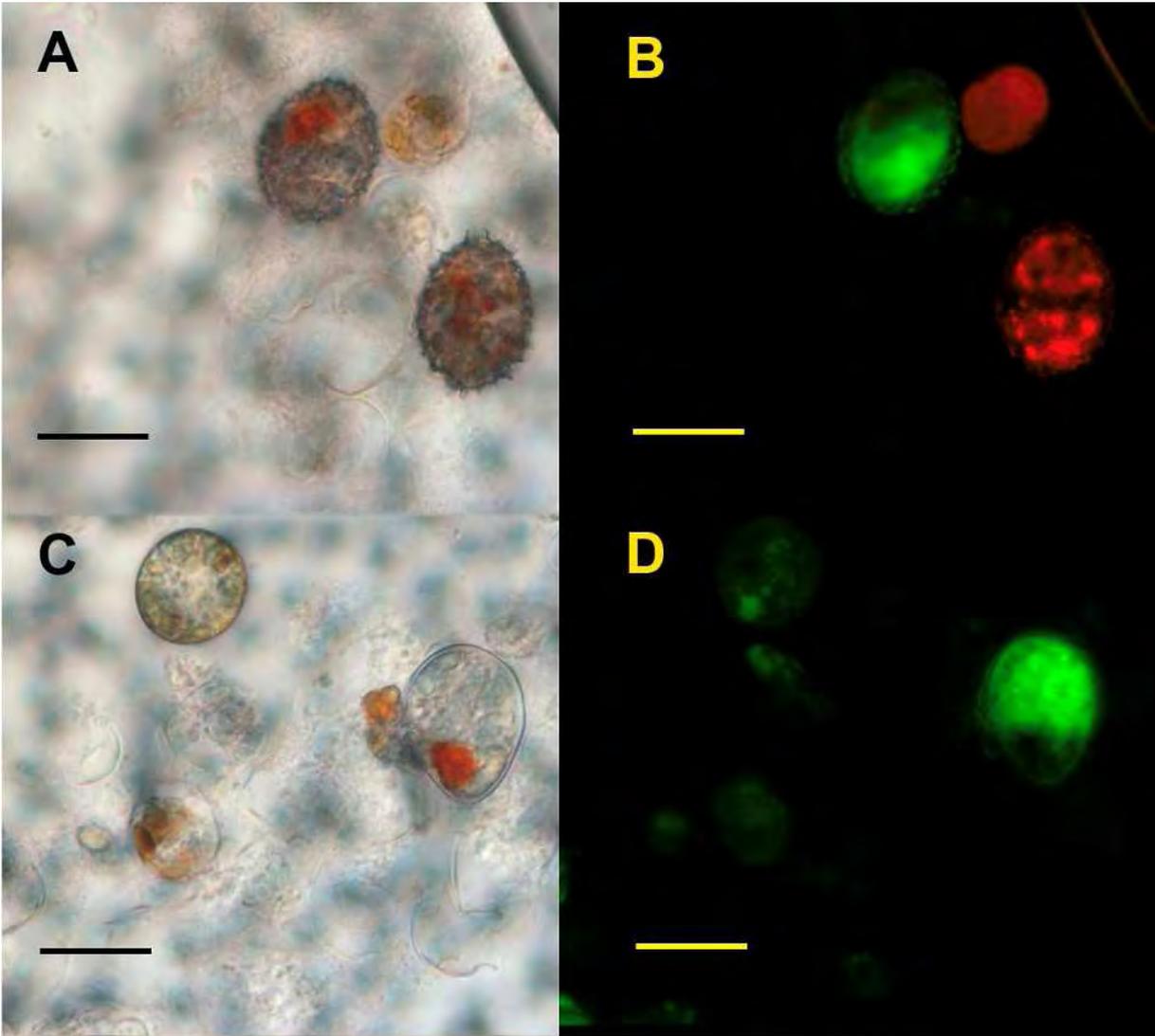


Figure 6

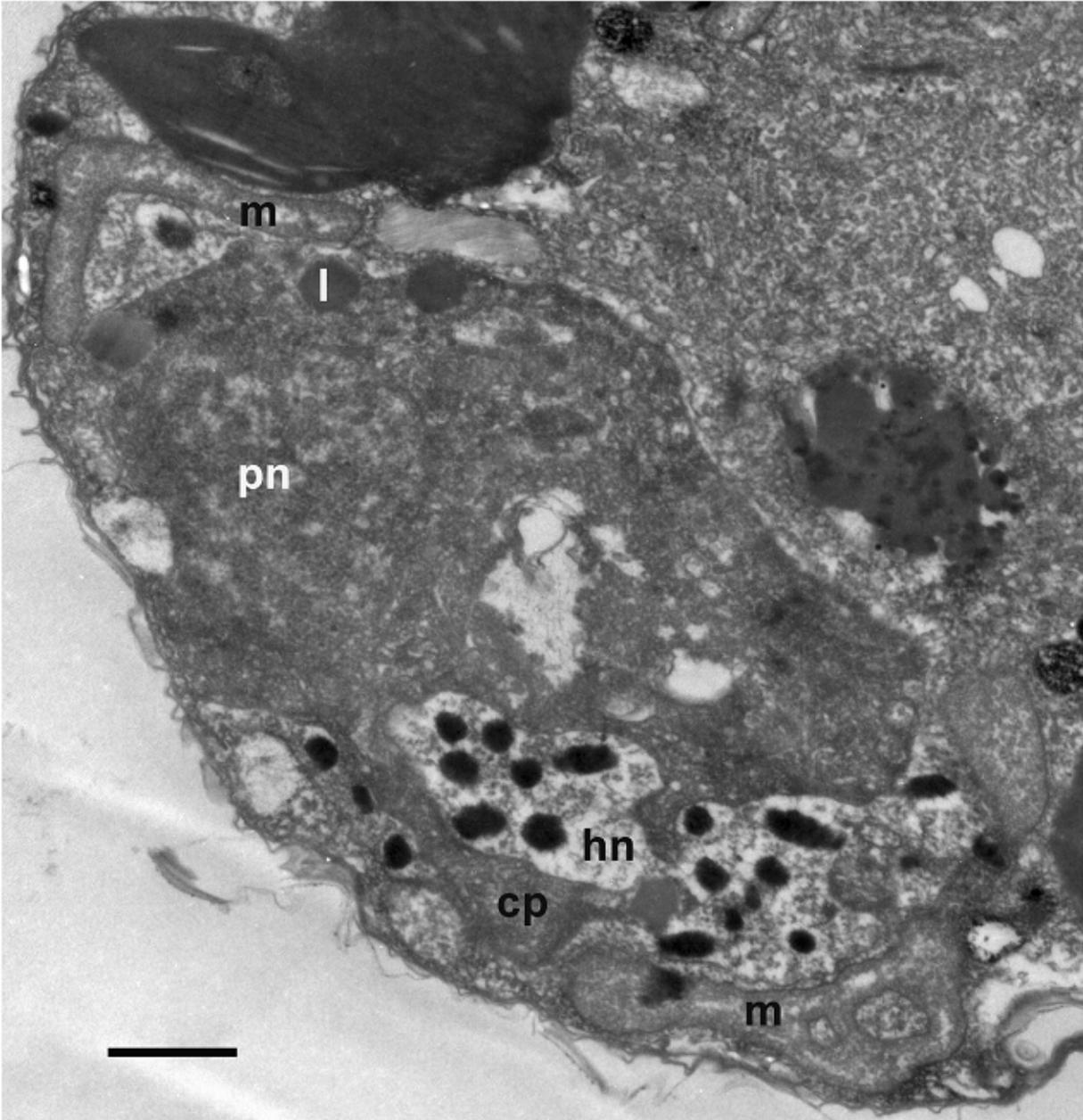


Figure 7

