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Dinoflagellate-infecting Zoosporic Parasitoids in the Baltic Sea

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Maailman meristä on uusien molekyylityökalujen avulla paljastunut laaja luokittelemattomien planktonloisten kirjo, eikä niiden tärkeää roolia merien ekosysteemidynamiikassa enää voida kieltää. Loiset ovat avainasemassa kasviplanktonyhdyskuntien muodon muokkaamisessa, esimerkiksi muuttamalla isäntä-populaatiokantojen tiheyttä. Työn tavoitteena oli selvittää, onko Itämeressä panssarisiimaleviä tartuttavia loisia, ylläpitää löydettyjä loiskantoja, tutkia loisten elinkaaria ja suorittaa alustava tutkimus loisten isäntäspesifisyydestä.

Näytteet kerättiin matalista merenlahdista Ahvenanmaan saaristossa kesällä 2016. Loisnäytteet analysoitiin valomikroskoopilla ja löydettyjen loislajien säilyttämistä varten tehtiin loisten elinkaarten tarkkaan havainnointiin perustuva ylläpitosuunnitelma.

Itämerestä löytyi kolme uutta panssarisiimaleviä tartuttavaa loislajia: kaksi *Parvilucifera*-lajia (*P. sp. nov. ja P. cf infectans*) ja yksi piiskasiimasienilaji (*Chytridiomycota*). Loisten lajikoostumuksessa oli eroja näytteenkeruukertojen välillä, mikä tarjoaa silmäyksen loisisäntävuorovaikutussuhteiden ja kasviplanktonyhdyskuntarakenteen väliseen dynamiikkaan. Loisilla havaittiin olevan eripituiset elinkaaret; lisäksi *Parvilucifera*-lajien myöhempien elinkaarivaiheiden ulkomuodoissa huomattiin eroavaisuuksia. Tehdyt tartuntakokeet viittasivat loisten tartuttavan osittain samoja isäntälajeja: *P. sp. nov*. tartutti kuitenkin testattuja isäntälajeja suppeammin kuin *P.cf infectans*. Tämän rinnakkaiselodynamiikan ymmärtämiseksi tarvitaan lisätutkimuksia.

Avainsanat	panssarisiimalevät, loiset, piiskasiimasieni, Parvilucifera, isäntä-loisdynamiikka, loisten elinkaari

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New molecular tools have revealed a great diversity of unclassified parasites in the world oceans and the important role marine parasitoids play in marine ecosystem dynamics can no longer be neglected. Parasites have a key role in shaping phytoplankton community structure, for instance, by altering host population densities. The aim of this study was to investigate if dinoflagellate-infecting parasites exist in the Baltic Sea, establish cultures of the found parasites, study their life cycles and conduct a preliminary study on their host ranges.

Field samples were collected in shallow inner bays in Åland Archipelago during summer 2016. Parasite samples were analyzed under light microscopy. In order to establish and maintain the discovered parasites their life cycles were studied thoroughly and a maintenance protocol was designed based on these observations.

Three new parasite species infecting dinoflagellates were found; two *Parvilucifera* species (*P. sp. nov.* and *P. cf infectans*) and one chytrid species. The composition of parasite species varied between the three sampling occasions which offers a glance to the interplay between host-parasite interactions and the phytoplankton community dynamics. The life cycles of the parasites varied in length and there were differences in the appearance of the later life stages of *Parvilucifera* species. Cross-infection experiments indicated that the *Parvilucifera* and chytrid species had similar host ranges. However, *P. sp. nov*. had more specialized host range than *P. cf infectans* and to better understand these coexistence dynamics more research is needed.

Keywords	dinoflagellate, parasitoid, chytrid, Parvilucifera, host-parasite dynamics, parasite life cycle

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Abbreviations

HAB	Harmful algal blooms. Algal blooms caused by toxic producing phyto-plankton species.
TEM	Transmission electron microscopy.

1 Introduction

The effects of host-parasite interactions on the ecosystem dynamics and evolution are extensive. Dinoflagellates are a diverse phytoplankton group, they are essential primary producers and they have a key ecological role in marine food webs (Reynolds, 2006). Hence, parasites infecting dinoflagellates play an important role in marine plankton food web dynamics (eg. Chambouvet et al. 2008). There are three recorded groups of parasitic eukaryotic microorganisms that infect photosynthetic and heterotrophic marine dinoflagellates: *Amoebophrya, Perkinsozoa* and chytrids. These zoospore producing parasites act as pathogens, host population regulators, food source and energy transfers. Due to their short generation times and abundant amount of zoospores produced by a single sporangium, zoosporic parasitoids can have a substantial impact on plankton communities (Jephcott et al. 2016). Parasitoids are parasites, which once the infection has occurred, consume and ultimately kill their host. The zoosporic parasitoids discussed in this study prevent the cell replication of their host, kill them and can thus have an important role in controlling the host population size. Also, by regulating the host population size, parasites maintain genetic polymorphism, diversity and resilience in marine phytoplankton communities (Chambouvet et al. 2008; Gleason et al. 2011). On the other hand, parasite zoospores serve as a food source for micro-zooplankton (Johansson and Coasts, 2002) and therefore also contribute to the growth of plankton communities. In addition, parasites infecting toxic plankton species transfer the otherwise inedible food source into edible zoospores and thereby return the organic matter to the food web and make it accessible for the higher trophic levels, i.e. mycoloop (Kagami et al. 2007).

Presumably most of the marine plankton groups are affected by parasites and since the range of host species in the marine environment is broad consequently the array of marine parasites infecting phytoplankton is also diverse (Chambouvet et al. 2008). Some parasites eg. *Parvilucifera sinerae* are generalists, infecting a wide range of host species, whereas some parasites eg. *Parvilucifera prorocentri* have a very specific host range (Garcés et al. 2013a; Leander and Hoppenrath 2008). However, host-parasite relationships are even more complex since parasites may change their behavior, generalists can become specialists and specialists can widen their host range and become generalists i.e. parasite paradox (Agosta et al. 2010). Studies have shown that fresh water fungal parasites attack different host species at different times of the year and sometimes same host species are infected by different parasite species depending on

the season (Holfed 1998). It is suggested that generalist parasitoids infecting multiple dinoflagellate species can potentially entirely change the ecosystem's dynamics (Dobson, 2004).

Clearly, the effects that parasites have on the planktonic community are widespread and depend on diverse factors, in particular on the host susceptibility and prevailing community structure (Alacid et al. 2016). For instance, some host species are more susceptible for parasite infections than others and the depleting effect parasites have on their host species benefit other concurrent species in the community. Furthermore, the prevailing community structure plays a role in the infectivity of the parasites since the parasite does not actively choose among hosts, but infects the most susceptible hosts available (Alacid et al. 2016). Also, high abundance of a highly susceptible host in the community can result into a heavy parasite load in the community which in turn affects the less susceptible host species (Woolhouse et al. 2001).

Studying the ecological effects of parasites infecting marine phytoplankton has historically been a neglected field and the importance of studying this field has only recently received the attention it deserves. Owing to new sequencing tools scientists have revealed that world oceans harvest a high diversity of unclassified parasites (de Vargas et al. 2015) which withhold an important role in the ecosystem dynamics. This study presents three parasite species which have not been previously discovered from the Baltic Sea, namely a new chytrid species and two new *Parvilucifera* species. The aim of this study was to investigate the presence of dinoflagellate parasites in the Baltic Sea, investigate the lifecycle of these parasites and conduct a preliminary study on their host range, in order to evaluate the coexistence dynamics of parasites with similar host preference.

2 Theoretical Background

2.1 Parasites and Biological Control of Toxic Algal Blooms

Under suitable environmental conditions various phytoplankton species can form massive blooms and often these blooms are formed by toxin producing species. Alarmingly, the distribution and frequency of these harmful algal blooms (HABs) have extended due to climate change, eutrophication and habitat modification (e.g. Anderson et al. 2012; Gilbert et al. 2014). Since toxins produced in blooms accumulate in food webs, toxic blooms can have serious consequences on marine ecosystems, aquacultures and human health (Zingone and Enevoldsen, 2000). In the Baltic Sea, cyanobacteria have been the main source of massive toxic blooms but additionally there are several dinoflagellate species that withhold the potential to cause toxic blooms such as *Alexandrium sp.*, *Prorocentrum sp.* and *Dinphysis sp*.. Toxic blooms of *Alexandrium sp.* have recently become more frequent and blooms of toxin producing *A. ostenfeldii* have been documented in the archipelago of Åland. (Hakanen et al. 2012)

Parasites are suggested to be adapted to blooming dynamics of their host species (Chambouvet et al. 2008) and parasitoid prevalence increases with increased host availability (Alacid et al. 2015) revealing the possibilities parasitoid activity withholds in controlling of toxic algal blooms (Coast and Park, 2002). Already in late 60's it has been proposed that the knowledge on parasite-host dynamics can be an important tool when planning the control of HAB's (Taylor, 1968). Since many of the parasite species infecting phytoplankton have only recently been discovered, the research on the host-parasite dynamics in marine environments is still in its infancy (eg. Jephcott et al 2016). Parasite specificity, infection mechanisms and eventual side effects of using parasites as biological control are some of the issues that need to be more profoundly investigated in order to better understand the dynamics (Anderson et al. 2009). Studies show that populations of invasive bloom forming dinoflagellate species, such as *Alexandrium minutum* in French estuaries, can become regulated by parasitoid activity over time (Chambouvet et al. 2008). It is also suggested that parasites have a key role as natural biological controllers of all marine plankton groups, but the capacity in which the parasites can impact a newly introduced invasive species is dependent on the parasite specificity and fitness (Chambouvet et al. 2008).

2.2 Parvilucifera

Parvilucifera species belong to the phylum Perkinsozoa with a sister group *Perkinsus* which in turn belong to the superphylum Alveolata including morphologically very distinctive subgroups: dinoflagellates, apicomplexan, marine alveolate clusters (MALV1,-2 and -4) and ciliates (fig. 1).

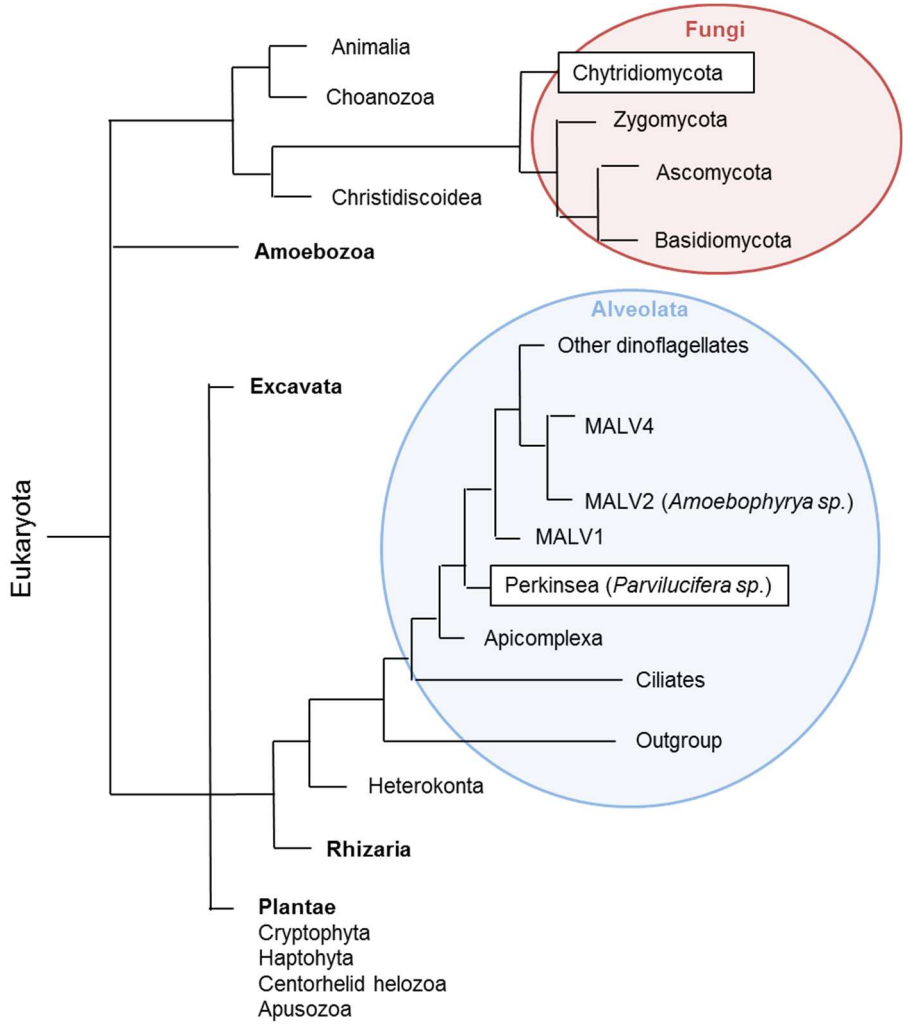

Figure 1. A simplified phylogenetic tree of Eukaryota (Ruggiero et al. 2015). The Alveolata phylogeny is based on the SSU rRNA sequencing by Lepelletier et al. 2014 (appendix 3).

To date, four species of the genus *Parvilucifera* have been identified and described: *P. infectans* (Norén et al., 1999), *P. sinerae* (Figuero et al., 2008), *P. prorocentri* (Leander and Hoppenrath, 2008) and *P. rostrata* (Lepelletier et al., 2014a). They are all endoparasites of dinoflagellates infecting a broad range of hosts, *P. prorocentri* being an exception since it specifically infects *Prorocentrum fukuyoi* (Leander and Hoppenrath

2008). In addition, all *Parvilucifera* species contain a reduced apical complex structure with rhoptries (secretory bodies), pseudo-conoid and microneme-like vesicles (e.g. Garces and Hoppenrath 2010; Leander and Hoppenrath 2008). In parasitic apicomplexans, these structures play a key role in the early stage infection processes and thus the same is presumed to be the case for *Parvilucifera* species (Alacid et al. 2015; Lepelletier et al., 2014a).

Parvilucifera life cycle consists of three stages: free-living motile stage (zoospore), intracellular stage (trophont) and free-living non-motile stage (sporocyst). One life cycle takes 1–5 days depending on the *Parvilucifera* species. The infection initiates when a free living biflagellate zoospore actively enters the host cell, where it will complete its life cycle. The zoospores are elongated, have two anterior flagella of which the other one is very short and are in general 4 µm long and 1–1,5 µm wide (fig 3B). Duple, triple and poly infections can occur (fig 2A) when several zoospores enter the same host (Garcés et al. 2013a).

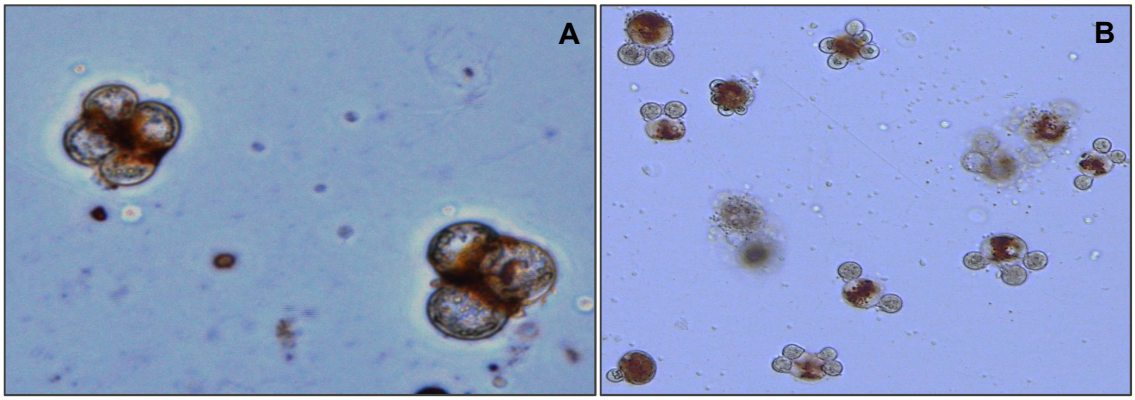

Figure 2. A) Multiple *Parvilucifera sp. nov*. infections in late trophocyte stage on *Kryptoperidinium foliaceum* host. B) Multiple chytrid infections on *Kryptoperidinium foliaceum.* Photos by Aurora Paloheimo.

When an infection takes place, the host cell drops its flagella, stagnates and sinks. Once in the host cytoplasm (intracellular stage), the parasitoid infects the hosts nucleus, forms a trophocyte inside a parasitophorous vacuole derived from the host cell membrane (Hausmann et al. 2003) and starts feeding on the hosts organelles. Early infections can be identified by this clear round body within the host cell (fig. 8C, fig. 9C and fig. 10 A1 and B1). The trophocyte grows and consumes the host until it occupies most of the cytoplasm and eventually detaches from the surrounding theca and enters the free-living non-motile stage. The late trophocyte is transparent, spherical and con-

tains vacuoles, starch granules and lipid droplets. (fig. 8D, fig. 9D and fig. 10 A3 and B3). During the replicative stage the sporangium gradually fills with zoospores starting from the periphery towards the center until the whole sporangia becomes packed with zoospores (fig. 8E, fig. 9f and fig. 10 A5 and B5).

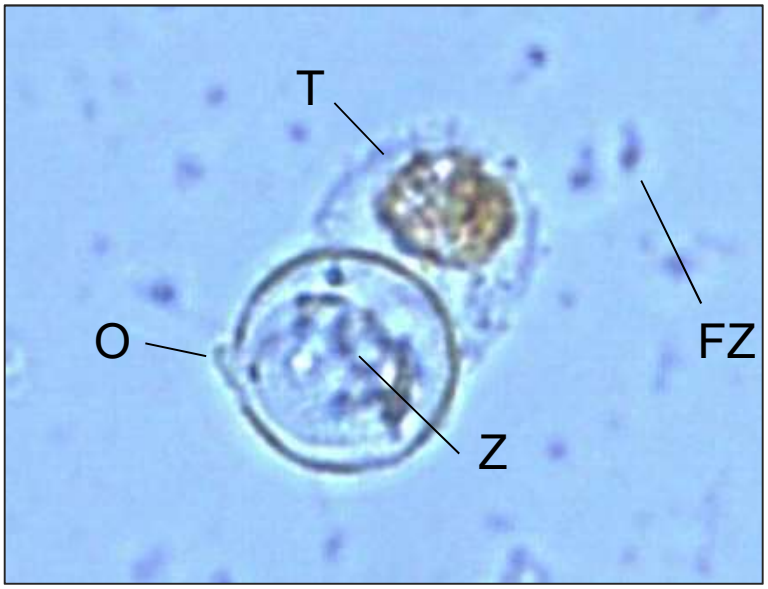

Figure 3. A) A *Parvilucifera sp. nov.* sporangium releasing zoospores. T = remains of the host theca, O = open operculum, Z = last zoospores circling inside the sporangium before leaving through the operculum and FZ = a free motile zoospore. Photo by Aurora Paloheimo.

Hundreds of zoospores (number depends on the host size), divided by schizogony, are produced in one sporangium. Finally, the zoospores are released to the environment through one or many opercula-covered orifices in its wall (except in *P. prorocentrum* zoospores are released through a germ tube) (fig. 3). This release typically occurs rapidly leaving behind only an empty sporangium (fig. 9G). How long the zoospores survive is unclear but they are considered short-lived; hours to days. (Alacid et al. 2015). The mature sporangium can remain in a resting dormant stage until it is activated by a chemical signal, namely a host-released dimethylsulfide (Garcés et al. 2013). These resting sporangia can be maintained in cold temperatures for months and are assumably used to survive periods of low host abundance and/or winter in nature (Lepelletier et al. 2014). Parasites surviving for several months in host cysts have also been recorded (Figuero et al. 2010)

2.3 Chytrid

The chytrid *Dinomyces sp.* belongs to a recently discovered genus Dinomycetaceae of which, to date; only one species (*Dinomyces arenysensis*) has been described. This zoosporic parasite is a true Fungi and belongs to the phylum of Chytridiomycota (fig. 1). Chytrids are found in diverse habitats from fresh water to soil ecosystems and many are parasites and/or saprobes, feeding on decaying organic matter. Thus, chytrids have an important function in the global energy transfer dynamics. Parasitic chytrids are broadly recorded in freshwater ecosystems but only a few species are known to be found in marine environment (Gleason et al. 2011). *D. arenysensis* is the first reported species to infect marine dinoflagellates (Lepelletier et al., 2014).

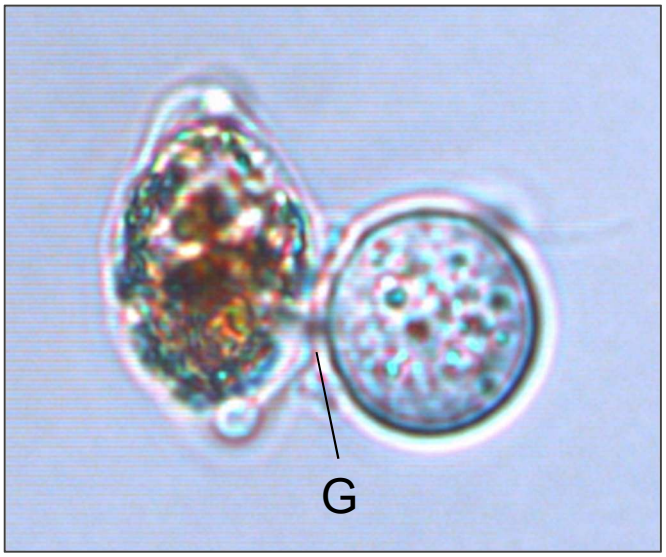

Figure 4. Chytrid feeding on *Hetercapsa triquetra* via a germ tube (G) penetrating the host. Photo by Aurora Paloheimo

Chytrids are ectoparasites, meaning they parasite extracellularly, forming a sac-like structure on the host. The life cycle of a chytrid consist of three stages: a unicellular flagellated stage (zoopores), a feeding stage (trophont) and a zoospore production stage (sporocyst). The infection initiates when a zoospore attaches to the host. Zoospores have one posteriorly directed flagellum, a characteristic of chytrids, that they resorb after attaching to the host. The attached parasite forms a thin cyst wall and produces a germ tube that penetrates the host through the gaps between the thecal plates (fig 4). The parasitoid remains outside the host and feeds on the host cytoplasm with branching rhizoids passing through the germ tube, grows and finally becomes a spo-

rangium (fig 12). Immature zoospores are produced in the sporangium by cytokinesis of the parasitoid body. When mature, the zoospores become rounded and are released to the surrounding environment through a break in the smooth sporangial wall. The size of a mature sack-formed chytrid sporangium depends on the number of infections on the same host. Polyinfections on one host cell are common (Fig. 2B). This lifecycle description is based on the lifecycle description of *D. arenysensis* provided by Lepelletier et al. 2014.

3 Materials and Method

3.1 Sampling and Study Area

The sampling was carried out in the Åland Archipelago during summer 2016. Samples were collected in 3 occasions: first 30th of June to 1st of July (S1), second 26th to 27th of July (S2) and last 18th to 19th of August (S3). Samples were taken from stations where *A. ostenfeldii* blooms were known to occur (Kremp et al. 2009 and observations by locals). During the first sampling (S1), samples were taken from 6 stations (fig. 5), in order to determine the most suitable sampling sites. The two later samplings (S2 and S3) were collected from two stations, station 3 and station 5 (fig. 5). The sampling sites were located in shallow inner bays (2–3 m deep) where sea water temperature can reach 24 ºC during the summer and salinity is typically 6–7 ppm (study area described by Kremp et al. 2009). The shallow sound of station 5 was heavily vegetated with macroflora (i. a. *Myriophyllum sp., Cladophora sp., Ranunculus sp. and Chara sp.*), especially during S2 and S3 (fig. 6).

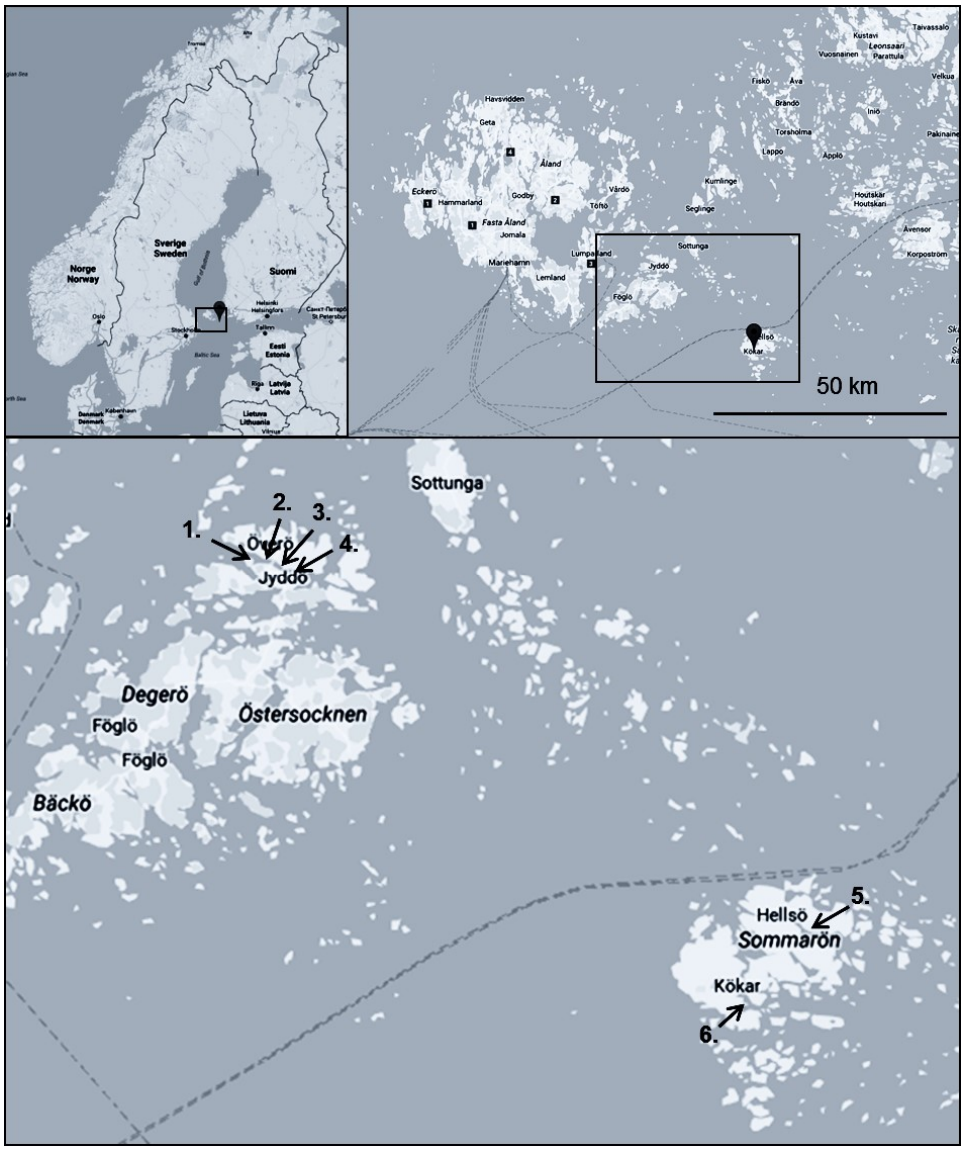

Figure 5. Sampling area. (A) Åland archipelago in the Baltic Sea with the inset indicating the showing the sampling stations (1-6). Maps are taken from GoogleMaps.

Two water samples, one natural surface sample (5 l) and one net sample (200 ml), were collected from a small boat at each station. The surface samples were collected with a jug from the water surface and sieved through a 76 µm mesh. The net samples were collected by slowly drawing the plankton net (10 µm mesh) close to the sea bottom and through the water column and then sieving the sample through 76 µm mesh into a jug. This was repeated 3 times before the sample was poured into a 200-ml polystyrene culture flask.

Figure 6. Station 5 during the second sampling, a shallow inner bay area in Kökar, Åland archipelago. Photo by Aurora Paloheimo

In field, three subsamples were preserved from the surface samples: one fixed with acidic Lugol's iodine solution and two fixed with formaldehyde solution (37%) in 200 ml culture flasks (one flask for bigger fractions (>10 µm) and one for smaller fractions (<10 µm). In addition, a mixture of suitable hosts was added to a flask with 50 ml of the net sample.

3.2 Laboratory Work

Net samples were observed under light microscope (Leica DMI3000B inverted research microscope) in order to detect the parasite. Surface samples from each station were concentrated and distributed into polystyrene well plates; S1 samples in 6 well plates, S2 and S3 samples in 12 well plates. For each station wells were prepared for bigger fractions (>10 µm) and for smaller fractions, zoospores, (<10µm) (Fig. 7). Depending on the phytoplankton density of the samples 2–5 l of the surface sample was filtered through a 10 µm mesh and 20 ml of the water concentrated in the mesh was collected (fractions >10 µm). Approximately 100 ml of the surface water, filtered through the 10 µm mesh, was filtered through a syringe with 0.2 µm Whatman polycarbonate filter paper and 10 ml of the concentrate was collected into a falcon tube. The filter paper was changed once during the process; both filter papers were placed into

the tubes and rinsed with the concentrate. Next, the concentrated samples were distributed into well plates and 1.5 ml of a host culture was added to each well (1 host/well). Altogether eight hosts were chosen and tested in order to determine suitable host strains for the parasite maintenance.

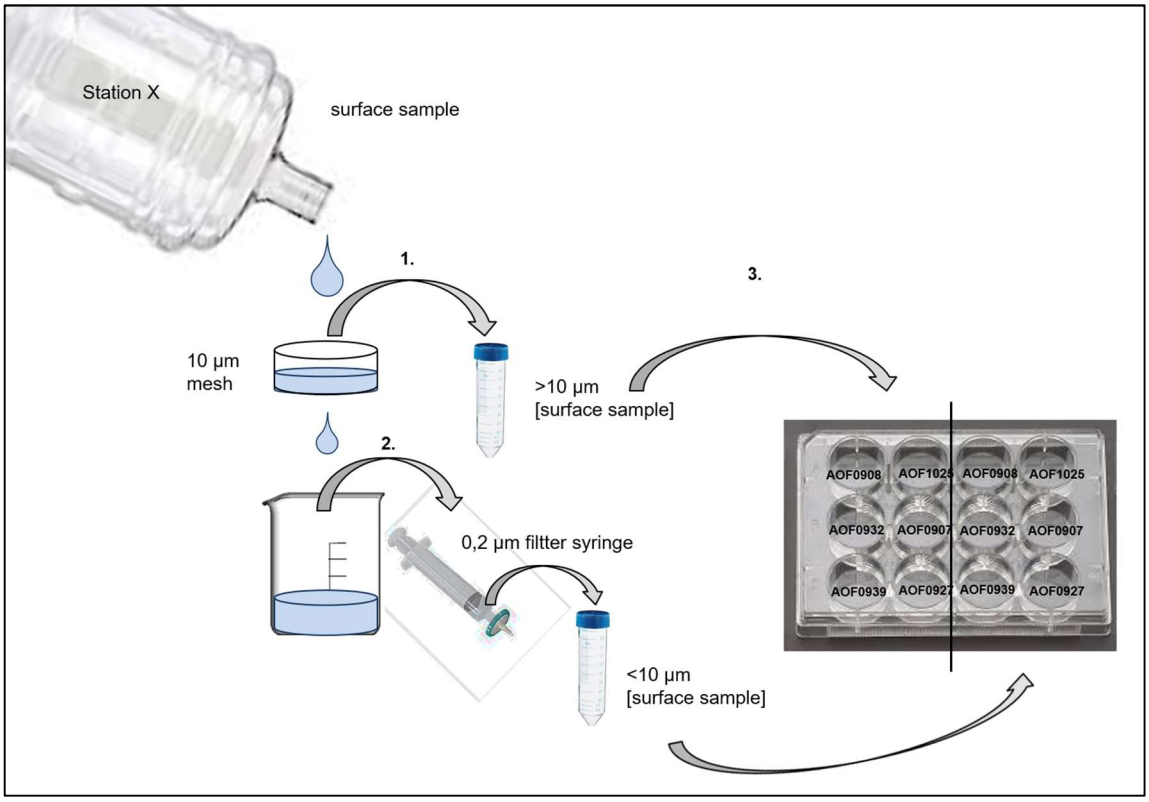

Figure 7. Concentration of the surface samples. 1. Surface water was concentrated into a 10 µm mesh and > 10 µm fractions were collected into a falcon tube. 2. The filtered surface water was concentrated into a syringe and < 10 µm fractions were collected into a falcon tube. 3. Concentrated samples were distributed into well plates and different hosts were added to each well. Example of a 12 well plate with *A. ostenfeldii* host strains.

Wells were observed daily under the microscope; the first aim was to find the parasite and then determine the most suitable host strain for maintenance. When the parasites were found from the net sample of the first sampling occasion (S1), 1 ml of concentrated (> 10 µm) net sample was added to each concentrated surface sample well. When parasites were not detected from the second samples (S2), net sample was added to concentrated surface sample wells. Net sample was not added to the concentrated surface well plates of the last sampling occasion (S3). All samples were preserved in 20 ºC climate room with 12:12 hour (dark:light) cycle. Sample types were evaluated based on the presence of the parasites in the different types of samples.

Plankton cultures used in this study were provided by Suomen Ympäristökeskuksen (SYKE) merentutkimuslaboratorio. Host cultures were maintained in exponential rate in 50 ml or 250 ml polystyrene tissue culture flasks, the volume depending on the consumption rate, with 6 psu f/2-Si growth medium.

3.3 Isolation, Establishment and Maintenance of the Parasite Strains

Parasites from the different sample types were isolated for culturing. A 96 well plate was prepared with one drop of sterile seawater growth medium (6 psu f/2-Si) in each well. Mature or in close to mature stage parasite sporangia were isolated by micropipetting one sporangium in each well. Then, under sterile conditions, 3 drops of the suitable host were added to the wells. Wells were observed after 2–3 days and when new infections were observed, preferably > 10 infected cells, the entire content of the well was transfer into a 24 well plate well. Infected parasites got easily stuck on the bottom of the well, so the wells were carefully spurted with a pipette and flushed several times to make sure that the parasites were transferred in the process. To finish, 1 ml of exponentially growing host culture was added to the new well. Newly established cultures were observed daily for new infections and the host-parasite ratio in the wells were evaluated. When infection and host concentration was low, more host culture was added and when the concentration of infected cells in the well grew denser, the parasite cultures were transferred into new wells.

Cultures were established in 24 well plates and host was added when majority of the infections in the wells were mature. In order to maintain the parasites in laboratory conditions, their lifecycle duration was observed under microscopy during several weeks. A host-feeding schedule was designed based on these observations. One third of the culture was moved into a new well after every second life cycle and fresh host culture was added both to the new and the old well. The cultures were cleaned by isolating 10 new sporangia of each strain and re-establishing the cultures from one of the isolates. Host cultures for the parasites were maintained in exponentially growing rate in culture flasks. Fresh host cultures were started every month and dense cultures were diluted with sterile seawater growth medium (6 psu f/2-Si).

After establishment of all the cultures a new long term maintenance schedule was designed where the time spend on maintenance was minimized (appendix 2). The host-

feeding was now timed so that the hosts were added to all the strains at the same time but still taking into account the different life lengths of the parasites. Small amount of the parasite strains (0.25 ml) were transferred into a new well once a week and dense host culture (1.5 ml) was added to ensure several life cycles without addition of new host. Also, the number of used host strains was reduced from six to two (appendix 1). Some behavioral tendencies of the parasite strains were noted during several months of maintenance.

To determine how long the parasites could be preserved, subsamples (late stage infections) were taken and conserved in 4 ºC for 4–6 week periods. The survival of the parasite subsamples was observed by inoculating the samples to host culture and observing if infections occurred.

3.4 Light Microscopy

Cells infected by the parasites were observed with Leica DMI3000B inverted light microscope, in which the examined object is illuminated above and the magnifying lenses are positioned under the examined object. This arrangement enables one to study living samples and culture dishes filled with media. Magnification of 10x was used to detect the parasites and magnification of 20x was used to study the finer details. Numerous digital images in color were taken for archives.

3.5 Life Cycle Analysis

The lifecycle lengths of the parasites were first estimated roughly by daily observations on the dominant life stages in the culture wells. The durance and appearance of the life stages of each parasite were documented and studied thoroughly by marking one spot in a culture plate and taking digital pictures of that spot at chosen time intervals. The initiation time of the infection was determined by first photographing a culture with mature sporangia and no host, then adding host and photographing the culture after one hour. Next, infection stages of the sunken host cells in the marked spots were followed and photographed during suitable time intervals. The appearances of different life stages of the two *Parvilucifera* species were compared.

3.6 Cross-Infection

Host ranges of the parasites were examined by conducting cross-infection experiments with 13 microplankton species from different algal groups (appendix 4). Host range of two chytrid strains, one *P. cf infectans* strain and one *P. sp. nov.* strain collected during sampling 1 from sampling station 5, were investigated.

The experiments were conducted in 24 well plates, where three replicates were set for each host. 1.5 ml of host culture was measured in each well followed by inoculation of 0.4 ml of a dense parasite culture. The chytrid cultures were in mature stage with plenty of free swarming zoospores and nearly no original host (*K. foliaceum*) present. The *Parvilucifera* cultures were relatively dense with sporangia in replicative/mature stage and with some original host (*A. ostenfeldii* and *K. foliaceum*) present. The host cultures used in chytrid cross-infection experiment were considerably old (2-3 months) and very dense whereas host cultures used in *Parvilucifera* cross-infection experiment were rather new (1 month) and recently diluted with fresh media. For chytrid host range experiment the wells were observed for infections 2, 3, 5, 7 and 9 days after inoculation. *Parvilucifera* wells were observed for infections 3, 6 and 10 days after inoculation. The percentage of infected host cells in the wells was roughly approximated to give a directive estimation of the susceptibility of the hosts. Infection responses of the hosts were classified into four categories (Lepelletier et al. 2014; Lepelletier et al. 2013): i) resistant, no infections were detected on the host ii) moderately resistant, some infections were detected but more than 10 host cells remained after 10 days, iii) moderately sensitive, infections were detected and less than 10 host cells were observed after 10 days and iv) sensitive, no host cells persist after 10 days. Infections were documented by taking digital images. Parasite infections on the different host species that were susceptible were documented.

3.7 Identification and Classification

The identification and characterization of the parasite species presented in this study will be completed later by the faculty of the department of Marin and Oceanography in Barcelona. Identification of the *Parvilucifera* species will be accomplished by sequencing the ribosomal internal transcriber spacer regions ITS1, 5.8S and ITS2. *Parvilucifera* species are separated from each other by comparison of SSU rRNA gene sequences

(Lepelletier et al. 2013). To complete the characterization, ultrastructure of the parasites life cycle stages will be studied under transmission electron microscopy (TEM). Samples for TEM were prepared by adding host to mature parasite cultures and fixing 5 ml of the samples with glutaraldehyde (final concentration 2 %) after 1 h, 6 h, 24 h, 30 h, 48 h and 72 h. Fixed samples were sent to the department of Marin and Oceanography in Barcelona for analysis.

3.8 Phytoplankton Community

The phytoplankton community of the lugol sample from the first sampling at station 5 was analyzed by A. Kremp (appendix 6). The phytoplankton communities of S2 and S3 were only briefly observed and precise data of that remains to be progressed.

4 Results

Three parasites were discovered from the field samples; one endoparasitic chytrid and two ectoparasites of genus *Parvilucifera (Parvilucifera cf infectans* and *Parvilucifera sp. nov.)*. Preliminary rRNA sequencing of the parasites conducted by the department of Marin and Oceanography in Barcelona indicate that all three parasites were unclassified species. Samples from the first and last sampling occasions contained the parasites but samples from the second sampling did not contain parasites. Parasites were found only from station 5 samples.

4.1 Sample Types

Overall the parasite abundance was highest in the net samples and lower in the concentrated surface samples (>10 µm). Parasites were not detected in any of the concentrated surface sample (<10 µm) wells. Distribution of the sample types in which the parasites were found is presented in table 1.

Table 1. Detected parasite infections in the different sample types from the 3 sampling occasions with notions on which samples the established strains were isolated.

Sample type			Sampling 1*			Sampling 2			Sampling 3		
Fraktion	Added host		*Parvilucifera*		Chytrid	*Parvilucifera*		Chytrid	*Parvilucifera*		Chytrid
	Species	Strain	*1*	*2*		*1*	*2*		*1*	*2*	
[surface < 10 µm]	*K. foliaceum*	KFF 1002	NA	NA	NA	-	-	-	-	-	x
[surface > 10 µm]	*K. foliaceum*	KFF 1002	x (E5, E10)	-	x (E12, FE5)	-	-	-	-	x (G10, D12, G3)	x (E3, B9)
	K. foliaceum	KFF 1003	x	-	x (E4)	-	-	-	-	x	x
[surface < 10 µm]	*A. ostenfeldii*	AOF 0908	-	-	-	-	-	-	-	-	-
	A. ostenfeldii	AOF 0932	-	-	-	-	-	-	-	-	-
	A. ostenfeldii	AOF 0939	-	-	-	-	-	-	-	-	-
	A. ostenfeldii	AOK 1025	-	-	-	-	-	-	-	-	-
	A. ostenfeldii	AOVA0907	-	-	-	-	-	-	-	-	-
	A. ostenfeldii	AOPL 0927	-	-	-	-	-	-	-	-	-
[surface > 10 µm]	*A. ostenfeldii*	AOF 0908	-	-	-	-	-	-	-	x	-
	A. ostenfeldii	AOF 0932	-	-	-	-	-	-	-	x	-
	A. ostenfeldii	AOF 0939	-	-	-	-	-	-	-	x (D5, E1)	-
	A. ostenfeldii	AOK 1025	-	x (D8)	-	-	-	-	-	x (D1, D7)	-
	A. ostenfeldii	AOVA 0907	-	-	-	-	-	-	-	x	-
	A. ostenfeldii	AOPL 0927	-	-	-	-	-	-	-	x	-
Prorocentrum sp.		Proro 1	NA	NA	NA	-	-	-	NA	NA	NA
Net sample			x (A12, B7)	-	x (C6, C7, C11)	-	-	-	-	x (D1)	x (A2, A9, A7, B3)

* For the first and second samples net sample was added to the surface well plates. 1 = *P.sp. nov.*, 2 = *P. cf infectans*, x =parasite infections were detected, - = parasite infections were not detected, NA = not done

The *Parvilucifera* composition of sampling 1 (S1) was different from sampling 3 (S3). *P. sp. nov.* was found only from S1 samples whereas the abundance of *P. cf infectans* was low in S1 but high in S3 samples. During isolation and establishment of new cultures, from the first sampling, most of the isolated sporangia infected *K. foliaceum* host,

whereas only one of the 40 isolates with *A. ostenfeldii* infected the host. In contrast, during the establishment of cultures from the last sampling, only three of the 50 isolates infected *K. foliaceum*, whilst establishment of cultures infecting *A.ostenfeldii* was found easy. The strains infecting *K. foliaceum* were identified as *P. cf infectans*. Chytrid strains were found and isolated from net samples and concentrated surface samples (>10 µm) with added *K. foliaceum* host.

P. sp nov. sub-sample strain A12 was not infective after one month preservation in 4 ºC but *P. sp nov.* sub-sample strain E10 was infective after a 6 weeks preservation period. *P. cf infectans* sub-sample strain D8 was not infective after 6 weeks preservation. All tested chytrid subsamples (C7, C6, E12) survived the 6 weeks preservation period in 4 ºC, but were not infective after 2 months of preservation.

4.2 Parasite Strain Maintenance

Eventually, 12 *Parvilucifera* strains and 12 chytrid strains were established and maintained (appendix 1) until the number was further reduced for long term maintenance. *P. cf infectans* was considered notably more virulent than *P. sp. nov.* This was distinguished as clearly more infected cells in the wells after every life cycle. The infection pattern of *P. sp. nov.* was found variable. In many occasions the newly added *K. foliaceum* host in the *P. sp. nov.* wells stagnated on the bottom and only a few infections were detected after one life cycle. Sometimes almost all the host cells were infected, with several multi-infections, after one life cycle. No reason for this infection behavior could be determined. Thus, the maintenance of *P. sp. nov.* required clearly more attention whereas the maintenance of *P. cf infectans* was found rather easy. The chytrid strains were also found rather virulent and able to infect almost 100 % of the added host after several life cycles. The infectivity of the chytrid strains seemed to decrease the longer the strains had been preserved in laboratory conditions.

4.3 Life Stages

Generation times of the two *Parvilucifera* species differed in length. One life cycle of *P. cf infectans* was approximately 48 hours. The trophocyte developed into a sporocyte approximately 24 hours after the infection had occurred and matured within the next 24 hours (fig. 8).

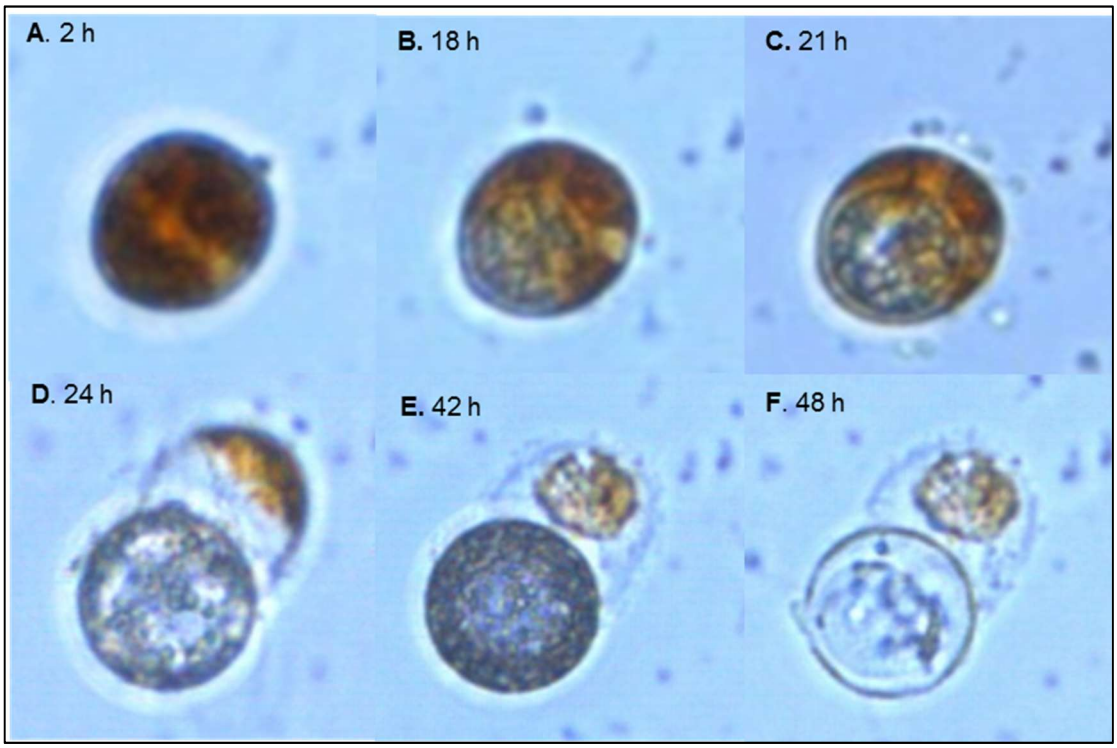

Figure 8. Life cycle of *Parvilucifera cf infectans* infecting *A. ostenfeldii.* (A) Newly infected host cell. (B and C) Early stages of infection identified by the round body (trophocyte) within the host cell. (D) A complete sporocyte with lipid-drops. Usually rests of the broken theca are in close proximity. (E) A late sporocyte (sporangium) filled with zoospores. (F) Sporangium releasing the infective zoospores.

The length of *P. sp. nov.* life cycle was approximately 72–90 hours. The trophocyte developed into a sporocyte approximately 42–48 hours after an infection and matured within the next 24–42 hours (fig. 9).

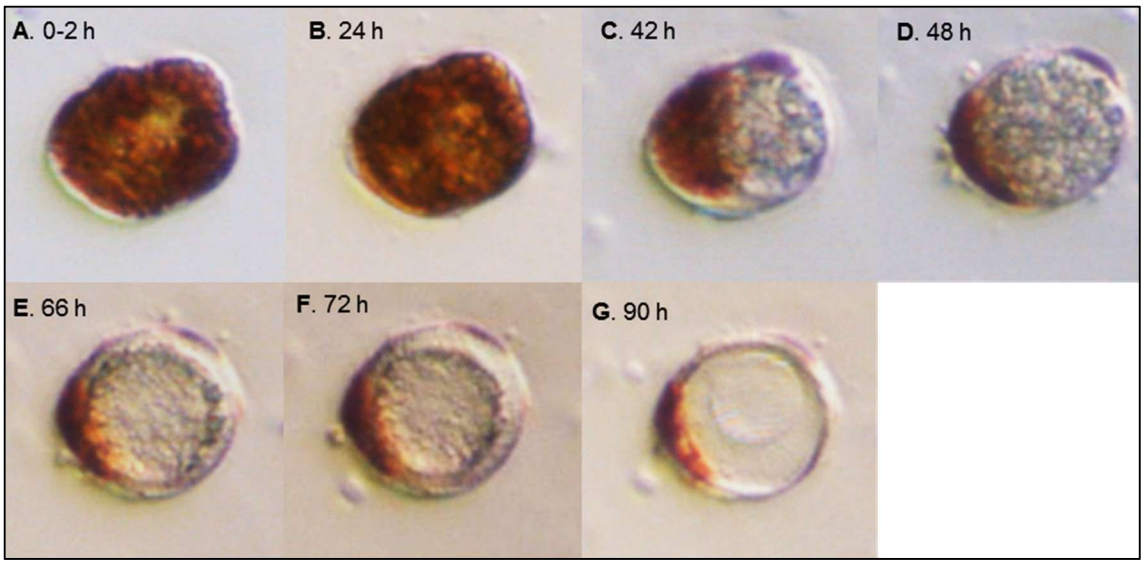

Figure 9. Life cycle of *Parvilucifera sp. nov.* infecting *K. foliaceum.* (A) Recently infected host cell. (B and C) Early stages of infection, a trophocyte develops within the host cell. (D) Late trophocyte, (E and F) a sporocyte in replicative stage with zoospores assembling from the periphery towards the middle. (G) An almost empty sporangium releasing infective zoospores.

The appearance of a trophont, a sporocyte and a mature sporangium of the two *Parvilucifera* species were observed to be different (fig. 10). Early stages of the infection were rather similar and at that stage the morphological differences were mainly dependent on the infected host species (fig. 10 A1 and B1, see also appendix 5). Although, round trophocyte bodies of *P. cf infectans* were considered sharper and easier to detect compared to *P. sp. nov.* trophocyte bodies. Almost without exception, sporocyst of *P. cf infectans* separated from the host theca in an early phase (fig. 8) whereas the host rests typically remained closely attached to the sporocyst throughout the development of *P. sp. nov*. (fig. 9 and fig. 10). Late stage trophocytes of the two species were different in appearance; *P. cf infectans* had distinct lipid globules (fig. 8D and fig. 10 B2–B4) whereas late trophocyte of *P. sp. nov*. was observed to have smaller, more evenly and densely packed globules (fig. 9D and fig. 10 A2–A4). The most significant difference was that the zoospores of *P.cf infectans* were clearly darker and slightly bigger than the zoospores of *P. sp. nov* which produced nearly opaque zoospores (fig. 10 A5 and B5). This was clearest when comparing the mature sporangia of the two species but the difference could also be detected in the younger sporangia.

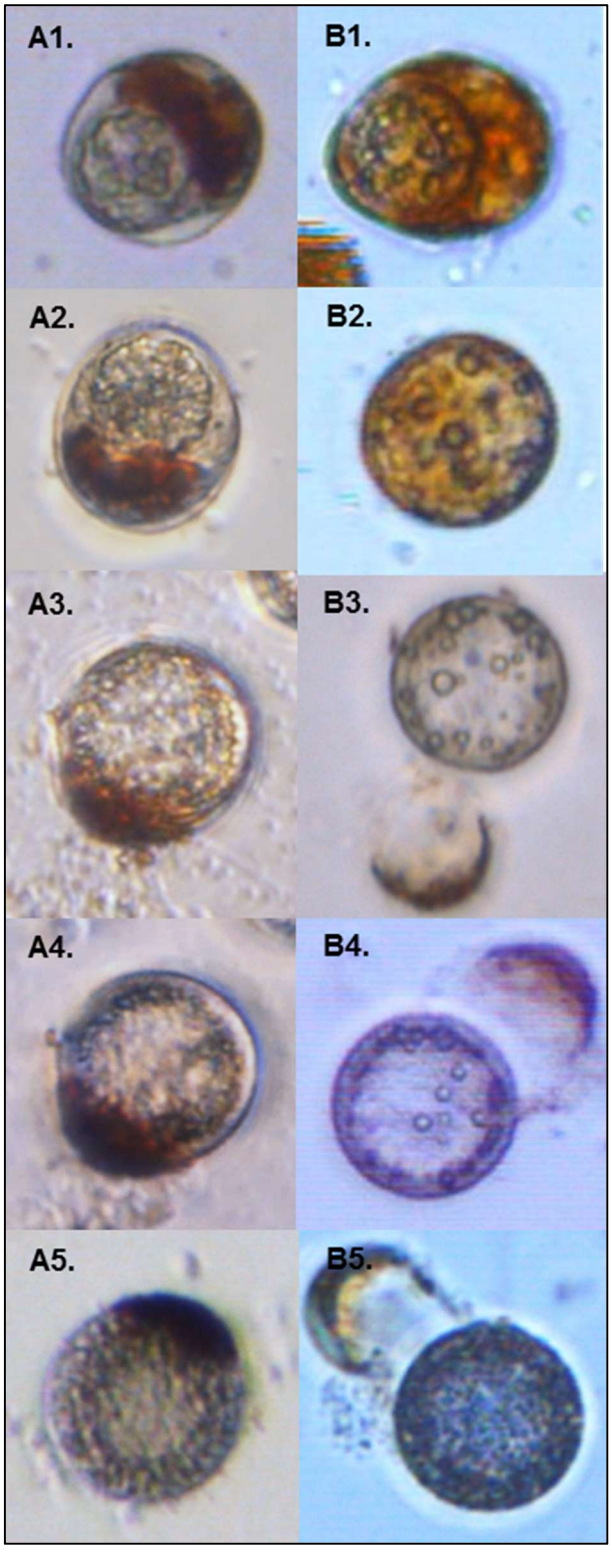

Figure 10. Life stage comparison of *P. sp. nov.* infecting *K. foliaceum* (A1–A5) and *P. cf infectans* infecting *A. ostenfeldii* (B1–B5). A1–A2 and B1–B2. Early infections, trophocytes growing and feeding within the hosts. A3–A4 and B3–B4. Zoospores multiplying within the sporocytes. A5 and B5. Mature sporangia filled with zoospores.

Life cycle length of the chytrid was estimated to be 28–36 hours depending on the number of infections on one host. Figure 12 presents the life cycle stages of a chytrid on *K. foliaceum*. After the zoospore had penetrated the host cell and formed a thin cyst wall, the parasite started to consume the host and grow approximately for 24 hours to become a sporangium. After the zoospores were released an empty sporangium attached to a degraded host cell was left behind to designate that an infection had occurred.

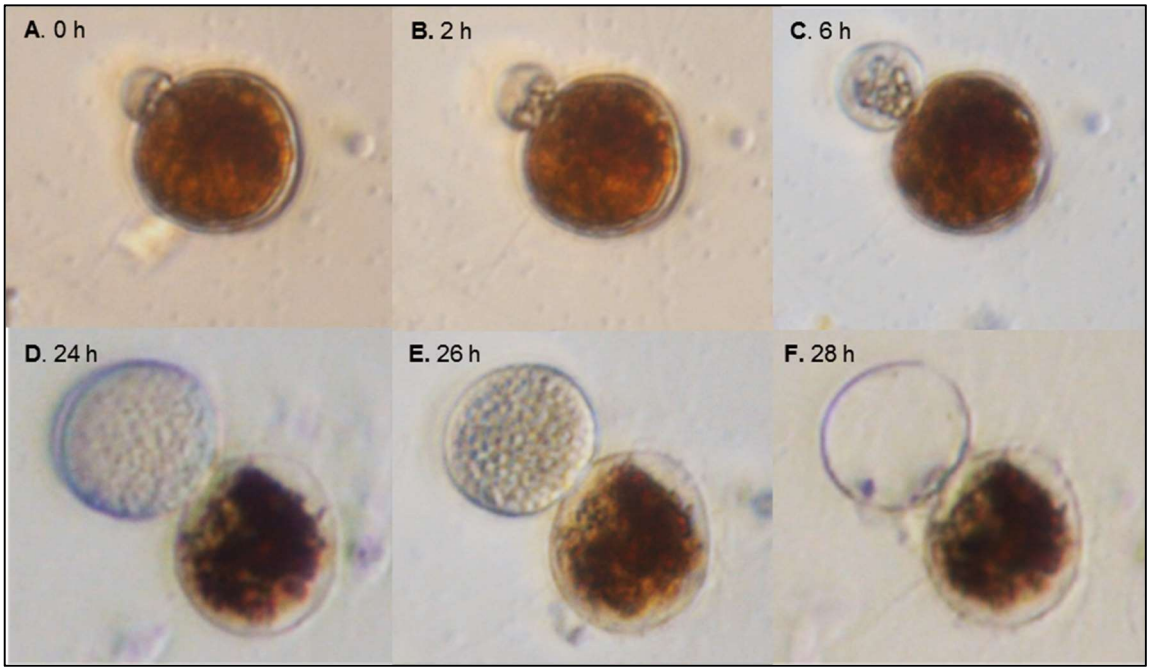

Figure 12. Life stages of the chytrid (on *K. foliaceum* host ~20 µm in diameter). A–C. Early stages of infection, the zoospore has attached and started to feed on the host. D–E Mature sporangium filled with zoospores attached to the degraded host cell. F. Empty sporangium left behind after the zoospores have been released.

4.4 Host Range

4.4.1 Parvilucifera

Of the 13 tested microplankton species only dinoflagellates were susceptible to *Parvilucifera* infections (table 2). Two species were susceptible to *Parvilucifera sp. nov* and four species were susceptible to *P. cf infectans.* Six days after inoculation *P. cf infectans* had infected approximately 70 % of the *A. ostenfeldii* (very dense culture), 85 % of the *H. triquetra, 40 %* of the *K. foliaceum* and 70 % of the *Levanderina fissa* host cells. Six days after inoculation *P. sp. nov.* had infected approximately 20 % of the *H. triquetra, 90 %* of the *K. foliaceum* cells. *P. sp nov.* was not found to infect *A ostenfeldii* or *L. fissa. A.ostenfeldii, H. triquetra* and *L. fissa* were classified as sensitive and *K. foliaceum* as moderately sensitive to *P. cf infectans. K. foliaceum* was classified as sensitive and *H. triquetra* as moderately sensitive to *P. sp nov.*

Table 2. Cross-infection of *Parvilucifera cf infectans* and *Parvilucifera sp. nov.* with different microplankton species. Compared with literature results of *P. infectans* and *P. rostrate* (Lepelletier et al. 2013).

Algal group	**Host species**	*Parvilucifera cf infectans*	*Parvilucifera sp. nov*	*Parvilucifera infectans**	*Parvilucifera rostrata**
Dinoflagellate	*Alexandrium ostenfeldii*	+	---	+/+++	+/+++
	Heterocapsa triquetra	+++	+	+++	+++
	Kryptoperidinium foliaceum	+	+++	+++	+++/-
	Levanderina fissa	+++	---	ND	ND
	Gymnodinium aureolum	ND	ND	+/+++	+++/-
	Prorocentrum sp.	---	---	---	---
	Karlodinium veneficum	---	---	---	---
	Pfiesteria piscicida	---	---	---	---

Resistant=---, moderately resistant=-, moderately sensitive=+ and sensitive=+++, ND=not done.
*literature (Lepelletier et al. 2013)

4.4.2 Chytrid

Two of the 13 tested species of microplankton were sensitive to chytrid infections. Approximately 99 % of *K. foliaceum* cells (in all 3 wells of both tested strains) were infected 5 days after inoculation of chytrid to the cultures. *K. foliaceum* was classified as

highly sensitive to chytrid infections. Approximately 80 % of *H. triquetra* cells were infected 10 days after chytrid inoculation. *H. triquetra* was classified as moderately sensitive to chytrid infections. Cross-infection results were compared with literature cross-infection results of *D. arenysensis* (Table 3).

Table 3. Cross-infection of the chytrid with different microplankton species compared with literature results of *D. arenysensis* cross-infections.

Algal group	**Host species**	***Chytrid, sp. nov.***	***Dinomyces arenysensis****
Dinoflagellate	*Alexandrium ostenfeldii*	---	+/+++
	Heterocapsa triquetra	+	+
	Kryptoperidinium foliaceum	+++	---
	Levanderina fissa	---	ND
	Prorocentrum sp.	---	---
	Karlodinium veneficum	---	---
	Pfiesteria piscicida	---	ND
Haptophyte	*Pleurochrysis sp.*	---	ND
Cryptophyte	*Rhodomonas sp*	---	ND
	Rhinomonas nottbeckii	---	ND
	Chryptophyceae	ND	---
Chlorophyte	*Chlorella pyrenoidosa*	---	ND
	Monoraphidium sp	---	ND
Cynobacteria	*Aphanizomenon sp*	---	ND

Resistant=---, moderately resistant=-, moderately sensitive=+ and sensitive=+++, ND=not done.
*(Lepelletier et al. 2014)

Infections on the different host species were briefly compared and digital images of the appearance were gathered in a table (appendix 5).

4.5 Phytoplankton Community

During S1 the phytoplankton community was dominated by *K. foliaceum;* possesing 78 % of the total biovolume (appendix 6). During S2 the phytoplankton community was in field estimated to be dominated by *Prorocentrum sp.* and during the last sampling *Alexandrium sp.*. *Heterocapsa sp.* were abundant during all samplings. *Alexandrium sp.* were more abundant during S2 and S3 than S1.

5 Discussion and Conclusions

Parvilucifera species are reported to be most active in marine sediments and infections are rarely found in water column samples (Chambouvet et al. 2014; Alacid et al. 2015). Therefore, it is not surprising that the abundance of parasites was found highest in the net samples, which seems to be the most effective way to collect the parasites. However, net samples contain a great variety of zooplankton preying on the dinoflagellate hosts and therefore adding extra host is advisable to ensure the survival of the parasites. Still, during denser blooms the surface samples may be more manageable and higher phytoplankton abundance may increase the abundance of parasite infections in the samples. In general, the surface samples are expected to contain parasite zoospores released by the infected dinoflagellates that have sunk to the bottom to mature after the infection has occurred. The absence of parasites in the surface samples could thus indicate the absence of zoospores in the surface water column. This raises a question whether the collected parasites found in the net samples were in resting stage and became activated in laboratory conditions. On the other hand, the density of zoospores is likely higher closer to the bottom of the sea and it is still unclear how far the zoospores move from the bottom after they have been released. Nevertheless, infections were found also in the surface samples (S1 and S3), indicating that active zoospores were in fact present in the studied communities. Consequently, also since sampling type could be an indicator of the parasite activity in the community, collection of both surface and net samples is advised when studying the parasite.

The different *Parvilucifera* composition of the three sampling occasions is probably explained mainly by seasonal variation of the phytoplankton community. Still, the presence of parasites in the community raises an interesting question on the extent of the impact the parasites have on the community structure and dynamics. These results suggest that *P. sp. nov.* was the dominating *Parvilucifera* species in the phytoplankton community of the first sampling occasion and *P. cf infectans* was dominant in the late summer samples. This could indicate that two species sharing a similar ecological niche can co-occur if only one of them is abundant. The abundance of these species seems to be phytoplankton community dependent, allowing one species to flourish while the other one is laying low in the background. Co-existence of closely related and morphologically similar parasitic species that compete for same host species in same ecosystem have been documented in past (*Amoebophrya sp.* (Salmon et al. 2003) and *Parvilucifera sp.* (Lepelletier et al. 2014a)) but the strategies and dynamics underlying

this kind of co-existence remains to be an unsolved ecologically interesting riddle. An additional factor to the dynamics brings the presence of a third parasite with similar host range, namely the chytrid, in the same community. It has been assumed that marine chytrids compete with other parasites of microplankton hosts (Richard et al. 2012). Co-occurance of other *Parvilucifera* species with *P. infectans* have also been reported in past (Lepelletier et al. 2014a).

Parvilucifera host range experiment results imply *P. sp. nov.* to have a more specific host range than *P. cf infectans* and, as determined in previous studies, *P. cf infectans* shows characteristics of a generalist parasitoid. The fact that *P. cf infectans* was considered more virulent than *P. sp. no.* may partly owe to the significantly shorter life cycle length of the species, which surely serves as a competitive advantage. Even though the studied *Parvilucifera* species have similar host range they seem to have slightly different host preferences among the shared host species which might be the key to their co-existence (Alacid et al. 2015). *P. sp. nov.* seems to prefer *K. foliaceum* as host whereas, even though *P. cf infectans* was able to infect *K. foliaceum*, it clearly seemed not to be the preferred host. The community composition of S1, where *K. foliaceum* (78 % of the biovolume) was dominant could have favored *P. sp. nov. Prorocentrum sp*. was resistant to the studied parasites and according to previous studies, of all four classified *Parvilucifera* species, only *P. prorocentri* can infect *Prorocentrum sp.* (Garcés et al. 2013). Interestingly, parasites were not found from the second sampling occasion when the phytoplankton community was dominated by *Prorocentrum sp*. (field observation, no data). Finally, the abundance of *P. cf infectans* in S3 samples could be due to the higher abundance of the preferred *A. ostenfeldii* host and/or the lower abundance of suitable hosts for *P. sp. nov.* These assumptions are supported by the studies on *P. sinera* infection strategies which have shown that in a community with two preferred host species the parasite infects the most abundant species and in a community composed of host species with different susceptibility, susceptibility overrules abundance and becomes the determining factor (Alacid et al. 2016). The cross-infection experiment conducted for this study gives only a directive for the host ranges of the studied parasites and in order to get a more comprehensive picture, more dinoflagellate species should be cross-infected. Also several strains should be tested since *Parvilucifera* species have a strong intraspecific variability (Lepelletier et al. 2014a). These host-parasite dynamics should be further explored since they might withhold important ecological clues on the community level.

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Established Parasite Strains

The parasite strains established and the host strains used for their maintenance are presented in the table. Strains kept for longterm maintenance are marked with grey background.

Parasite	Sampling	Strain ID	Original host	Host	Life cycle (days)	Sent for sequencing	Sent for TEM
P. cf infectans	S1	**D8**	AOK 1025	AOF 0939	2	x	x
		D1	AOK 1025	AOF 0939	2		
		D7	AOK 1025	AOF 0939	2	x	
		D5	AOF 0939	AOF 0939	2	x	
		E1	AOF 0939	AOF 0939	2		
	S2	G10	KFF 1002	KFF 1002	2		
		D12	KFF 1002	KFF 1002	2		
		G3	KFF 1002	KFF 1002	2		
P. sp. nov.		**A12**	KFF 1002	KFF 1002	3-4	x	x
		B7	KFF 1002	KFF 1002	3-4		
		E5	KFF 1002	KFF 1002	3-4	x	
		E10	KFF 1002	KFF 1002	3-4	x	
Chytrid	S1	C6	KFF 1003	KFF 1002	1-2		
		C7	KFF 1003	KFF 1002	1-2		
		C11	KFF 1003	KFF 1002	1-2		
		E4	KFF 1002	KFF 1002	1-2	x	x
		E12	KFF 1003	KFF 1002	1-2	x	
		FE5	KFF 1002	KFF 1002	1-2		
	S2	A2	HTF 1001	KFF 1002	1-2		
		A9	HTF 1001	KFF 1002	1-2		
		B3	KFF 1002	KFF 1002	1-2		
		E3	KFF 1002	KFF 1002	1-2		
		A7	HTF 1002	KFF 1002	1-2		
		B9	KFF 1003	KFF 1002	1-2		

Long-term Maintenance Protocol for the Parasite Strains

The host-feeding schedule is based on the generation times of the parasites which vary between the three species from 1–4 days (table 1). The cultures are established in 24 well plates, more host is added every 4th day and the cultures are moved into new wells once a week (figure 1.). Preferably, most of the infections should be in mature stage when they are transferred into new wells.

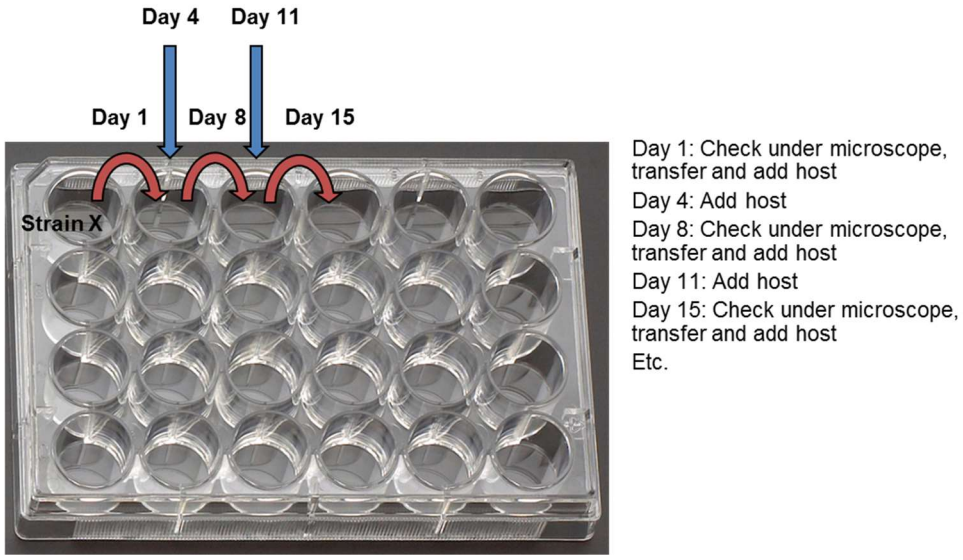


Figure 1. Maintenance schedule

Before transfer, the condition of the parasite cultures should be evaluated under the microscope.

i. If the culture is dense with infections, 0.5 ml of the infected culture is moved into a new well and 1 ml of fresh and dense host culture is added to the new well (add host also to the old well to have a back-up). This is the typical situation.
ii. If the culture is extremely dense with infected cells, transfer only 0.25 ml of the infected culture to the new well and add 1-1.5 ml of the host culture.
iii. If the number of infections is low (<20 infected cells/well), a larger amount of the infected culture (up to 1 ml) should be moved to the new well.
iv. If there are only a few infections in the well, new host should be added and the transfer postponed until there are a minimum of 20 infected cells in the well. In this case the older wells should be checked for infections and if a suitable amount of infections (>20) appear in one of the previous wells the transfer can be made from there.

Infected parasites get easily stuck on the bottom of the well and therefore the well should be carefully spurted with a pipette to make sure that the parasites are transferred in the process. If the well is full when more host should be added, remove some of the top layer of the media with a pipette (infected cells sit on the bottom) and replace it with a fresh host culture. Host addition is conducted in the lamina with an automate pipette.

Host cultures for the parasites are maintained in culture flasks (200 ml) in an exponentially growing rate. Fresh host cultures are started every 4–6 weeks by filling ¾ of a 200 ml culture flask with sterile seawater growth medium (6 psu f/2-Si) and adding 20–50 ml of a dense culture to the flask (depending on the density of the culture). When half of a dense culture has been used, it can be diluted by adding new growth media to replace the consumed host culture. Approximately 3 ml of host culture per each strain is needed on weekly bases (1ml for first addition and 2 ml for addition to the old and new well after the transfer). Thus, 24 ml of dense *A. ostenfeldii* culture should be ready for the 8 *P. cf infectans* strains and 48 ml of *K. foliaceum* culture should be ready to use for the 12 chytrid and 4 *P. sp. nov.* strains every week.

Subsamples of the cultures are taken by transferring 2 ml of a dense culture, preferable in mature stage, into an Eppendorf and preserving it in 4 ^{0}C and in dark. In case a parasite strain is lost, a new culture can be established from a sub-sample.

Phylogenetic Tree

Phylogeny of *Parvilucifera* species and the closely related clades (Lepelletier et al. 2014).

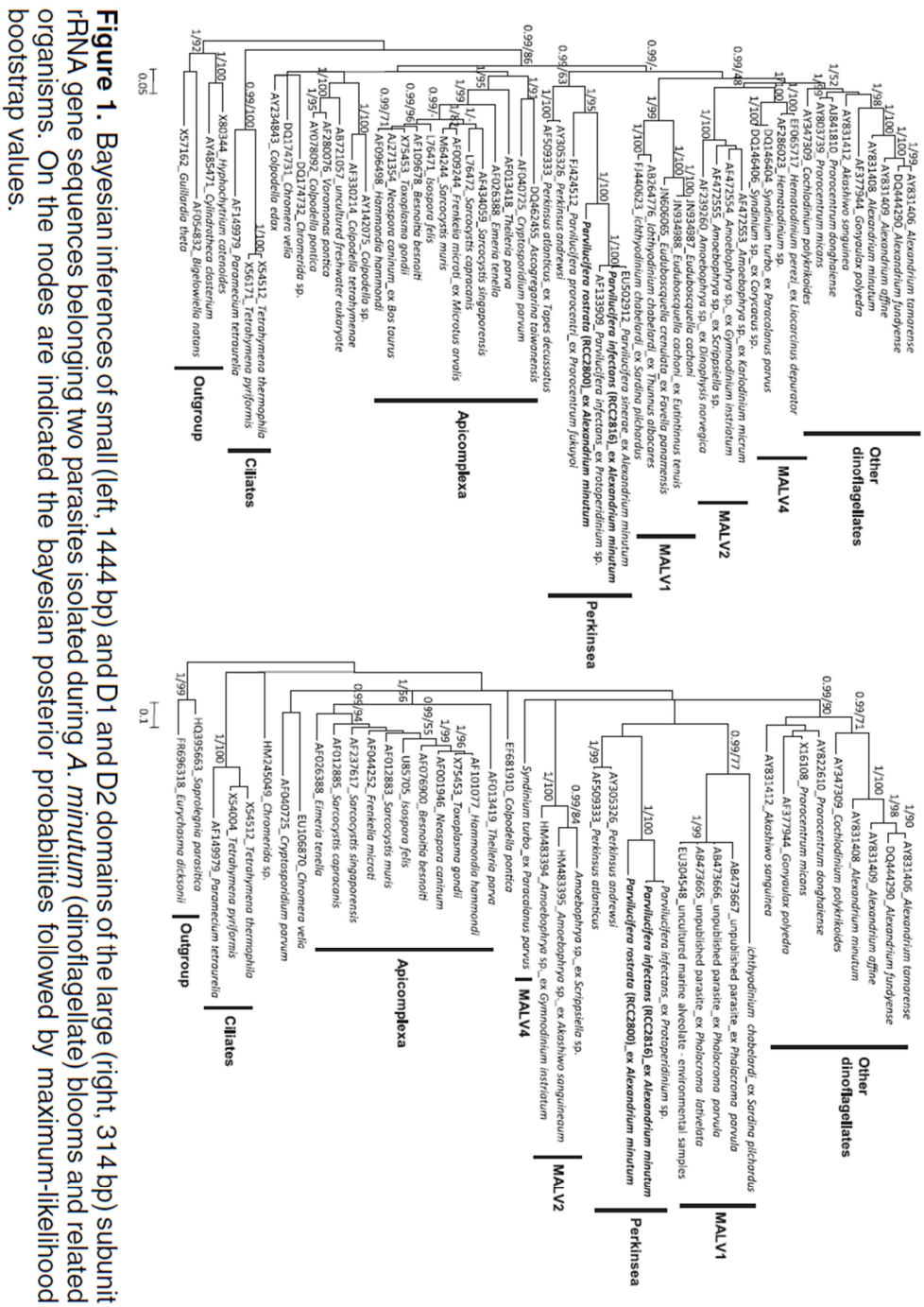


Figure 1. Bayesian inferences of small (left, 1444 bp) and D1 and D2 domains of the large (right, 314 bp) subunit rRNA gene sequences belonging two parasites isolated during *A. minutum* (dinoflagellate) blooms and related organisms. On the nodes are indicated the bayesian posterior probabilities followed by maximum-likelihood bootstrap values.

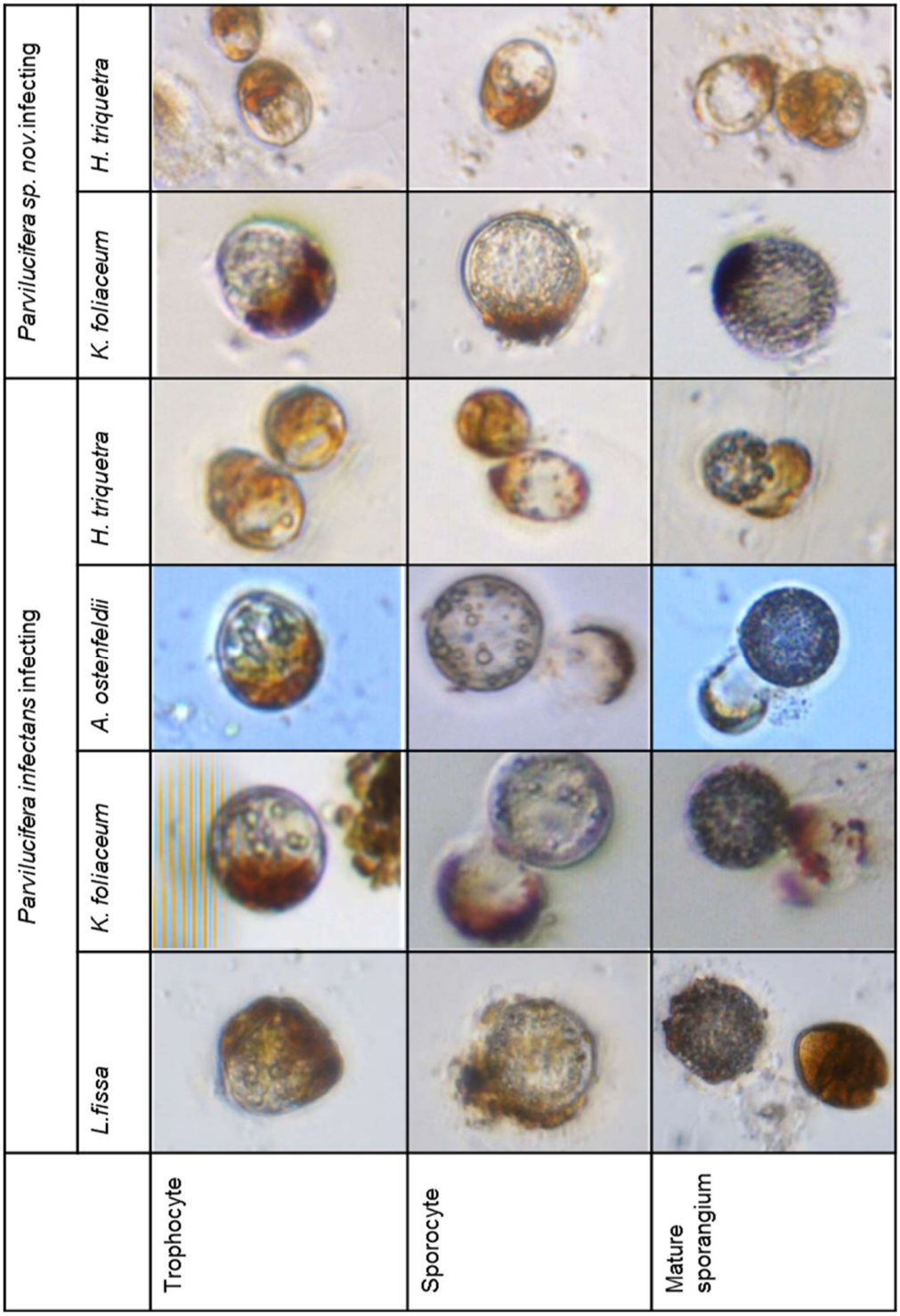
Host Range Strains

Microplankton strains used for the cross infection experiment.

Species	Strain designation	Algal group
Alexandrium ostenfeldii	AOF0908	Dinoflagellate
Heterocapsa triquetra	HTF1002	Dinoflagellate
Kryptoperidinium foliaceum	KFF1002	Dinoflagellate
Levanderina fissa	GFF1101	Dinoflagellate
Prorocentrum sp.	Proro 1	Dinoflagellate
Karlodinium veneficum	KVDAN31	Dinoflagellate
Pfiesteria piscicida	PPF02	Dinoflagellate
Pleurochrysis sp.	Cocco 3	Haptophyte
Rhodomonas sp	Crypto07B1	Cryptophyte
Rhinomonas nottbeckii	Crypto07B6	Cryptophyte
Chlorella pyrenoidosa	TV216	Chlorophyte
Monoraphidium sp	TV70	Chlorophyte
Aphanizomenon sp	KAC28	Cyanobacteria

Parvilucifera Infections on Different Dinoflagellate Hosts

Parvilucifera infections on the different dinoflagellate species tested in the cross infection experiment.



Community Structure S1

The phytoplankton community structure of station 5 during S1.

St.5	S1			
species	cells per L	Biovolume/L	%Biovolume	% cells
Heterocapsa triquetra	27741,11	30515222,02	2,482336724	6,21669
Heterocapsa rotundata	162483,65	21447841,76	1,744728097	36,41204
Durinskia baltica	6340,83	21235424,11	1,727448455	1,420958
Kryptoperidinium foliaceum	91149,36	966000964,66	78,581754	20,42627
Alexandrium ostenfeldii	3963,02	83912897,53	6,82610361	0,888099
heterotr. Dinoflagellate 20um	3170,41	6670548,27	0,54263236	0,710479
heterotr. Dinoflagellate 10um	3170,41	3328933,31	0,27080037	0,710479
Phaeopolykrikos sp.	792,60	39820383,23	3,239288234	0,17762
Levanderina fissa	6340,83	29415088,82	2,392843649	1,420958
Cryptophytes (Teleaulax sp)	141083,3641	26946922,55	2,192064518	31,61631
TOTAL	446235,5843	1229294226		