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Key Ecological Roles for Zoosporic True Fungi in Aquatic Habitats

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INTRODUCTION

Phylogeny of Zoosporic True Fungi and Fungus-Like Microorganisms

The "Aquatic Phycomycetes" (*sensu* Sparrow) (1) constitutes an ecologically and economically important assemblage of eukaryotic microorganisms that share many morphological traits and ecological functions and interact with each other in the same aquatic ecosystems. There is molecular and structural evidence that the aquatic phycomycetes is a diverse, polyphyletic assemblage of species. For many years little research has been conducted with the aquatic phycomycetes, possibly because they were thought to be ecologically and commercially insignificant, but this perception has recently changed. Many of these species have been found to play key roles in biomass conversion in food webs (Fig. 1) and in the carbon cycle (2).

Baldauf (3, 4) divided eukaryotic organisms into eight supergroups based on a consensus phylogeny. The fungus-like species described in the "Aquatic Phycomycetes" *sensu* Sparrow (1) have been reassigned by Baldauf (3, 4) into four of these eight supergroups: the Opisthokonta, Straminipilia, Alveolata, and Rhizaria. The true fungi, the fungus-like organisms with uniflagellate zoospores (including chytrids, rumen chytrids, rosellids, and aphelids that are closely related to the true fungi), the mesomycetozoa (distantly related to the true fungi), the choanozoa, the slime molds, several groups of uniflagellate amoebae, and the phyla of multicellular animals were all placed in the Opisthokonta. One whiplash flagellum is usually present on zoospores of those species in the Opisthokonta, which produce motile stages for dispersal in their life cycles. Baldauf (3, 4) placed the fungus-like species with biflagellate zoospores into the supergroups Alveolata, Rhizaria, and Straminipilia. Many of the fungus-like species have heterokont zoospores with one anterior tinsel flagellum with mastigonemes and one posterior smooth whiplash flagellum without mastigonemes, but other species have zoospores with two whiplash flagella or one tinsel flagellum (5).

Ruggiero et al. (6) recently proposed a new higher-level classification of all living organisms. In this new proposal the eight supergroups of eukaryotes used by Baldauf (3, 4) have been replaced by five kingdoms: Animalia, Chromista, Fungi, Plantae, and Protozoa. The microorganisms within the "Aquatic Phycomycetes" *sensu* Sparrow (1) have been split by Ruggerio et al. (6) among three kingdoms: the kingdoms Chromista, Fungi, and Protozoa. The organisms within the supergroup Opisthokonta have been split among three kingdoms: the kingdoms Animalia, Fungi, and Protozoa. The microorganisms in the supergroups Amoebozoa, Discicristates, and Excavates have been placed into the kingdom Protozoa, and the organisms in the supergroups Alveolata, Rhizaria, and Straminipilia into the kingdom Chromista by Ruggerio et al. (6). Furthermore, some of the names for phyla have been changed by Ruggiero et al. (6). For example, the phylum Oomycota has been replaced by the phylum Pseudofungi.

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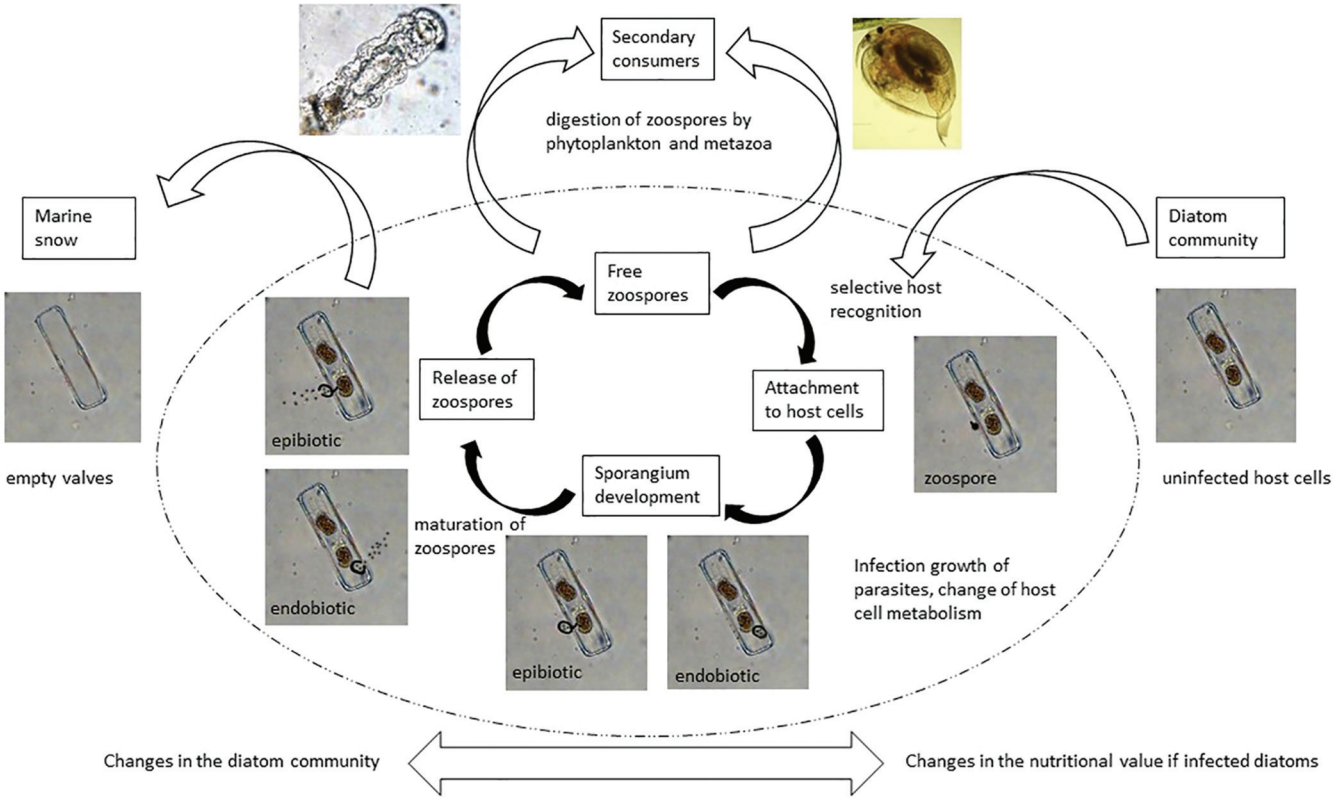

Figure 1 Schematic life cycle of endo- and epibiotic zoosporic parasites infecting marine diatoms. Besides the main cycle (solid black arrows), ecological effects on the marine planktonic and benthic community compositions, as well as interactions, are also depicted (unfilled outlined arrows).

Most fungus-like opisthokonts (Table 1) have a single, basal, whiplash, smooth flagellum on reproductive cells (zoospores and gametes), cell walls composed of chitin and plate-like mitochondrial cristae (1, 7–9). Most of the zoosporic true fungi, which Sparrow (1) and Barr (7) originally placed into the phylum Chytridiomycota (*sensu* Barr), have recently been reassigned into four newly described phyla (Blastocladiomycota, Chytridiomycota, Monoblepharidimycota, and Neocallimastigomycota) and the Olpidium clade (9–11). Sparrow (1) included *Rozella* in the phylum Chytridiomycota, but recently its species have been transferred to their own phylum, the Cryptomycota or Rozellida (12). Also, two new phyla of fungus-like microorganisms, which were unknown to Sparrow, have been recently added to the Opisthokonta supergroup: Aphelidea (9) and Mesomycetozoea (13). Busk et al. (14) reported about low sequence similarity between, and high overall diversity of, the genes encoding for secreted biomass-degrading enzymes produced by species of Blastocladiomycota, Chytridiomycota, Monoblepharidimycota, and Neocallimastigomycota.

Data from the sequences of rDNA and other genes have been used to describe a new phylum, the Rosallida (Cryptomycota), found in freshwater bog samples as a basal fungal clade (15) and the estimation of the molecular diversity of this clade as equivalent to that of the entire fungal kingdom (12). Species of Rosallida (Cryptomycota) are parasites of host species in the Chytridiomycota and Oomycota. Sparrow (1) described many species of *Rozella* based on their preferred hosts. The aphelids (phylum Aphelidea) are a small group of intracellular parasitoids of common species of eukaryotic phytoplankton with three known genera (*Aphelidium, Amoeboaphelidium*, and *Pseudaphelidium*) and 10 valid species that form, along with related environmental sequences, a very diversified group as well (16). The phylum Aphelidea is also new. The species in these two related phyla could easily be mistaken as chytrids because of similar morphology.

The use of software programs to compare DNA sequences of ribosomal and other conservative genes in living organisms has provided new insights into phylogeny and evolution. However, many questions have risen

Table 1 Currently described phyla in the supergroup Opisthokonta[a]

Phyla	Superphyla	Group	Cluster	Propagules
Basidiomycota	Holomycota	Eumycota	Dikarya	Thick-walled spores
Ascomycota				
Zygomycota			Sporangiospores	
			Zygospores	
Glomeromycota				
Olpidium clade[b]			Zoosporic	Zoospores
Monoblepharidomycota[b]			True fungi	(amoebae rarely)
Blastocladiomycota[b]				
Neocallimastigomycota[b]				
Chytridiomycota[b]				
Microsporidia		Opisthosporidia	True fungi	Walled spores
Rozellomycota[b]			Fungus-like	Zoospores
Aphelidea		Zoospores and amoebae		
Nucleariida				Amoebae
Fonticulida (MAFO)[c]				
Mesomycetozoea	Holozoa	Mesomycetozoea	Fungus-like	Thick-walled endospores
Cl. Dermocystida[b]				Zoospores
Cl. Ichthyophonida		Amoebae		
Acanthoecida		Choanozoa		Zoospores
Craspedia				
Fistasterea				
Animal phyla	Metazoa		Multicellular	

[a]Unifying characteristics are (i) uniflagellate zoospores usually, (ii) plate-like mitochondrial cristae; and (iii) if present, cell walls composed of chitin.
[b]Only the groups marked [b] were part of the "aquatic phycomycetes" as defined by Sparrow (1). The groups in bold fonts are zoosporic true fungi and will be considered in this article.
[c]MAFO, marine Fonticulida.

about phylogenetic relationships and evolutionary history from molecular studies, especially at the higher taxonomic levels, and remain unanswered. It is hoped that when complete genomic sequences of many more organisms become available, we will have a clearer understanding of phylogenetic relationships among all species of living organisms. The phylogenetic patterns proposed by Baldauf (3, 4), Adl et al. (17), and Ruggiero et al. (6) are based primarily on vertical gene transfer. Horizontal gene transfer has occurred between many species of both prokaryotes and eukaryotes, including fungi, and recent studies comparing complete genomes suggest that horizontal gene transfer has been more common than previously thought (18–20). Horizontal gene transfer must be considered in evolutionary history as well as vertical gene transfer. The precise phylogenetic relationships between many of the phyla of eukaryotic microorganisms are not clearly understood at present, and these higher-level classifications will probably be changed periodically in the near future as more sequence data become available.

In the past, lack of knowledge about aquatic zoosporic fungi and their ecological functioning has been compounded by misidentification of fungal zooflagellates, for example, as phagotrophic nanoflagellates (21). The identification of isolates of zoosporic true fungi and fungus-like microorganisms is complex, because of the lack of morphological characters visible in the light microscope, and often requires ultrastructural characterization of zoospores and other structures (22–24). Also chytrid sporangia and rhizoids exhibit phenotypic plasticity with morphological changes due to nutrient or other environmental conditions (25, 26). Furthermore, the parasitic species can be either epibiotic, with the zoosporangium on the surface and the rhizoids inside the host cell, or endobiotic, with both the zoosporangium and the rhizoids inside the host cell (27). Endobiotic parasites are very difficult to see under the light microscope. Recently, SSU rDNA sequences have been useful for understanding the phylogeny of zoosporic fungi (28).

Objectives of This Review

In this article, only the phyla that are closely related to the true fungi, which have adapted to aquatic environments and which produce motile zoospores usually with one whiplash flagellum, are considered (Table 1). All these species are within the supergroup Opistho-

konta (3, 4). Species of true fungi that produce thick-walled, nonmotile spores, but have adapted to aquatic environments, such as the aquatic hyphomycetes, are not considered in this review. Finally, the fungus-like organisms in the supergroups Alveolata, Rhizaria, and Straminipilia (or the kingdom Chromista) are not considered here either, because they are not considered to be true fungi, even though they share many morphological and physiological characteristics with true fungi and often inhabit similar environments. Some of these characteristics could have come across from species of true fungi to fungus-like species by lateral gene transfer during evolutionary history. For example, Richards et al. (19) and Richards and Talbot (20) discussed exchange of genes between groups of protists, in general, and specifically the lateral transfer of genes for pathogenicity from Ascomycota to Oomycota since these groups originally evolved. Although the Mesomycetozoea have some fungus-like characteristics, they are more closely related to the Choanozoa than the true fungi and will not be considered here.

This review focuses on some of the recent research on the ecology of zoosporic true fungal and fungus-like species in four phyla, Aphelidea, Chytridiomycota, Neocallimastigomycota, and Rosallida (Cryptomycota), and the interactions between these species and their substrates. Most of these species tend to be monocentric (unicellular), but there are polycentric (filamentous) species. Their lifestyles are considered based on the nature of symbiosis in aquatic habitats: saprotrophic, parasitic, and mutualistic lifestyles. It is essential for a thorough understanding of ecological processes that we correctly identify all species in all food webs in each ecosystem. Consideration of only species in the kingdom Fungi in ecosystems causes a dilemma, but our investigations have to begin somewhere, and thus, for this article, the scope of the topics covered must be limited.

DESTRUCTIVE MECHANISMS IN THE LIFE CYCLE OF ZOOSPORIC TRUE FUNGI

Zoospores

Zoosporic parasites produce motile zoospores. Some aspects of the physiology and ecology of zoospores have been reviewed by Fuller and Jaworski (29) and Gleason and Lilje (30). Motile zoospores are released from zoosporangia in large numbers into water and can swim for several hours. They are capable of navigating quickly through chemical gradients to their substrates (chemotaxis). After attachment, they encyst, germinate, and produce rhizoids, which penetrate their hosts and release digestive enzymes. The process of chemotaxis has only been studied in detail in the frog parasite *Batrachochytrium dendrobatidis* (31), in *Rhizophydium littoreum* (32), and in chytrid parasites of diatoms (33). In contrast, walled spores and cysts of other groups of parasites are carried by air or water currents to their hosts by chance or by vectors, such as animals.

Recent research into the chemotaxis of chytrid zoospores (33), using four marine chytrid-diatom tandem cultures in combination with different potential triggers, has shown that whole-cell extracts of the light-stressed hosts attracted the highest numbers of zoospores (86%), followed by the combined carbohydrate standard solution (76%), while all other compounds acted as weak triggers only. The triggers include aqueous host extracts grown under different stress conditions and standards of eight carbohydrates, six amino acids, five fatty acids, and three compounds known as compatible solutes (in individual and mixed solutions). In light of the results, it is tempting to hypothesize that pathogen zoospores were preferentially attracted to the fast-growing cells by photosynthesis-derived carbohydrate exudates. The chytrid-diatom tandem cultures comprised *Chytridium* sp./*Navicula* sp., *Rhizophydium* type I/*Nitzschia* sp., *Rhizophydium* type IIa/*Rhizosolenia* sp., and *Rhizophydium* type IIb/*Chaetoceros* sp., and could not be further isolated by the use of conventional methods (e.g., pollen) (33).

Rhizoids

The functions of rhizoids include penetration of the substrate, delivery of extracellular digestive enzymes, and absorption of food sources. Rhizoids exhibit phenotypic plasticity with morphological changes due to nutrient or other environmental conditions (19, 20).

Extracellular Enzymes

Proteases

Many species of zoosporic true fungi and fungus-like organisms can grow on substrates containing protein (1). Krarup et al. (34) demonstrated that some saprotrophic species of zoosporic true fungi also produce proteases. These extracellular enzymes are presumably released by rhizoids, which penetrate into utilizable nonliving substrates. Piotrowski et al. (35) found that the parasite *B. dendrobatidis* also produced proteases. Joneson et al. (36) describe significant lineage-specific expansions in three protease families (metallo, serine, and aspartyl proteases) in *B. dendrobatidis*. They show that expansion of these protease gene families occurred after the divergence of *B. dendrobatidis* from its an-

cestors. Finally, they demonstrated that the timing of these expansions predates the emergence of *B. dendrobatidis* as a globally important amphibian pathogen. Most likely, other groups of zoosporic parasites also use a variety of proteases in different families to digest the tissues in their animal hosts.

Cellulases

Rhizophlyctis rosea is a common chytrid, which grows quickly on plant debris containing cellulose in soil and aquatic ecosystems. In culture, this species is capable of the digestion of crystalline cellulose in the form of lens paper, filter paper, and powdered filter paper, and grows well with noncrystalline carboxymethyl cellulose or cellobiose, but cannot use starch or maltose as sole carbon sources in liquid and on solid media (37). Lange et al. (38) discovered that *R. rosea* zoospores excrete endocellulase GH45, which is thermostable.

The nature of cellulases excreted by other fungi is currently under study.

Cellulose, hemicellulose, and pectin are the polysaccharides in plant cell walls (39); cellulose has a linear structure of β-1,4-linked D-glucose that can be degraded to oligomers by the combined action of cellobiohydrolases, endocellulases, and lytic polysaccharide monooxygenases (LPMO) and further degraded to glucose by β-glucosidases (14). In higher plant species, cellulose is protected from degradation by the complex structures of plant cell walls including both covalent links between cellulose and hemicellulose and entanglement of the cellulose with other macromolecules. These structures make enzymatic digestion of cellulose difficult (14). Busk et al. (14) used PPR to classify GH families 1 to 131 and the AA families 9 to 11 (i.e., the LPMOs) into subfamilies. They found that enzymes with different properties are necessary for degradation of cellulose in different complex substrates. Also, that clustering of the fungi based on their predicted enzymes indicated that Ascomycota and Basidiomycota use the same enzymatic activities to degrade plant cell walls. They also found that the subfamilies could be used not only to predict the function of known GH-encoding genes but also to find all GH- and LPMO-encoding genes in a fungal genome and predict their functions (14).

GLOBAL DISTRIBUTION OF FRESHWATER AND MARINE TRUE FUNGI AND FUNGUS-LIKE MICROORGANISMS

Zoosporic true fungi (within the Eumycota and Opisthosporidia groups) (Table 1) are found in most aquatic and wet terrestrial ecosystems throughout the world. The phylum Chytridiomycota is one of the largest taxa of fungi (within the superphylum Holomycota) found in aquatic ecosystems (40). Since the 1960s, many species of freshwater zoosporic true fungi have been described (1, 7, 41, 42). However, ecological studies on these species have been limited. Phytoplankton and heterotrophic flagellates have traditionally been considered of primary importance in aquatic environments, while fungal diversity has been largely neglected (43). In recent years, discoveries in molecular methods of rDNA sequencing of small-subunit (SSU) rRNA genes (9, 44, 45), combined with broad meta-analysis of fungal populations in lacustrine, high-altitude soils under glaciers, and Arctic freshwater habitats (43, 46, 47), have revealed that zoosporic true fungi are predominant in these ecosystems and are likely to perform crucial ecological roles, like the degradation of pollen by saprotrophic freshwater zoosporic fungi (47).

Freshwater zoosporic fungi in freshwater ecosystems may be important parasites of phytoplankton and zooplankton. Planktonic chytrids are both parasitic and saprobic on the freshwater phytoplankton of Lake Inba, Japan (48). Chytridiomycota-like sequences with many novel lineages dominate the fungal diversity of temperate Lake Tahoe, United States, and freshwater Arctic habitats. Zoosporic fungi were positively correlated with either total chlorophyll a concentrations or with proportions of diatom sequences, indicating the likely parasitism of algae (43). *Olpidium gregarium* in the *Olpidium* clade was associated with declines in populations of rotifers (freshwater zooplankton) in the Rio Grande Reservoir, United States (49). In mesotrophic Lake Bourget in eastern France, zoosporic fungi in the *Rhizophydium* and *Nowakowskiella* clades were strongly represented among the fungi during May when the Chlorophyta and Bacillariophyta were also abundant (45). Zoosporic fungi may also be the primary decomposers of fine organic particulates and pollen grains in some freshwater environments (1). In the lakes of northeastern Germany, four fungal phyla were identified attached to pollen grains; 49% of the sequences were from the Chytridiomycota, predominantly the order Rhizophydiales. The zoosporic fungi in this study often appeared to be particle attached rather than free living (47). Pollen grains, leaves, insect and crustacean exoskeletons, pieces of snake skin, and live host species are excellent substrates for many chytrid species and have been used in the past to isolate chytrids into pure culture (1).

Historically, only a few species of zoosporic true fungi have been found in marine ecosystems, which led to the belief that these microorganisms were mainly lo-

cated in freshwater (50). Recent research has challenged this conclusion because of the discovery of many new marine species (discussed later).

SAPROTROPHS

The Effect of Environmental Factors on Growth and Development

Freshwater zoosporic true fungi encounter variations in physical conditions such as pH, soluble metals, temperature, and salinity. Although the temperature range of growth in aquatic zoosporic fungi has been little studied, many saprotrophic zoosporic fungi found in the soil have a maximum temperature for growth at 30°C, some species at 35°C and 37°C, and a few species at 40°C, but none survived incubation in liquid media above 45°C (51–53).

Fluctuations in pH are common in freshwater aquatic systems because of acid rainfall and runoff, sediments and fertilizers, algal blooms, and aquatic plant respiration. Zoosporic true fungi appear to tolerate extremely low pH, but not extremely high pH (1, 54). DNA sequences attributed to zoosporic fungi have been reported from the Rio Tinto River in Spain, which, as well as historically highly polluted with heavy metals, has been recorded as low as pH 2 (55). However, DNA sequences from the Rio Tinto River give no clues whether the inoculum was alive or dead; in addition, it cannot be excluded that the inoculum came from a tributary with higher pH. In comparison, zoospores from seven species of the plant parasitic *Phytophthora* (Oomycota), isolated from one reservoir, had optimal growth rates at acidic, neutral, or alkaline pH. However, some zoospores from all seven species survived at pH 3 and 11 (56).

Toxic metals affect many freshwater zoosporic fungi and fungal-like organisms with effects dependent on the type of metal and stage of the organism within its life cycle. Zoospores, which have a cell membrane rather than a cell wall, are more sensitive than any other stage. For example, gold is toxic to zoospores of the oomycete genus *Phytophthora* at 50 ppb (57). Some effects of metals on zoospores include increased encystment (58) and increased germination (59–61). A stimulatory effect on zoospore release at low levels of copper (10 ppm), lead (60 ppm), and zinc (10 ppm) was demonstrated for four isolates of zoosporic true fungi (62).

The level of osmolarity tolerated by zoosporic fungi varies based on their environment of origin. For example, the tolerance of an estuarine ecotype of *R. littoreum* to sodium, potassium, magnesium, and calcium ions was higher than freshwater ecotypes but lower than those in seawater (63), while maximum growth rate of the same ecotype in the presence of sodium ions was at 237 mM, approximately one-half the concentration of seawater (64). Zoosporic true fungi soil and freshwater isolates from several different genera were tested for growth at different salinities (65). All 20 isolates of zoosporic fungi in the orders Blastocladiales, Chytridiales, Cladochytriales, Rhizophydiales, Rhizophlyctidales, and Spizellomycetales grew on complex solid media supplemented with 170 mM NaCl, but not with 340 mM NaCl, indicating they would not survive in seawater.

Until recently, there have been relatively few studies on identifying marine zoosporic true fungi and fungus-like microorganisms, as opposed to freshwater zoosporic true fungi, which have been extensively studied (50). Those few studies mostly focused on chytrid parasites of dinoflagellates (50, 66) and diatoms (33). The identification of marine of zoosporic fungi has been problematic, because identification of species relied largely on morphology, which, in the case of some zoosporic fungi, with inconspicuous structural components, is extremely difficult (1, 67). However, the use of rDNA sequencing over the past few years has improved the species identification process, enabling researchers to quantitatively measure and compare the diversity and abundance of zoosporic fungi in marine environments, including the water column (9, 28, 44). Taylor and Cunliffe (68) recently used fungi-specific high-throughput sequencing and quantitative PCR analysis of plankton DNA samples collected over 6 years from the coastal site off Plymouth, United Kingdom, and assessed changes in the temporal variability of mycoplankton diversity (mainly Ascomycota, Basidiomycota, and Chytridiomycota) and abundance in relation to co-occurring environmental variables. Repeating mycoplankton blooms were linked to nitrogen availability and temperature, and specific relationships were found between mycoplankton and other plankton groups, for example, seasonal chytrid blooms matching diatom blooms in consecutive years. The study identified possible environmental drivers for mycoplankton diversity and abundance, with both diversity and abundance increasing with reduced salinity, and also when substrate availability was increased (68). With efficient and accurate methods of species identification now readily accessible to more researchers, we would expect that many more studies on the influence of environmental factors on diversity and distribution of marine zoosporic true fungi and fungus-like microorganisms will be undertaken in the near future.

Colonization Strategy

Saprotrophic zoosporic fungi colonize solid substrates in freshwater and soil ecosystems by using rhizoids, which release extracellular enzymes in order to penetrate the substrate to consume plant debris and fibers (1, 30, 69). (The colonization of fibrous plant material by fungi in the rumen is discussed in detail later in this review.) These enzymes are essential for the decomposition of organic matter in aquatic systems, because of their ability to degrade cellulose, the major component of vegetable debris (70, 71). Of interest is the recent discovery of an aquatic saprobe, *Synchytrium microbalum*, occurring within a genus of over 200 otherwise parasitic terrestrial species of zoosporic fungi (72).

PARASITES

Parasites of Marine Phytoplankton

Recent research revealed the presence of parasitic chytrids in marine coastal habitats. Several microalgae species, besides some dinoflagellates (66) and cyanobacteria (e.g., *Nodularia*), first and foremost bacillariophytes (diatoms), such as *Pseudo-nitzschia pungens* (73), *Chaetoceros*, *Amphora ovalis, Rhizosolenia, Biddulphia* (27, 74–76) (Fig. 2), *Thalassiosira, Skeletonema* (77), *Bellerochea*, and *Cylindrotheca* (78), were identified as host species for such zoosporic pathogens. Overall, only one chytrid, *Dinomyces arenysensis* infecting various dinoflagellates including toxic species of *Alexandrium*, has been identified and properly characterized by Lepelletier et al. (66, 79).

Traditionally, species description has been based on morphology of host and parasite (1, 67), which has been extended by discoveries in rDNA sequencing over the past years (9, 44). In addition, chytrids can show considerable morphological differences caused by nutrient availability or environmental conditions (25, 26). Also, many species might have been described previously, but their taxonomy has never been resolved or updated to fit our current phylogenetic concepts.

In general, molecular methods based on the amplification, cloning, and sequencing of SSU rRNA genes are a powerful tool to study the diversity of prokaryotic and eukaryotic microorganisms for which morphological features are not conspicuous (80). While DNA

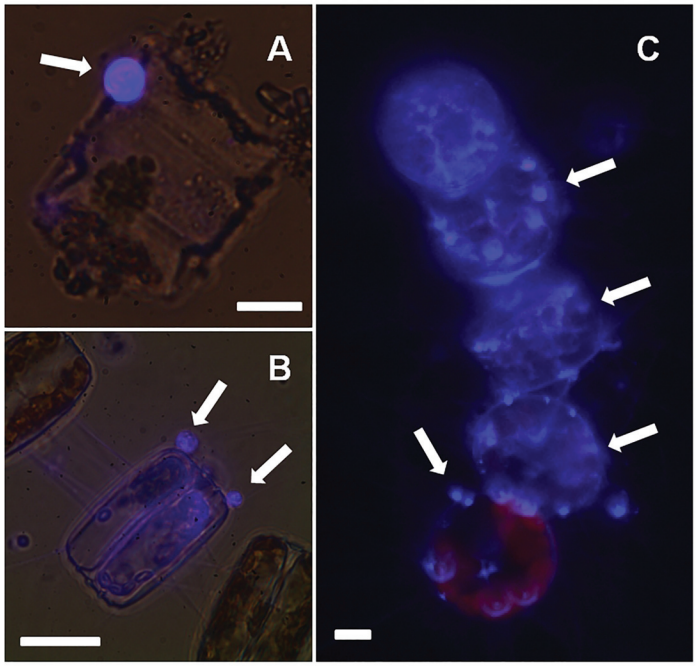

Figure 2 Representatives of the chytridiomycota infecting marine diatoms in phytoplankton net samples collected from the Skagaströnd area (northwest Iceland). *Odontella* (**A**) and *Thalassiosira* (**B**) single cell with multiple chytrid sporangia and colony with multiple infections (**C**). Pathogens were visualized by using calcofluor white stain in combination with transmission light and fluorescence excitation (UV light, 330 to 380 nm). Image by B. Scholz. Bar, 100 μm.

barcodes for terrestrial oomycetes are available and widely used (81), and genome regions for potential fungal barcodes have been identified (82), DNA barcodes for most other zoosporic parasites are, despite considerable effort (83), missing or not conclusive and the existing ones rarely allow identification, even at genus level (44). In all cases, further difficulties of DNA-based methods are primer bias within mixed samples going hand in hand with the troublesome establishment and maintenance of "pure" dual cultures of host and chytrid (44). However, current high-throughput sequencing approaches have revealed an unappreciated diversity and abundance of eukaryote parasites in the marine environment (de Vargas et al. [84]). Although reported as low in abundance and diversity (28, 84), marine true fungal sequences retrieved in metagenomic surveys are in most cases new to science (85). The phylum Chytridiomycota alone accounted for more than 60% of the reads in six near-shore sites around Europe (28, 86). Taking into account another fungal taxon likely to include parasites, such as the CRA (Cryptomycota/Rozell[ida]/Aphelid) group (28), this percentage increases by another 5%. Furthermore, the phylogenetic analysis of the same data set, together with other eukaryotic environmental sequences, suggested the existence of a whole clade of unknown marine chytrids and reported two clades branching close to known parasite species of phytoplankters (*Chytridium polisiphoniae* and *Amoeboaphelidium protococcarum*) (28).

In general, chytrid parasites may impact marine planktonic and microphytobenthic populations; therefore, large-scale, targeted approaches to monitor different habitats will result in increased knowledge about these parasites. In order to quantify chytrid infections in marine samples, the use of calcofluor white stain in combination with epifluorescence according to the protocol given by Rasconi et al. (87) for phytoplankton samples, as well as the much more cumbersome protocol for microphytobenthic samples (74), has shown the first acceptable results (27, 76). Another detection protocol involves the use of wheat germ agglutinin staining of chitin in environmental samples (28), with the side effect of staining Gram-positive bacteria due to its affinity for *N*-acetylglucosamine. The clear disadvantage of these methods is that they are time-consuming and do not lead to a secured identification of the parasite.

Although our knowledge of marine infectious disease dynamics is limited and often biased toward an economic point of view, there is much evidence to suggest that the occurrence of marine infectious diseases will increase with currently observed climatic trends (88), such as the alteration of epidemiology through effects on pathogen growth rates, transmission, virulence, and susceptibility, with potentially devastating results (79). Thus, it is necessary to draw attention to the importance of the host-parasite system in the marine environment, and emphasize the increasingly crucial role this system will play as, for example, aquaculture becomes more important in order to sustain the growing human population (79). Since infection by zoosporic parasites may not always significantly affect population size of hosts or microbial succession, in general, it is important to apply and develop new qualitative and quantitative techniques to study host-parasite interactions.

Parasites of Freshwater Phytoplankton

The infection of freshwater phytoplankton by chytrid parasites has been documented for a variety of dinoflagellates, diatoms (Fig. 3), and cyanobacteria and in a variety of environments. There is accumulating evidence that the pelagic zone of lakes is housing a significant unexplored diversity in chytrid species (89). These discoveries are particularly interesting when the system concerned is exhibiting significant ecological and environmental change. For example, Leshem et al. (90) recently characterized a new species of chytrid parasit-

Figure 3 Chytrid parasites infecting a freshwater diatom (*Pinnularia* sp.) collected from a freshwater pond in Centennial Park, Sydney, Australia. Image by D.J. Macarthur. Bar, 50 μm.

izing the dinoflagellate, *Peridinium gatunense*, in Lake Kinneret, Israel, a system that has been the subject of studies detailing its pronounced shifts in phytoplankton community structure (91). Similarly, chytrid infection of the cyanobacteria *Planktothrix* has been extensively analyzed in Lake Kolbotnvannet, Norway, a system that has experienced increased urbanization over the past 35 years (92). In this study, it was found that improved water quality conditions, due to better water quality management practices, favored a single *Planktothrix* chemotype. It was hypothesized that the loss of diversity would allow the chytrid species known to parasitize *Planktothrix* to dominate and therefore reduce the dominance of the single chemotype and return the system to a diverse equilibrium. Contrary to the hypothesis, it was found that the chemotype-chytrid relationship remained in equilibrium. It was therefore further hypothesized that this was due to the *Planktothrix* moving deeper in the water column, taking advantage of increased light penetration as a result of the improved water quality. As a result of this movement, environmental stresses, such as decreased temperature, have increased for the chytrids and limited their ability to dominate. These contextual studies are beginning to add a further dimension into our perspective on the anthropogenic effects of ecological interactions. Elucidated host-parasite relationships are emerging as excellent models for garnering insight into the traditionally opaque ecological dynamics of aquatic food webs (93). This insight has been experimentally confirmed and is known informally as the "Mycoloop" (94).

The Mycoloop is a trophic transfer loop, where the release of zoospores by chytrid parasites of phytoplankton provides an additional and potentially very important source of food for grazer zooplankton (94). This dynamic is particularly crucial where phytoplankton species are not a suitable food source for zooplankton, such as the large diatom *Asterionella* (95). The significance of the presence of chytrid zoospores in food webs has been explored mathematically by Grami et al. (96), who did extensive sampling of Lake Pavin in France and quantified bacteria, heterotrophic nanoflagellates, nanoplankton and microphytoplankton, ciliates, metazooplankton, and chytrids. These data combined with physiochemical measurements of water were used to construct a pelagic food web model. The inclusion of chytrids in this model resulted in significant increases in lake carbon flows, suggesting a mathematical replication of the conceptual Mycoloop. In addition, the presence of chytrids in the system increased the number of trophic links and the length of trophic pathways, which contribute to the overall stability of the system (2).

Much research has been committed to attempting to explain and manage the potentially devastating toxins produced by cyanobacteria, particularly in light of global elevated temperatures and stratification in water bodies favoring the physiological strategies employed by these prokaryotes (97, 98). Studying the relationship between the filamentous toxin-producing cyanobacteria *Planktothrix* and its chytrid parasite *Rhizophydium megarrhizum* revealed that the synthesis of microcystins in *Planktothrix* provided a significant defensive aid against infection, whereas mutants of *Planktothrix* with toxin-synthesis genes knocked out exhibited an enhanced vulnerability to parasitic infection (99). This study is arguably the most concrete in discerning the ecological function of toxins synthesized by phytoplankton, an area that is still characterized by ambiguity.

Evidence is emerging that the interactions between the chytrid parasite and its host may involve strong phenotypic selection pressures. An unpublished study by D. J. Macarthur (NSW Environmental Trust 2006/RDS/0007) involved the inoculation of cultures of the bloom-forming cyanobacteria *Anabaena circinalis* and *Microcystis aeruginosa* with diatoms infected with chytrids and with pure cultures of chytrids in the Rhizophydiales. One of these chytrids, which was isolated in pure culture from a phytoplankton sample containing infected diatoms (by E. Lefèvre, Department of Civil and Environmental Engineering, Duke University, Durham, NC) and was tentatively identified as *Rhizophydium* sp., adapted to the new host environments by parasitizing both species of cyanobacteria. These results support the theory of Kagami et al. (100) that the host range of chytrid parasites, often thought to be host-specific, could be altered by environmental stress. More interesting, however, was an apparent enhancement of growth and survival of *A. circinalis* cultures (101). Apparently, there were benefits to both the chytrid parasite and the cyanobacteria host. However, more data are required to unravel the complicated interactions between the chytrid parasite and its host. The growth and survival of chytrids are quite sensitive to physical factors, such as moisture, temperature, salinity and dissolved oxygen (102), and possibly toxic chemicals. With global warming and increased environmental deterioration, more research is required on the ecology of the chytrids in aquatic ecosystems. There is growing evidence that the survival of many aquatic ecosystems may depend on the activities of chytrids along with other groups of microorganisms.

More recently, Frenken et al. (103) demonstrated that warming can significantly interact with chytrid-

bloom dynamics. Mesocosms with a blooming population of the diatom *Asterionella*, which was also being parasitized by chytrids resembling *Zygorhizidium planktonicum*, exhibited earlier bloom termination times in response to a warming treatment of 4°C. This result was accompanied by cascade effects in the zooplankton population; *Keratella* numbers peaked earlier and at a higher density, but exhibited a more rapid and dramatic decline in the postbloom stages of the mesocosms. These results hint at the potentially huge significance chytrid parasites may have in aquatic ecosystems.

A key but contentious theory in evolutionary ecology, the Red Queen Hypothesis (104) has also been explored in the context of chytrid parasites of freshwater phytoplankton. The theory states that host-parasite relationships are evolutionary balancing acts, where hosts and parasites are coevolving to resist infection, or infect at a higher incidence, respectively. The theory is conceptually pleasing as a driver of rapid microbial evolution, but Gokhale et al. (105) found that a mathematical representation of the theory, based on classic Lotka-Volterra dynamics, resulted in frequent system collapse rather than a stable persisting relationship. However, a field study examining the long-term relationship between a genetically monotonous population of *Planktothrix* and their chytrid parasite in sediment cores from Lake Kolbotnvannet, Norway, showed a very stable relationship over many years (92). As discussed previously, there are several plausible reasons for this, most notably the seasonal patterns of the lake providing intermittent "thermal refugia," where the temperature drops below the tolerable threshold for the chytrid, but not for *Planktothrix*, allowing the host population to stabilize. The presence of hyperparasites, such as the zoosporic *Rozella* sp., in the system could also theoretically act as an additional pressure on the parasite population (106), but the influence of these enigmatic organisms is still very poorly characterized.

Similar to the paradigm of negativity surrounding blooms of phytoplankton, which may be perfectly natural and even essential biological "resetting" events, there is a negativity associated with diseases and parasitism, mostly derived from their potentially devastating economic effects. This dynamic has been associated with chytrid infection of commercial algae crops (107), which, because of rigorous strain selection, suffer substantially from infection. However, being a component of the so-called biological "dark matter" (108), these fungi should be considered as evidence of further uncharted levels of biodiversity, which deserves a certain respect and appreciation.

SYMBIONTS

Distribution

Fungi that thrived in the absence of oxygen were first detected in the form of motile zoospores in the rumen fluid of ruminant animals by Liebetanz and Braune as early as 1910 (109, 110). At the time, these remarkable organisms were mistakenly described as ciliated protozoa. More than 6 decades later, Orpin consistently observed vegetative fungal growth while attempting to isolate ciliated protozoa from the sheep rumen (111, 112). The morphology of these microorganisms and the presence of chitin in their cell walls provided further proof that they were in fact spores of a new lineage of true fungi (111, 113, 114). Since their original discovery in sheep, anaerobic gut fungi have been isolated from and detected based on microscopy in the rumen, hindgut, and feces of at least 24 different host genera representing eight different animal families (115, 116). Among these are all foregut fermenters, including the ruminants (families Bovidae and Cervidae), pseudoruminants (e.g., hippopotamus, camel, llama, alpaca, and vicugna) and foregut nonruminants (e.g., marsupials), as well as some hindgut fermenters (e.g., elephant, horse, and rhinoceros). Anaerobic fungi have further been identified in the intestine of laboratory mice (117), in guinea pigs (118), and in some larger herbivorous rodents, e.g., the mara (*Dolichotis patagonum*) (119), but not in some other small hindgut-fermenting animals (presumably because of the shorter duration time of ingested plant material in their smaller cecum) (116). The panda, which lacks foregut and hindgut fermentation chambers, has also not been found to harbor anaerobic fungi (120), and this has been attributed to the simplicity of its alimentary tract (116). Anaerobic fungi were, however, detected in the gut of the marine iguana (*Amblyrhynchus cristatus*) (121, 122). These herbivorous reptiles feed on marine algae and have fermentative digestion processes similar to those in herbivorous mammals. However, some other herbivorous reptiles (i.e., tortoises) do not seem to be colonized by anaerobic fungi (122). In the sea urchin, *Echinocardium cordatum*, zoosporic true fungi have been observed to make up part of the microflora in the anterior cecum, intestinal cecum, and coelomic fluid (123). Some parts of the digestive system are thought to be relatively anoxic and the microflora to be obligately anaerobic. This species of sea urchin digs and feeds in anaerobic muddy substrates in marine environments. Sea urchins are herbivores and feed primarily on marine macroalgae. Whether the zoosporic true fungi observed in *E. cordatum* are phylogenetically related to rumen fungi

found in herbivorous mammals and reptiles is not yet known because of the lack of molecular data.

Life Cycle and Ecology

The strictly anaerobic fungi (phylum Neocallimastigomycota, order Neocallimastigales) (124) play a pivotal role in the functioning of the alimentary tract of herbivorous mammals and reptiles (125), and so it is not surprising that anaerobic fungi comprise some 20% of the microbial biomass of sheep feeding on high-forage diets (126). Forages mainly consist of crystalline cellulose and noncrystalline hemicellulose from plant cell walls and represent the most important carbon and energy sources for terrestrial herbivorous mammals and reptiles. In a mutualistic relationship with their host, anaerobic fungi, together with bacteria, archaea, and protozoa, realize the degradation of the ingested plant material, which the host could otherwise not use (114, 125). While sexual reproduction has not been reported for anaerobic fungi of the phylum Neocallimastigomycota, asexual reproduction occurs through the production of flagellated zoospores from sporangia (127). The formation of sporangia, zoospore differentiation, and their subsequent maturation are thought to be induced by heme and other related porphyrins, which are released from ingested plant material (111, 112, 118, 128, 129). Two types of zoospores have been observed: monoflagellate and polyflagellate zoospores. During this motile stage, anaerobic fungi rapidly locate surfaces for colonization *via* chemotaxis toward soluble sugars that leak from damaged plant material (111, 112, 130). Upon attachment to the fiber, the zoospores shed their flagella and form a cyst (111, 114). Development of the cyst varies depending on whether the fungus is monocentric (one sporangium develops from single zoospore) or polycentric (many sporangia develop from a single zoospore) (131). In monocentric taxa, the nucleus remains within the cyst (endogenous), which enlarges to form a zoosporangium. Thus, the rhizoids remain anucleate. In polycentric taxa, the nuclei migrate into the rhizoidal system (exogenous), thereby enabling the formation of multiple sporangia on each thallus (125, 129). The genera *Caecomyces* and *Cyllamyces* form bulbous holdfasts and appear to represent intermediate forms (132). In both genera, nuclei are observed in the holdfast, consistent with exogenous development, and, in the case of *Cyllamyces*, also in the branched sporangiophores. However, the development of these thalli, while not as strictly determinate as the monocentric/rhizoidal *Neocallimastix* and *Piromyces*, is clearly more limited than in the polycentric/rhizoidal *Anaeromyces* and *Orpinomyces* (116). The rhizomycelium of the anaerobic fungi is characterized as being either filamentous or bulbous, the latter possessing spherical holdfasts (132, 133). Distinct intercalary rhizoidal swellings are occasionally observed in the genus *Oontomyces* (134). The developing rhizoids physically penetrate rigid, undamaged plant cell walls using an appressorium-like structure (113, 135–138). The so revealed internal plant tissues become accessible to enzymatic breakdown and provide nutrients that enable the development and maturation of the sporangia, which may then produce and release zoospores before the cycle repeats (139, 140). The initial attack of plant fiber by anaerobic fungi appears to facilitate a more rapid breakdown of forage feed by fibrolytic bacteria, which have difficulty penetrating large food particles (141, 142). In addition to using physical force, anaerobic fungi also produce cellulolytic and hemi-cellulolytic enzymes, in contrast to their mammalian and reptilian hosts that mediate the breakdown of plant cell wall carbohydrates, especially lignocellulose (143–147), thereby supplying reducing equivalents in the form of hydrogen to the bacterial and archaeal communities (148–150). The presence of hydrogen- and ATP-generating hydrogenosomes in anaerobic fungi was first demonstrated in the rumen anaerobic fungus *Neocallimastix patriciarum* (151). Axenic cultures of fungal species within this genus produced hydrogen during a (bacterial-type) mixed-acid fermentation of carbohydrates (152, 153), but the mechanism of hydrogen production had not been established and the presence of specialized redox organelles not reported. Yarlett et al. (151) showed hydrogenase activity in both the motile zoospore stage and the nonmotile vegetative reproductive stage of the fungus. Since then, the mitochondrial origin of anaerobic fungal hydrogenosomes has been demonstrated (154, 155), and their presence confirmed in several other genera of anaerobic fungi (143, 156). The end products of microbial fermentation, including short-chain fatty acids such as acetate, propionate, and butyrate, are taken up by the host via the epithelium (148–150). Anaerobic fungi successfully disperse between hosts, and it is believed that transition occurs via the formation of aerotolerant life stages, such as the two- to four-chambered spores formed by some *Anaeromyces* spp. (132, 157), although the processes whereby these develop and later germinate remain to be elucidated (116).

Taxonomy

Currently, the anaerobic fungi are classified in a single order (Neocallimastigales) within the recently erected phylum Neocallimastigomycota (124) and are most closely related to the Chytridiomycota. Within this

order, eight genera of anaerobic fungi are recognized: *Anaeromyces* (158), *Buwchfawromyces* (159), *Caecomyces* (160), *Cyllamyces* (132), *Neocallimastix* (161), *Oontomyces* (134), *Orpinomyces* (162), and *Piromyces* (160). Since 18S rRNA genes of anaerobic fungi are too conserved to distinguish between different genera and species, the internal transcribed spacer 1 (ITS1) has been recommended as molecular barcode marker (82, 157, 163). Several studies used this gene region to evaluate anaerobic fungal diversity in the alimentary tracts of a wide range of wild and domesticated, ruminant and nonruminant herbivores (122, 164–172). Some of these studies reported the existence of several as yet uncultivated novel groups of anaerobic fungi (122, 168, 169). Based on anaerobic fungal ITS1 sequence and secondary structure information, Koetschan et al. (173) established a comprehensive and stable phylogeny and pragmatic taxonomy of the Neocallimastigomycota, which may be used for highly resolved taxonomic assignment of high-throughput sequence data. Studies using next-generation sequencing technologies have recently allowed comprehensive new insights into the diversity and community structure of anaerobic fungi in the guts of a wide range of herbivorous animals (122) or in the rumens of cattle (171, 174), deer (175), or sheep feeding on different diets (176) or exhibiting different methane emission phenotypes (177). It appears that even hosts from highly distant families that feed on rather unique diets can harbor large numbers of shared sequence types (122), and that there are no (apparent) geographical restrictions in the occurrence of anaerobic fungal sequence types (168). It is likely, however, that some anaerobic fungal species are indeed host specific, as has been suggested for some of the novel as-yet uncultivated clades (122) and the recently described species *Oontomyces anksri*, which was isolated from the Indian camel (134). The recently conducted sequence-based surveys have broadened our understanding of the ecology of anaerobic fungi and provided a glimpse into their global genus-level diversity. Interestingly, the relative lack of overlap between the novel lineages identified in the different studies suggests that additional, yet-unknown novel candidate genera may exist in nature (116).

CONCLUSIONS

The diversity and abundance of zoosporic true fungi have been recently analyzed by using fungal sequence libraries and advances in molecular methods, such as high-throughput sequencing. This review focuses on four phyla, the Aphelidea, Chytridiomycota, Neocallimastigomycota, and Rosellida (Cryptomycota). Zoosporic fungi appear to be both abundant and diverse in many aquatic habitats around the world, with abundance exceeding other fungal phyla and numerous novel genetic sequences identified. Zoosporic fungi are able to survive extreme conditions, such as high and extremely low pH, but more work remains to be done. They appear to have an important role as saprobes in the decomposition of particulate organic substrates such as pollen, plant litter, and dead animals; as parasites of zooplankton and algae; as parasites of vertebrate animals (such as frogs); and as symbionts in the digestive tracts of mammals. Some chytrids cause economically important disease of plants and animals. They also regulate sizes of phytoplankton populations. Further metagenomics surveys of aquatic ecosystems are expected to enlarge our knowledge of the diversity of the zoosporic true fungi. Coupled with studies on their functional ecology, we are moving closer to unraveling the role of zoosporic fungi in carbon cycling and the impact of climate change on zoosporic fungal populations.

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