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Diversité phylogénétique et fonctionnelle des Eumycètes dans les écosystèmes pélagiques

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« *Etre grand n'est pas nécessairement une bonne chose: la plupart des organismes sont des bactéries et très peu sont des éléphants* »

Le Gène Egoïste. R. Dawkins.

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Résumé

Les microorganismes jouent un rôle prépondérant dans le fonctionnement des écosystèmes aquatiques, où ils sont à la base de la minéralisation et du recyclage de la matière organique. Les « vrais » champignons, ou Eumycètes, font partie de ces microorganismes hétérotrophes qui permettent le renouvellement de la matière organique dans les écosystèmes. Pourtant, la diversité et l'importance quantitative et fonctionnelle des champignons restent très largement méconnues dans les milieux pélagiques. Récemment, l'utilisation de méthodes moléculaires pour étudier la diversité des picoeucaryotes (de taille < 5 µm) lacustres a mis en évidence l'importance des champignons microscopiques avec, notamment, la présence de chytridiomycètes (chytrides). Cette découverte, en conjonction avec le rôle important connu des Eumycètes dans d'autres écosystèmes naturels, nous a amené à poser l'hypothèse d'une diversité et d'un rôle fonctionnel importants des champignons dans les écosystèmes pélagiques. Ce travail vise à préciser la diversité globale, la structure génétique et l'importance quantitative des différentes divisions du règne des Eumycota dans les écosystèmes pélagiques lacustres, et à proposer des outils méthodologiques pour l'étude écologique de ces peuplements. La diversité phylogénétique et l'importance des champignons de taille comprise entre 0,6 et 150 µm ont été analysées dans trois milieux pélagiques différents. Une étude de clonage-séquençage de l'ADNr 18S et de l'ITS a été réalisée au printemps 2007 dans les lacs Pavin (oligomésotrophe), Aydat (eutrophe) et Vassivière (mésotrophe, humique). L'affiliation phylogénétique des séquences a permis, non seulement de confirmer la présence d'une importante diversité de chytridiomycètes parasites du phytoplancton, mais aussi de mettre en évidence la présence non négligeable d'ascomycètes et de basidiomycètes, potentiellement saprophytes. L'étude de la dynamique saisonnière de la structure des peuplements (par TRFLP) et de l'importance quantitative de différentes divisions (par PCR quantitative) de la communauté fongique ont permis de déceler des différences en fonction des saisons et de l'écosystème. Ces différences ont été reliées à la dynamique des peuplements phytoplanctoniques, avec une influence des apports allochtones, principalement dans le lac eutrophe d'Aydat. De plus, les séquences moléculaires générées au cours de ces dernières années ont permis l'élaboration d'amorces ciblant des clades de champignons microscopiques d'intérêt, pour une étude écologique de la dynamique des peuplements, par des approches PCR à temps réel et FISH (*fluorescent in situ hybridization*). Enfin, nous considérons que l'acquisition de données complémentaires permettra d'intégrer les champignons saprophytes et parasites dans les flux de matière et d'énergie qui transitent par les écosystèmes pélagiques et les cycles biogéochimiques associés.

Abstract

Microorganisms play major roles in aquatic ecosystems, primarily as the main actors for organic matter mineralization and recycling. “True” fungi (i.e Eumycota) are among heterotrophic microorganisms that are highly efficient in recycling organic materials in natural ecosystems. However, the overall diversity of fungi and their quantitative and functional importance remain largely unknown in typical pelagic ecosystems. Environmental 18S rDNA surveys have recently highlighted the importance of microscopic fungi in the diversity of picoeukaryotes (size < 5 µm) in lake ecosystems, including particularly the members of chytridiomycetes (i.e. chytrids) as the dominant phyla. These studies and the known major roles of fungi in natural ecosystems such as soils have leaded us to venture the hypothesis that fungal diversity and functional roles are important structuring factors in pelagic ecosystems. The main aims of the thesis were to examine the overall diversity, genetic structure and quantitative importance of various phyla belonging to the Kingdom Fungi in freshwater pelagic ecosystems. Methodological tools were also developed for ecological investigations of fungal populations of interest. Phylogenetic diversity and quantitative importance of fungi (size classe: 0.6 and 150 µm) were analysed in three contrasting pelagic lakes. Environmental 18S and ITS rDNA surveys were performed during spring 2007 in the oligomesotrophic Lake Pavin, the eutrophic Lake Aydat, and the mesotrophic and humic Lake Vassivière, all located in the French Massif Central. Phylogenetic affiliation of sequences confirmed the presence and the substantial diversity of chytridiomycetes, known as parasites of primarily phytoplankton. We also have unveiled a sizeable number of sequences belonging to the fungal lineages of ascomycetes and basidiomycetes, mainly known as saprophytes. The seasonal dynamics of fungal community structure (essayed by TRFLP), and the quantitative importance of various taxonomic divisions (estimates by real time quantitative PCR or qPCR), revealed significant differences with seasons and with ecosystems. These differences were linked to phytoplankton composition and population successions, with at times the influence of allochthonous inputs, primarily for the eutrophic Lake Aydat. Finally, molecular sequences obtained during the few past years allowed the development of primers for targeting microscopic fungal lineages of interest, and the ecological study of their *in situ* dynamics using qPCR and FISH (*fluorescent in situ hybridization*) approaches. Overall, we consider that the acquisition of complementary data is necessary to allow the inclusion of fungi and their main functions (i.e. saprophytisms and parasitism) in the energy and matter fluxes in pelagics ecosystems, and the related biogeochemical cycling.

INTRODUCTION GENERALE : CONTEXTE SCIENTIFIQUE

Les « vrais » champignons ou Eumycètes représentent un règne à part entière dans le monde du vivant, qui a depuis longtemps fasciné et intéressé les chercheurs. Leur diversité globale dans la biosphère est estimée à près d'un million et demi d'espèces (Hawksworth, 2001 ; Schmitt et Mueller, 2004), alors que seules 74 000 à 120 000 espèces sont connues (Hawksworth, 2001). Ils comprennent quatre grandes divisions : les Ascomycètes, les Basidiomycètes, les Chytridiomycètes, et les Zygomycètes. Malgré leur diversité et leur importance dans le fonctionnement des écosystèmes naturels comme le sol, l'étude des champignons dans les milieux aquatiques, notamment pélagiques, reste très limitée.

L'importance de l'étude des champignons dans les milieux pélagiques vient sans doute, principalement, de leurs rôles en tant que parasites et saprophytes. En effet, dans ces milieux, le concept de boucle microbienne (Pomeroy, 1974 ; Azam *et al.*, 1983) met en avant l'importance des microorganismes dans le recyclage de la matière organique dissoute et particulaire (MOD et MOP), provenant du phytoplancton et du zooplancton mais aussi d'apports allochtones. Cette matière organique est consommée par les bactéries, elles-mêmes consommées par les organismes bactérivores ciliés et flagellés. Le zooplancton se nourrit à son tour des microorganismes bactérivores, réintroduisant ainsi la matière organique dissoute et détritique dans la chaîne alimentaire linéaire classique : phytoplancton > zooplancton > poissons (Figure 1). Dans ce contexte, les microorganismes flagellés hétérotrophes, en tant que bactérivores, ont un rôle important dans les transferts de matière, notamment de la biomasse bactérienne, aux niveaux trophiques supérieurs. La présence des champignons dans les écosystèmes pélagiques remet en question l'importance de certains maillons trophiques de cette boucle microbienne. En effet, les champignons saprophytes dégradent la matière organique morte au même titre que les bactéries (Findlay *et al.*, 2002). Cependant, les champignons ne peuvent sans doute pas être consommés par les picoeucaryotes flagellés bactérivores, du fait notamment de leur taille, mais aussi de leur morphologie et de leur paroi rigide composée de chitine pour les stades de vie végétatifs. Une partie de la matière organique consommée par les champignons ne transiterait donc pas par la boucle microbienne.

Par ailleurs, la présence et l'importance de champignons parasites, notamment du phytoplancton, jouent également un rôle prépondérant dans les flux de matière au sein de l'écosystème. Ces champignons parasites ont un impact sur les populations algales et plus particulièrement sur les espèces dites « *inédibles* », en raison de leur grande taille qui constitue un véritable refuge vis-à-vis de la prédation (Kagami *et al.*, 2007 ; Rasconi, 2010). Les principaux champignons parasites du phytoplancton connus (les chytrides) appartiennent à la division des Chytridiomycota, dont les espèces développent au cours de leur cycle de vie une spore flagellée (zoospore) de taille généralement inférieure à 5 µm. Cette forme flagellée des spores et l'absence de caractéristiques morphologiques spécifiques, ont sans doute amené à une confusion de ces champignons avec d'autres eucaryotes unicellulaires flagellés, notamment les protistes bactérivores.

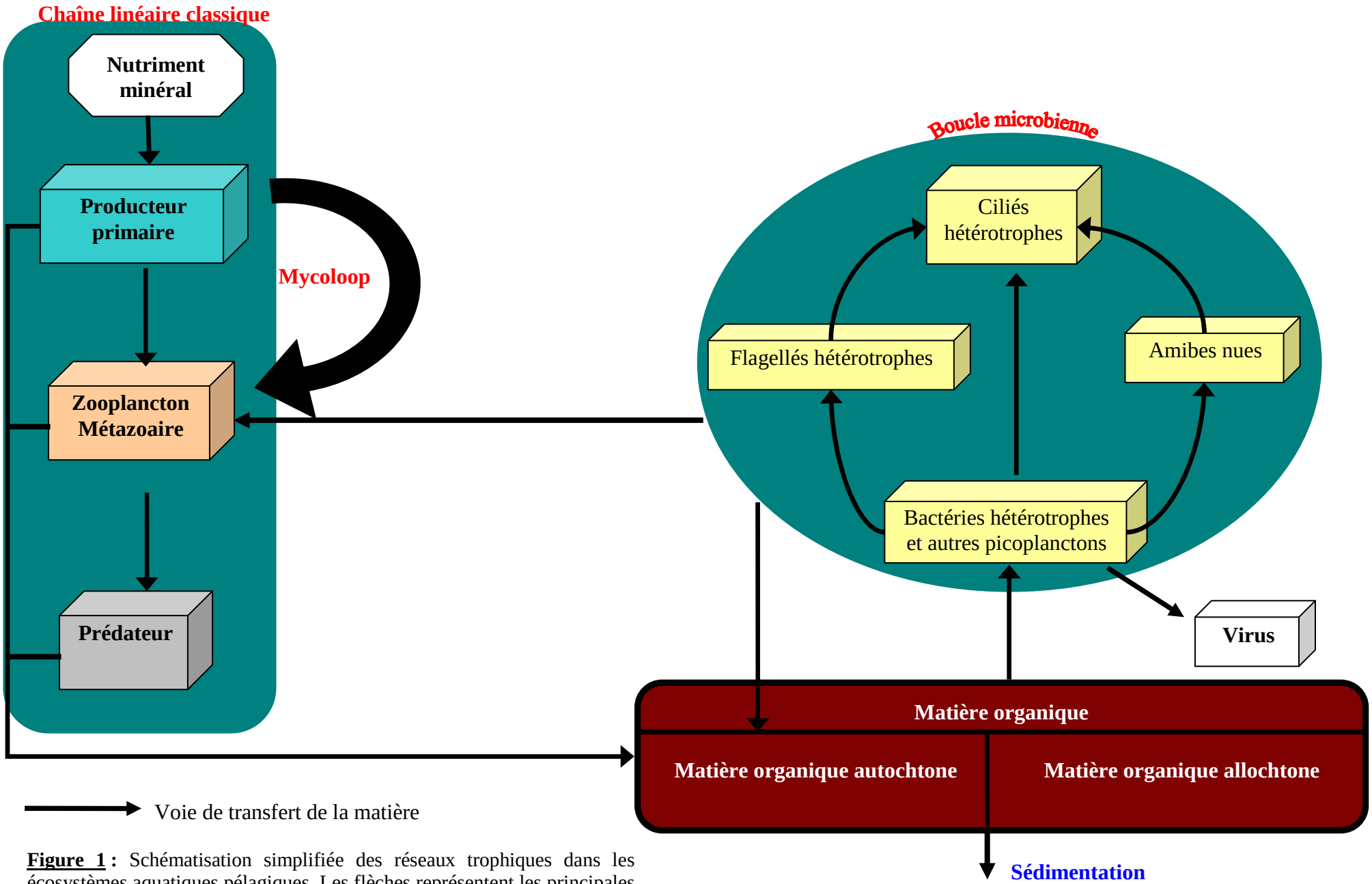

Figure 1 : Schématisation simplifiée des réseaux trophiques dans les écosystèmes aquatiques pélagiques. Les flèches représentent les principales voies de transfert de carbone entre les différents compartiments du réseau trophique.

Or, les zoospores de chytridiomycètes sont des structures de dispersion du champignon et par conséquent n'exploitent pas les ressources bactériennes. En outre, ces spores, au même titre que les flagellés bactérivores, peuvent être consommées par le zooplancton, créant ainsi un lien trophique entre le phytoplancton (particulièrement le phytoplancton de grande taille) et le zooplancton. Ce lien trophique s'est récemment traduit par le concept de « *Mycoloop* », proposé par Kagami et ses collaborateurs à partir d'études dans les écosystèmes pélagiques lacustres (Kagami *et al.*, 2007).

L'étude des champignons aquatiques se heurte à des difficultés méthodologiques, puisque ces organismes diffèrent largement des autres microorganismes étudiés en milieu aquatique. En effet, la plupart des méthodes utilisées pour détecter et quantifier les microorganismes aquatiques, comme la microscopie ou encore les méthodes culturales, sont difficilement applicables à l'étude des champignons. Contrairement aux autres microorganismes colonisant les lacs, les champignons possèdent des contraintes particulières. La complexité morphologique, avec la formation d'un appareil végétatif multicellulaire (phase végétative) et la production de spores ou de conidies (phase dispersive), fait qu'il est très difficile de détecter ou de quantifier les individus fongiques, les méthodes adaptées à ces deux formes n'existant pas. Les méthodes de détection et de quantification par microscopie s'avèrent efficaces pour la phase végétative des espèces de champignons chytridiomycètes, en raison de la présence de chitine pouvant être ciblée par des fluorochromes comme le calcofluor white (Rasconi *et al.*, 2009). La phase de dispersion (zoospore) des chytridiomycètes n'est, en revanche, pas détectable en microscopie, en raison de l'absence de membrane chitineuse. Les chytridiomycètes sont principalement des exoparasites, présentant une forme végétative (le sporange) qui vit attachée à son hôte, et une forme dispersive dont la taille (2 et 5 µm) et l'absence de caractéristiques morphologiques rendent difficile leur identification précise dans l'environnement. Ces deux formes peuvent être facilement assimilées à des protistes flagellés phagotrophes libres ou vivant fixés (exemple : les choanoflagellés qui sont les ancêtres phylétiques les plus proches des chytrides). Par ailleurs, les espèces de champignons saprophytes sont difficiles à observer au microscope, en raison d'un mode de vie cryptique. Leur forme mycélienne, qui est un atout majeur dans la colonisation et la recherche du substrat, présente en revanche un mode de développement intraparticulaire qui rend le mycélium pratiquement invisible en microscopie classique (Raghukuma, 2004).

Les approches de biologie moléculaire pour l'étude des microorganismes aquatiques permettent de pallier aux difficultés microscopiques ou culturales, en ciblant des gènes conservés. Ces approches ciblent en particulier le gène de la petite sous-unité de l'ARN ribosomique (ARNr 18S pour les eucaryotes). Ce gène est présent chez tous les organismes vivants où il effectue la même fonction, ce qui explique un degré de conservation élevé qui est à la base de son utilisation comme marqueur phylogénétique (Amann *et al.*, 1995 ; Amman et Ludwig, 2000). Ces approches moléculaires, une fois

mises au point, sont rapides et permettent de cibler les champignons, indépendamment de leur stade de vie (végétatif ou dispersif). Aujourd'hui, des méthodes moléculaires d'étude de la diversité microbienne (clonage-séquençage, pyroséquençage), de la structure génétique des peuplements (*terminal restriction fragment lenght polymorphism*, *Automated ribosomal intergenic spacer analysis*), ou de l'importance quantitative des différents phyla ou clades (PCR quantitative en temps réel (qPCR), *Fluorescent In Situ Hybridization* (FISH)), sont couramment utilisées en écologie microbienne aquatique. Cela a permis la mise à jour d'une diversité insoupçonnée de microorganismes eucaryotes, parmi lesquels la détection d'une quantité importante de séquences fongiques (Diez *et al.*, 2001 ; Lefèvre *et al.*, 2007, 2008 ; Lefranc *et al.*, 2006 ; Lepère *et al.*, 2008 ; López Garcia *et al.*, 2006 ; Nikolcheva *et al.*, 2003 ; Nikolcheva et Bärlocher, 2004). Récemment, l'étude de la diversité moléculaire des microorganismes eucaryotes lacustres a, en effet, révélé la présence de champignons pélagiques (Lefèvre *et al.*, 2007, 2008 ; Lefranc *et al.*, 2005 ; Lepère *et al.*, 2008). Ces études ont mis en évidence une diversité importante au sein du groupe des Eumycètes, avec une dynamique saisonnière marquée. A titre d'exemple, dans le lac Pavin, les Eumycètes représentent 23% de la diversité totale des picoeucaryotes potentiellement hétérotrophes en automne (Lefèvre *et al.*, 2007) et 19% pendant la période printanière (Lefèvre *et al.*, 2008). Les champignons détectés appartiennent majoritairement à la division Chytridiomycota, et représentent 60% des séquences fongiques en automne, et jusqu'à 75% au printemps. Cependant, ces études n'ont ciblé que les eucaryotes de taille picoplanctonique (< 5-10 µm), éliminant sans doute une grande partie de la diversité des champignons présents dans le milieu naturel.

La présence et l'importance d'autres divisions de champignons en milieux aquatiques (par exemple : Ascomycètes dans les rivières, Basidiomycètes dans les milieux marins) nous a amené à poser les hypothèses **(i) que la diversité et l'importance des champignons dans les écosystèmes pélagiques sont sous estimées**, et **(ii) qu'une étude globale de cette diversité est source de nouvelles données cognitives en écologie microbienne**, notamment dans le domaine du fonctionnement des écosystèmes aquatiques. C'est dans ce cadre scientifique de la connaissance d'une ressource génétique importante mais négligée, que se situe l'objectif principal de ce travail qui vise à une étude globale de la diversité fongique en milieux pélagiques, afin de répondre à des questions d'intérêt écologique.

Il est donc essentiel de faire un état de l'art sur ces questions, afin de préciser **(i) la signification écologique potentielle des champignons en milieux pélagiques, (ii) le rôle des différents stades de vie dans les transferts de matière et d'énergie et, de manière générale, (iii) l'importance du parasitisme et du saprophytisme dans le contexte des réseaux trophiques aquatiques et des cycles biogéochimiques associés.**

CHAPITRE I

SYNTHESE BIBLIOGRAPHIQUE

Diversity and functions of microscopic fungi: a missing component in pelagic food webs

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Short Title: Fungi in pelagic ecosystems

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Abstract

Fungi are a highly complex group of organisms of the kingdom Eumycota (i.e. the true-fungi) and other fungus-like organisms traditionally studied by mycologists, such as slime molds (Myxomycota) and oomycota (Straminopiles or Heterokonts). They constitute a significant proportion of the as yet undiscovered biota that is crucial in ecological processes and human well-being, through at least three main trophic modes: saprophytism, parasitism, or symbiosis. In addition to direct benefit (sources of antibiotics) or adverse effects (agents of disease), fungi can impact many environmental processes, particularly those associated with the decomposition of organic matter. They are present in almost all regions and climates, even under extreme conditions. However, studies have focussed mostly on economically interesting species, and knowledge of their diversity and functions is mainly restricted to soil, rhizosphere, mangrove, and lotic ecosystems. In this study, we review the diversity and potential functions of microscopic fungi in aquatic ecosystems, with focus on the pelagic environments where they often are regarded as allochthonous material, of low ecological significance for food web processes. Recent environmental 18S-rDNA surveys of microbial eukaryotes have (i) unveiled a large reservoir of unexpected fungal diversity in pelagic systems, (ii) emphasized their ecological potentials for ecosystem functioning, and (iii) opened new perspectives in the context of food web dynamics. In spite of persisting methodological difficulties, we conclude that a better documentation of the diversity and quantitative and functional importance of fungi will improve our understanding of pelagic processes and biogeochemical cycling.

Keywords: Fungi, Diversity, Microbial Ecology, Food web dynamics, Aquatic ecosystems.

Introduction

Microscopic fungi are amongst the most diverse organisms in the world, with an estimated 0.71 (Schmit and Mueller, 2007) to 1.5 millions species (*cf.* Hawksworth, 2001). They constitute a significant proportion of the yet undiscovered biota that is crucial to the maintenance of ecological processes and human well-being. One could speculate for example that, without fungi, major bottlenecks in nutrient cycling would occur, plants would lack the nutrients that fungi liberate for them from the soil, many animals would be without food, wood would not be broken down, some insects and other animals would not be able to digest plant materials, and even soil structure would change (Hawksworth, 2004). The term Fungi globally embraces all organisms that belong to the kingdom Eumycota (i.e. the so-called true-fungi), while the term fungi also includes other microorganisms (i.e. fungus-like organisms) traditionally studied by mycologists such as members of Myxomycota (Amobozoa) also called slime molds, and of Oomycota (Heterokonts) also called water molds, as well as mushrooms and other molds (Figure 1). All these organisms share similar trophic strategies, namely saprophytism, parasitism or symbiosis, and can occur in the same ecosystem. In this review, we mainly focus on the monophyletic Kingdom Eumycota that which groups the following 4 divisions: Ascomycota, Basidiomycota, Zygomycota and Chytridiomycota (James *et al.*, 2006) (Figure 1).

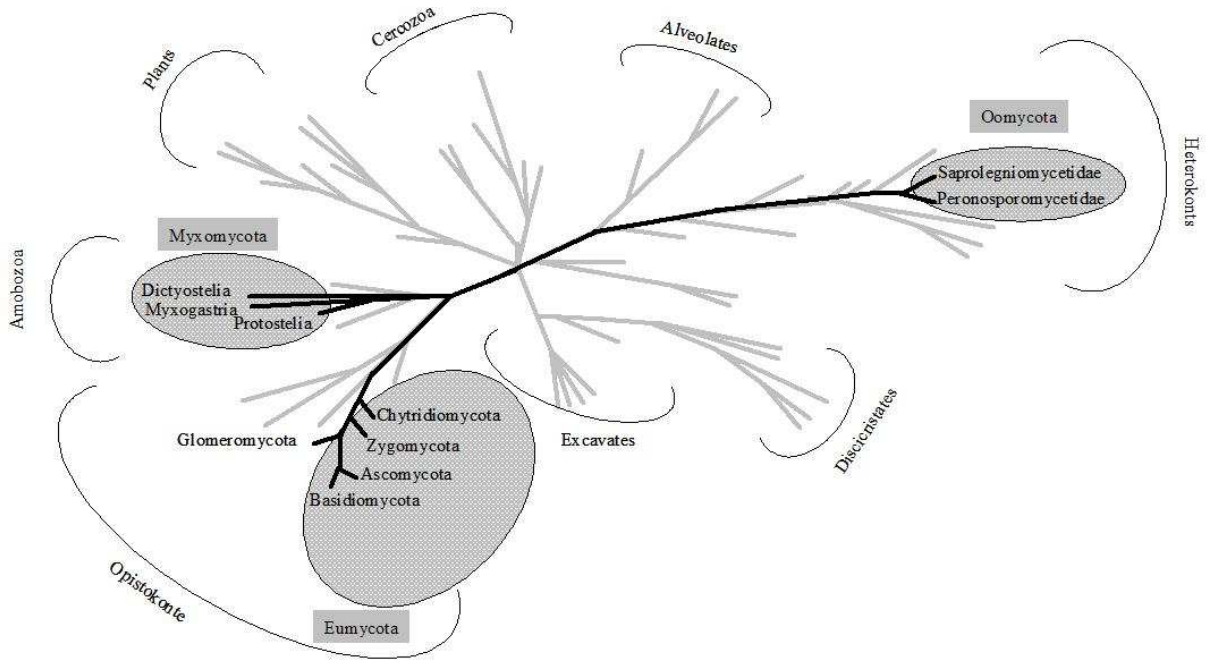

Figure 1: Microscopic fungi and fungus-like phyla in the phylogenetic tree of eukaryotes. The grey circles highlight fungi and fungus-like organisms recovered in aquatic ecosystems. Modified from Baldauf (2003).

Eumycota also include a particular group of microorganisms phylogenetically affiliated to the divisions Ascomycota and Basidiomycota (i.e. the so-called Deuteromycota), known as ‘‘fungi imperfecti’’ because only asexual forms (or anamorphs) have been observed during their life cycle. This group contains the particularly well-studied members of fungi in running waters, i.e. the hyphomycetes (Shearer *et al.*, 2007).

The manifold trophic modes, combined with complex and diverse phenotypes, are key factors explaining the omnipresence of fungi in natural environments, including extreme environments such as deep-sea hydrothermal ecosystems (Le Calvez *et al.*, 2009) (Table 1). In aquatic ecosystems, fungi are mainly known from lotic systems, mangroves and wetlands (Zettler *et al.*, 2002; Seena *et al.*, 2008) as the main decomposers of leaves, wood, detritus and other recalcitrant organic particles (Gulis *et al.*, 2008), and in pelagic ecosystems as phytoplankton parasites (Canter, 1951). Typical pelagic (i.e. floating) species, defined as those that rely on free water phase for whole or part of their life cycle, have received less attention (Goh and Hyde, 1996), compared to some economically and ecologically relevant species. For example, chytridiomycosis from *Batrachochytrium dendrobatidis* that causes amphibian mortality associated with population declines worldwide has been well studied (Berger *et al.*, 1998; Longcore, 1999), leading to a great deal of publicity. The same observation can be made for the rumen chytrids (i.e. in the Order Neocallimastigales), which are essential in the anaerobic degradation of lignocellulose (Kamra, 2005).

Table 1: Divisions and phenotypes of fungi encountered in varying conditions in aquatic habitats. Oomycota, i.e. fungus-like organisms, are not included.

Ecosystem	Environmental condition	Division	Phenotype	Examples of species	Reference
Lotic	Aerobic	Ascomycota, Deuteromycota	Filamentous	*Tetracladium sp*	Nicholcheva et al., 2003; 2004; Bärlocher et al., 2006; Bärlocher, 2007
	Acidic	Ascomycota Zygomycota Basisiomycota	Filamentous Yeast	*Penicillium sp* *Mortierella sp, Mucor sp* *Rhodothorula sp, Cryptococcus sp*	López-Archilla et al., 2004; Gadanho and Sampaio, 2006
Sediments, lotic	Freshwater, weakly aerobic	Deuteromycota	Filamentous	*Fusarium sp*	Medeiros et al., 2009
Deep sea	Salted waters, hydrothermal conditions	Ascomycota Basidiomycota Chytridiomycota	Filamentous Yeast Zoosporic	*Cladosporium sp* *Rhodosporidium sp, Cryptococcus sp* *Chytridium polysiphoniae*	Le Calvez et al., 2009; Burgaud et al., 2009; Lopez-Garcia et al., 2006
Riparian and marine	Anoxic (Arthropod gut)	Zygomycota (Trichomycetes)		*Asellaria aselli, Harpella sp*	Lichtwardt et al., 2003
Baltic sea (Gotland deep)	Marine, suboxic and anoxic	Ascomycota Basidiomycota Uncultured fungus	Filamentous Yeast	*Penicillium sp* *Rhodothorula sp* *LKM*	Stock et al., 2009
Marine (Atalande Basin in Méditerranée)	Hypersaline, anoxic	Ascomycota Basidiomycota	Filamentous Yeast	*Penicillium* *Rhodothorula*	Alexander et al., 2009
High-altitude Lake	Cold	Basidiomycota	Yeast	*Rhodothorula sp, Cryptococcus sp*	Libkind et al., 2009

The importance and impact of fungi has not been quantified extensively in pelagic ecosystems, mainly because their occurrence in these systems is often regarded as allochthonous (i.e. from soils) and of minor importance in food web dynamics (Kurtzman and Fell, 2004). Furthermore, there are technical difficulties in assessing their diversity as well as quantitative and functional ecology (Rasconi *et al.*, 2009). For example, in routine methods for assessment of molecular diversity (i.e. SSU rDNA), there is a lack of specific primers to target all fungi in aquatic environments (Lefèvre *et al.*, 2007). Staining with fluorochromes such as calcofluor white also is a routine method for microscopic observation and counting of chitinous tissues such as sporangia or mycelium, but fungal zoospores typically have no cell wall and lack chitin (Rasconi *et al.*, 2009). Similarly, the routine assessment of fungal biomass based on ergosterol measurements does not target typical pelagic fungi (i.e. chytrids) that lack ergosterol (Gessner, 1997). Damare and Raghukumar (2008) presented evidence that poor detection of fungi in sediments is due to their cryptic presence in macroaggregates, similar to freshwater parasites or symbionts (e.g. trichomycetes, a fungal class affiliated to the division Zygomycota) that are associated with living organisms, including arthropods, crustaceans, or algae (Lichtwardt *et al.*, 2003; Sigee, 2005; Rasconi *et al.*, 2009). According to Lichtwardt and coauthors (2003), trichomycetes can be collected in virtually all regions of the world and represent a major source of undiscovered fungal diversity (Hawksworth and Rossman, 1997), but they typically remain undetected until their hosts are dissected. Recent observations of host associated fungi such as phytoplankton parasites (Rasconi *et al.*, 2009) and zooplankton symbionts (Lichtwardt *et al.*, 2003), confirm the cryptic presence of fungi in pelagic ecosystems. Indeed, the growth of fungal mycelium inside organic particles or living hosts tends to make fungi undetectable by classical direct microscopy.

From studies in soil systems, we know that fungi are vital in recycling nutrients through the metabolism of complex organic materials. In pelagic ecosystems, it does not seem unreasonable to postulate that their saprophytic activity also may be of importance in many ecosystem processes (Watling, 2005; Treseder, 2005). However, our ecological knowledge of fungi in pelagic ecosystems is scant. We know little of the potential impact of human activities on natural fungal communities, or the responses of fungi to ecosystem perturbations. Nonetheless, recent research, primarily in freshwater pelagial, has demonstrated that fungi are important heterotrophic microbial eukaryotes (Gessner *et al.*, 2007; Jorgensen and Stepanauskas, 2009), and that some typical planktonic life forms (e.g. chytrids as free-living zoospores and fixed sporangia) have probably been overlooked or misidentified as protistan eukaryotes in the past (Lefèvre *et al.*, 2007, 2008). A review on the importance of fungi is thus warranted, based on the hypothesis that these microorganisms are well adapted to thrive in aquatic ecosystems where they potentially play diverse roles. In this paper, we attempt to summarize the available knowledge on the diversity and putative functions of fungi in aquatic systems, with a particular attention to pelagic habitats. Other recent reviews have focused on

fungi associated with submerged natural substrates in lotic and wetland ecosystems, or attached to leaf litter or decaying wood (Shearer *et al.*, 2007; Gessner *et al.*, 2007; Gulis *et al.*, 2008).

Living in aquatic systems: oxygen availability and dispersal

Oxygen concentration. Water-borne fungi have to face various difficulties and constraints characteristics of aquatic habitats, among which oxygen availability perhaps may be one of the most restrictive parameters. Fungal biomass and sporulation can sharply decrease with dissolved oxygen concentration (Medeiros *et al.*, 2009). Nevertheless, in some anaerobic environments such as sulphide rich springs and the anoxic deep sea, an unexpected diversity of fungi was encountered (despite the general low sampling effort), mostly restricted to Ascomycota and Basidiomycota phyla. However, the diversity appears lower than that found in oxic waters (Nikolcheva and Bärlocher, 2004; Luo *et al.*, 2005; Stock *et al.*, 2009; Alexander *et al.*, 2009). Investigations of the tolerance to anoxia in the chytrids *Rhizophydium sphaerotheca* and *Phlyctochytrium punctatum* have revealed that these fungi are facultative anaerobes (Goldstein, 1960). Other aquatic species, which include the so-called aeroaquatic fungi, exploit decaying leaves in streams and ponds for their vegetative growth, but need exposure to ambient air to form propagules (Shearer *et al.*, 2007). They are encountered in shallow stagnant to slow-flowing freshwater bodies where they can withstand prolonged periods of oxygen depletion (Field and Webster, 1983; Shearer *et al.*, 2007).

In addition, some terrestrial fungi can colonise aquatic habitats. For example, the genera *Fusarium* or *Aspergillus* (i.e. anamorphs of Ascomycota species) are common soil-borne fungi that can live and grow in aquatic conditions (Shearer *et al.*, 2007; Seena *et al.*, 2008; Damare *et al.*, 2008). The main ecological implication is that fungi from allochthonous inputs can remain active in pelagic waters and thus contribute to the food web dynamics and nutrient cycling. This is an important information since a sizeable proportion of fungi recovered in pelagic areas may come from soil. Recently, Jorgensen and Stepanauskas (2009) found that the biomass of fungi in a pelagic ecosystem was positively correlated with terrestrial high-molecular-weight dissolved organic matter. Although these authors also concluded that fungi were of minor importance in river ecosystems at the sampling time, it is likely that fungi represent a potential link between terrestrial and aquatic environments.

Dispersal. Adaptation to dispersal in water is typical of some fungi. Turbulence in lotic systems provides oxygenation for the growth of fungi but can interfere with fungal attachment. To overcome this environmental challenge, some fungi have developed spores with particular morphology allowing successful attachment on substrates in flowing waters (Ingold, 1975; Shearer *et al.*, 2007). The asexually produced propagules, e.g. conidia of aquatic hyphomycetes, are relatively large and complex. They are multiradiate (tetraradiate or quadriradiate) or sigmoid, which increases the

probability of attachment to detritus in flowing waters (Kearns and Bärlocher, 2008; Sigee, 2005). Ingold (1953) speculated that these complex morphologies might delay settling in the water column, thereby ensuring adequate dispersal. Alternatively, they might also act as ‘‘anchors’’ for the attachment of individuals to suitable substrata (Kearns and Bärlocher, 2008). The complex phenotypes of hyphomycete spores probably have arisen from convergent evolution in several lineages as a secondary adaptation to aquatic life (Ingold, 1942), as recently confirmed by molecular markers (Belliveau and Bärlocher, 2005; Baschien *et al.*, 2006). In fact, aquatic hyphomycetes (i.e. Ingoldian hyphomycetes) are a highly specialized group of fungi, particularly adapted to colonize flowing water environments.

Within true fungi encountered in aquatic ecosystems, Chytridiomycota (chytrids) represent the sole phylum with species producing flagellated cells in their life cycle (i.e. zoospores). These temporary swimming life stages are particularly well adapted to dispersal in aquatic medium (Gleason and Lilje, 2009), and are released into the environment from sporangia as the main means for actively finding new host cells (Suberkropp and Cantino, 1973; Lilje and Lilje, 2008; Gleason and Lilje, 2009). Although chytrids are also common in wet soils (Lozupone and Klein, 2002), they are considered typical of pelagic systems where they represent the best studied group of fungi, primarily in lakes where they occur mainly as phytoplankton parasites (Rasconi *et al.*, 2009; Ibelings *et al.*, 2004).

Interestingly, specific morphological adaptations are not absolutely necessary conditions for fungi to colonize aquatic ecosystems. Indeed, fungi with no particular phenotypic modification have been reported in aquatic habitats. They are often called miscellaneous freshwater mitosporic Ascomycota fungi, whose conidia are not distinctively modified for the aquatic environment (Shearer *et al.*, 2007). It is thus likely that the general lack of dispersal limitation in aquatic microorganisms, i.e. in the context of the hypothesis that ‘everything is everywhere, but the environment selects’ (*cf.* Wit and Bouvier, 2006), is also valid for fungi, suggesting that this group of organisms is a functional component of pelagic systems, although their ecological significance remains to be fully established.

Diversity

Phylogenetically, organisms studied by mycologists (i.e. fungi) typically belong to three main groups: the true fungi of interest (i.e. the four phyla of Eumycota), and subgroups of Heterokonts (Oomycota) and of Amoebozoa (Myxomycota) (Figure 1). In aquatic habitats, including marine, freshwater, benthic, and lotic ecosystems, these organisms are mostly zoosporic fungi (i.e. Chytridiomycota), Ascomycota and Basidiomycota, although fungi are considered cosmopolitan (Tsui and Hyde, 2003). Recent surveys have recorded a great diversity of fungi in various aquatic ecosystems (Bärlocher *et al.*, 2006; Bärlocher, 2007; Lefèvre *et al.*, 2007, 2008; Burgaud *et al.*, 2009;

Jorgensen and Stepanauskas, 2009; Rasconi *et al.*, 2009). More than 600 species of freshwater fungi have been described, including about 300 species of anamorphic fungi and 300 species of Ascomycota followed by few Basidiomycota and Chytridiomycota species (Wong *et al.*, 1998; Nikolcheva and Bärlocher, 2004; Shearer *et al.*, 2007; Gulis *et al.*, 2008), but new taxa are being discovered every year at an increasing rate (Letcher *et al.*, 2008; Raja and Shearer, 2008; Hoffman *et al.*, 2008). The apparent dominance of Ascomycota affiliated fungi may be due to the fact that most surveys on fungal diversity and ecology have been conducted in running waters, with a main focus on the well-studied litter-decomposing hyphomycetes. This group is mainly affiliated with the Ascomycota, with only a small percentage related to Basidiomycota (Nikolcheva *et al.*, 2003; Belliveau and Bärlocher, 2005; Shearer *et al.*, 2007). In addition, aquatic fungal surveys are often restricted to harvesting and identifying fungal propagules present in water. This method provides a better detection approach for large propagules like hyphomycete conidia and Ascomycota spores, while yeast and chytrids can escape detection due to their smaller size (Table 2). Recent introduction of molecular approaches to investigate fungal diversity in the environment has documented the presence of more aquatic fungi, including yeast-forming Basidiomycota and zoosporic Chytridiomycota species (Seena *et al.*, 2008; Lefèvre *et al.*, 2008).

Table 2: Fungal divisions found in diversity surveys of small eukaryotes by cloning sequencing of differing size-fractions of pelagic samples from lake and marine ecosystems. Retrieved fungal sequences are restricted to zoosporic and yeast-forming organisms, belonging to Chytridiomycota and Basidiomycota. Closest relatives are given by the authors.

Sample		Fraction of sample (µm)	Division	Closest clade relative	Reference
Site	Condition				
					Chen et al., 2008
Lake systems	Subtropical	0.8-20	Chytridiomycota	*Chytriomyces angularis, Chytridium polysiphoniae, Hyaloraphidium curvatum, Allomyces macrogynus, Triparticalcar arcticum*	
	Temperate freshwater	0.6-5	Chytridiomycota	*Rhizophydium, Rhizophydiales*	Lefèvre et al., 2007; 2008
		0.2-5	Chytridiomycota	*Spizellomyces, Nowakowskiella*	Lefranc et al., 2005; Lepère et al., 2006; 2007; 2008
Marine systems	Antartica	0.2-2	Basidiomycota	*Taphrina*	Diez et al., 2001
	Temperate coastal	0.2-3	Basidiomycota Chytridiomycota	*Bullera sp, Rhodothorula sp* *Spizellomyces sp*	Massana et al., 2004
	Temperate coastal	0.45-3	Ascomycota Chytridiomycota	*Byssothecium sp* *Spizellomyces*	Romari and Vaulot, 2004
	Suboxic and anoxic	>0.2	Ascomycota Basidiomycota Uncultured	*Penicillium, Taphrina* *Rhodotorula* LKM	

Fungi are influenced by environmental conditions and aspects of their diversity are specific to certain aquatic environments. In marine habitats, about 500 fungal species have been described (Kis-Papo, 2005), with a large majority of yeast-forming fungi belonging to Ascomycota phylum (e.g. *Candida, Debaryomyces, Kluyveromyces, Pichia,* and *Saccharomyces)* which are common in shallow waters, while those belonging to the Basidiomycota are most common in deep waters (Munn, 2004). In more extreme conditions, fungi belonging to the Ascomycota have mainly been recovered from deep-sea hydrothermal vents (Burgaud *et al.*, 2009; Le Calvez *et al.*, 2009), or in anoxic oligotrophic marine environments (Takishita *et al.*, 2005; Golubic *et al.*, 2005). Moreover, a great diversity of Basidiomycota yeasts adapted to extremely cold environments (ice, snow and sea ice) has been recovered (Margesin *et al.*, 2002; De Garcia *et al.*, 2007; Libkind *et al.*, 2009). In pelagic areas, particularly in freshwaters, knowledge of fungal diversity is very scant and is often reduced to accidental detection during eukaryotic diversity investigations (Table 2). Recent molecular studies of picoeukaryotes in pelagic systems have highlighted that these systems are particularly undersampled for fungal diversity (Lefèvre *et al.*, 2007, 2008), and this is why little knowledge is available on fungal communities in pelagic systems and their related food web dynamics (Gleason *et al.*, 2008). This is not satisfactory from a scientific point of view because pelagic systems contain a major part of the total biodiversity on the Earth (Hawksworth, 2006).

Chytrids are the most consistently documented group of fungi in pelagic freshwater lakes, when overall eukaryotic microbial diversity investigations are undertaken (Diez et al., 2001; Lefranc *et al.*, 2005; Lepère *et al.*, 2006; Lefèvre *et al.*, 2007, 2008; Lepère *et al.*, 2008). However, these 18S rDNA surveys mostly have analysed the diversity of picoeukaryotic microorganisms, using plankton samples where organisms above 5 µm have been filter-removed. Chytrids whose zoospore size is about 2-5 µm diameter (Kudoh and Takahashi, 1990) were recovered this way, but filamentous fungi were most likely overlooked in these studies (Table 2). This is corroborated by our recent observation of fungal hyphae colonizing organic particulates and macroaggregates harvested from pelagic lake samples (Figure 2). Fungal adaptability and the detection of fungi in many aquatic ecosystems in sizeable diversity lead us to hypothesize that fungal diversity and function are underestimated in the pelagial at the present time.

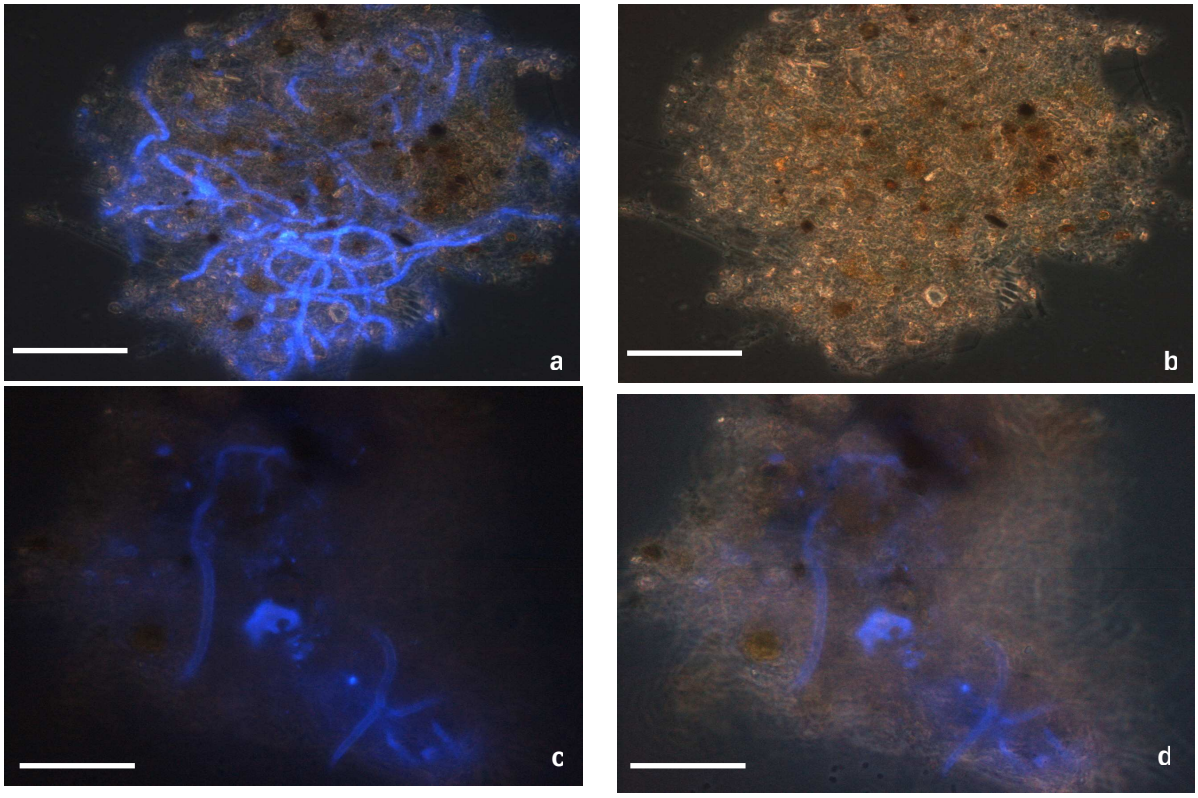

Figure 2: Micrographs of particulate organic matter (POM) aggregates from the surface waters of Lake Aydat (France) observed under epifluorescence microscope, for evidence of fungal hyphal colonization. Samples were stained with calcofluor white following the protocol described in Rasconi *et al.* (2009). Two different POM aggregates were observed under UV light revealing fungal hyphae (a, c), after visualization using transmitted light (b, d). The hyphae diameters are larger than 1 µm, which is typical of true fungi hyphae. Scale bar = 20 µm.

Potential functions

Fungi associated with detritus and dead hosts: saprophytism. Decomposition of organic matter is an important process in aquatic ecosystems, allowing mineralization and ensuring the availability of essential elements (e.g. C, N, P) for primary producers. Degradation of high molecular weight and recalcitrant organic polymers is usually the rate-limiting step in mineralization, while the monomer derivatives are rapidly incorporated by the microbial communities (Sigee, 2005). Grazers and lytic viruses are considered the main actors for organic matter release and the related biochemical catalysis, while heterotrophic prokaryotes are the main mineralisers (Hoppe *et al.*, 1993; Weinbauer and Rassoulzadegan, 2004; Sime-Ngando and Colombet, 2009). To our knowledge, there is no information about the importance of fungi in the processing of organic matter in pelagic areas in either fresh- or marine waters. In addition to parasitic chytrids which have been shown to impact phytoplankton communities and food-web dynamics in few lakes (Ibelings *et al.*, 2004; Kagami *et al.*, 2007; Gleason *et al.*, 2008; Rasconi *et al.*, 2009), other groups of fungi also occur in pelagic systems and deserve more attention because of the significance of their potential functions.

Decomposition of organic matter is a community process which, in the nature, implies the intervention of different microbial populations. No single population can express the entire range of enzymes needed to degrade complex substrates such as cell wall polymers (Osono and Takena, 2002). Bacteria participate actively in organic compound degradation and the presence of both fungi and bacteria on freshwater aquatic detritus can lead to neutral, synergistic or antagonistic interactions (Mille-Lindblom *et al.*, 2006). In most situations, bacterial production gradually increases throughout the decomposition sequence and peaks during the terminal processing phase, contrasting with fungal activity (Suberkropp and Klug, 1976; Sigee, 2005). Indeed, fungi are very efficient in the breakdown of complex and recalcitrant compounds such as leaf litter biomass, enhancing both the palatability of the substrate and the release of more labile substrates that, subsequently, favour bacterial growth and respiration (Mansfield, 2005; Gessner *et al.*, 2007). Resource acquisition is a critical factor that fungi have to face in aquatic systems. This is facilitated by their capabilities to produce various extracellular hydrolytic enzymes that degrade complex molecules such as lignin, cellulose, pectin, protein or other polymers (Abdel-Raheem and Ali, 2004; Mansfield, 2005; Gulis *et al.*, 2008). In addition, due to their hyphal organization, fungi are more efficient than bacteria in colonizing and breaking down large organic particles (Raghukumar, 2004; Sinsabaugh, 2005). Hyphae of most true fungi are typically about 3-5 µm in diameter and often are organized as branching mycelium, representing a highly successful vegetative structure for parasitic or saprophytic solubilisation of living and non-living organic particles.

Enhanced bacterial metabolism, at least partly due to fungal release of labile substrates, can make unproductive lakes and estuaries net heterotrophic systems, i.e. the total carbon processed by bacteria exceeds the carbon fixed by primary producers (Del Giorgio *et al.*, 1997). However, the importance of fungi has never been taken into account in this context, although it is widely accepted that fungi are the main actors in the decomposition of refractory organic matter. Such refractory materials accumulate in the pelagial as an important reservoir of carbon, and are also characteristics of allochthonous inputs (Jorgensen and Stepanauskas, 2009). We believe that fungi are responsible for a substantial proportion of CO_2 release (Baldy *et al.*, 1995; Kuehn *et al.*, 2004; Gessner *et al.*, 2007) but are virtually ignored in the context of mass and energy balance in pelagic systems. Excluding fungi from carbon cycling processes in pelagic ecosystems undoubtedly leads to a poor estimation of CO_2 release from these ecosystems (and at a more global scale as well), and the question of the relative importance of fungi and bacteria in this estimation remains largely unanswered.

There is compelling evidence that fungi are critically important, if not the main decomposers of plant materials in coastal marine and freshwater ecosystems, although most studies have been conducted in benthic and stream habitats (Bärlocher, 1992; Baldy *et al.*, 1995, 2002; Bärlocher, 2005; Gulis *et al.*, 2006; Raghukumar, 2006). The majority of aquatic hyphomycetes thriving in lotic

ecosystems are saprophytes, able to break down recalcitrant substrates like plant materials rich in cellulose. On aquatic macrophytes and leaf surface areas, fungal biomass generally exceeds that of bacteria, and typically represents about 90% of the total microbial biomass (Newell *et al.*, 1989; Baldy *et al.*, 1995; Kominkova *et al.*, 2000; Findlay *et al.*, 2002). Chytridiomycota have also been recovered as saprophytes and can degrade polymers from a variety of plant and animal debris (Czeczuga and Mazalska, 2000; Czezuga *et al.*, 2005), including algae, pollen (Shaerer *et al.*, 2004), seeds, fruits, dead insects, and insect exuvia (Sparrow, 1960; Czeczuga *et al.*, 2000, 2002). The saprophytic species encountered depend to some extent on the nature of the substrates colonized. For example, *Nowakowskiella ramosa* is found primarily on cellulosic plant materials, while *Chytriomyces hyalinus* grows in freshwaters on the exuvia of may flies and fragments of chitin (Sigee, 2005). The presence of these fungi in pelagic food webs is largely uninvestigated and yet, saprophytic fungi and particularly chytridiomycetes, quite possibly represent a missing trophic link between organic matter in the food web. This may be crucial in the processing of production from phytoplankton blooms that could provide important amounts of dead organic particles for fungal solubilisation (Halemejko and Chrost, 1986). Recently, we observed substantial hyphal colonization of detritus particles following a late summer bloom of the cyanobacteria *Anabaenae* sp in the productive Lake Aydat, France (Figure 2). Fungi colonizing aggregates, and particles such as pollen or zooplankton carcasses have also been observed in a few studies, but their functional importance has never been investigated (Simon *et al.*, 2002; Raghukumar, 2004; Tang *et al.*, 2006). Moreover, allochthonous materials in lake and coastal waters certainly are associated with telluric fungi which can, at least transiently, survive in aquatic habitats, leading to the release of a sizeable amount of allochthonous dissolved substances before colonization of particles by autochthonous microheterotrophs.

Fungi associated with living hosts: Parasitism. As parasites, fungi can cause serious damages to living organisms, including both autotrophs (cyanobacteria, algae, macrophytes, plants) and heterotrophs (other fungi, protists, invertebrates, vertebrates). Most of the described fungal parasites in pelagic systems belong to the zoosporic Chytridiomycota and are able to attack phytoplankton and zooplankton. The most sensitive hosts seem to be autotrophic prokaryotes and eukaryotes (Table 3), and chytrids are thought to be important regulators of the dynamics of these populations (Kagami *et al.*, 2007; Lefèvre *et al.*, 2007, 2008). Mainly based on laboratory studies, chytrids are known to have major effects on algal growth efficiency (Van Donk, 1989; Sigee, 2005), and are thought to be a major agent leading to the decline of phytoplankton populations in nature (Van Donk and Bruning, 1992; Sigee, 2005). It has been shown that fungal parasites can decimate a wide range of both prokaryotic and eukaryotic algal host populations (Van Donk and Ringelberg, 1983; Kudoh and Takahashi, 1990; Gons *et al.*, 2002), leading to the discharge of a large amount of organic matter available to saprophytic fungi and bacteria. In the context of pelagic environments, such activity may prevent

sedimentation of host populations and increase the retention time and heterotrophic recycling of organic matter in the water column (Lefèvre *et al.*, 2008).

Table 3: Examples of chytrids parasites and their hosts in freshwaters. Note that the diatom *A. formosa* can be infected by different species of chytrid parasites.

Host trophic strategy	Host		Fungal parasite	Habitat	% of total host infected	Reference
	Group	Species				
Autotrophic						
Procaryote	Cyanobacteria	*Microcystis* sp.	*Rhizidium microcystidis*	Freshwater lake	15-90	Sen (1988)
		Anabaena floquae	*Rhizosiphon* sp.		<1	Rasconi et al. (2009)
Eukaryote	Diatom	*Asterionella formasa*	*Zhygorhizidium* sp.		2-30	Kudoh and Takahashi (1990)
			Rhizophydium planktonicum		0-80	
		Stephanodiscus alpinus	*Zhygorhizidium* sp.		70	Bruning et al. (1992)
						Holfeld (2000)
	Green algae	*Haematococcus pluvialis*	*Blastocladiomycota*	Freshwater pond		Gromov et al. (1999a, b)
			Rhizophydium algavorum			
		Kirchneriella obese				
		Ankistrodesmus sp.				
		Chlorella sp.				
		Chlorococcum sp.				
		Scenedesmus sp.				
	Dinoflagellate	*Peridinium gatunense*	*Phlyctochytrium* sp.		1-30	Alster and Zohary (2000)
			Amphicypellus elegans			
		Ceratium spp.				Canter and Heaney (1984)
Heterotrophic						
Eukaryote	Cladocera	*Daphnia pulicaria*	*Polycarym leave*	Freshwater lake	1-34	Johnson et al. (2009)
		Daphnia pulicaria	*Polycarym leave*		80	Johnson et al. (2006)
		Daphnia sp.	*Metschnikova bicuspidate*			Lampert and Sommer (2007)
	Fungus	*Allomyces arbuscula*	*Rosella allomycis*			Held (1972)
	Amphibian	*Mixophyes fasciolatus*	*Batrachochytrium dendrobatidis*			Boyle et al. (2004)

The probability of epidemic development of chytrid parasites in pelagic systems is considerable because these microorganisms exhibit typical r-strategist features, with simple vegetative stage (i.e. sporangium) that allows rapid exploitation of resources, and the production of large numbers of swimming zoospores that can actively find and colonize new hosts, at times aided by host-secreted specific signals (Sigee, 2005; Van Donk, 2006, 2007; Kagami *et al.*, 2007). Chytrid epidemics can thus spread rapidly within an exponentially growing host population, affecting both healthy and metabolically-weakened or moribund host cells (Canter and Lund, 1948; Holfeld, 2000). In the latter, the transition between parasitism and saprophytism is foggy because the two processes overlap. The general lack of massive parasite colonization of individual host cells (Holfeld, 2000; Rasconi *et al.*, 2009) means that even a single parasite can kill its host cell, which substantially increases the functional impact of fungal parasites on host populations. However, the prevalence of chytrid infections in phytoplankton populations sometimes is less than 30% (Table 3), comparable to other mortality sources (i.e. grazing, viral lysis). This highlights the complexity of chytridiomycosis in plankton communities. Zooplankton such as *Daphnia* have been demonstrated to feed on fungal zoospores in the absence of other suitable food sources (Kagami *et al.*, 2004), which may help explain

the overall moderate level of the prevalence of chytrid infections in plankton, but established these organisms as potential key intermediates in food web dynamics.

Fungal parasitism thus seems to be a complex process in pelagic ecosystems, linking multiple interactions between various microorganisms (Table 3), because their action on a single population or trophic level might impact several others through food-chain cascades. However, very little is known about the integration of fungal parasites into food web dynamics (Gleason *et al.*, 2008). For example, unavailable nutrients contained in large inedible but parasitized phytoplankton species (i.e. whose fate is supposed to be sinking to the bottom) can be converted back to the water column food chain toward zooplankton grazing of fungal zoospores (Lefèvre *et al.*, 2008), hence the concept of the "Mycoloop" (Kagami *et al.*, 2007). In reality, this mycoloop is an underestimate because the infection intensity of small-size edible algae is largely underestimated due to grazing (Rasconi *et al.*, 2009), and it is absolutely unknown if grazing can represent a source of fungal infection of grazers. The introduction of parasitism in food web investigations is thus essential information (Marcogliese and Cone, 1997; Wood, 2007; Lafferty *et al.*, 2008; Byers, 2009), potentially representing a source of new knowledge and enhanced understanding in the context of aquatic microbial ecology.

Fungi associated with living hosts: mutualism. The third major trophic strategy of fungi is longstanding interactions with other organism, leading to mutual advantages for both partners. Knowledge about mutualistic fungi in pelagic ecosystems is scarce due to their cryptic presence and related methodological difficulties. For example, Trichomycete fungi (an order belonging to the Zygomycota phylum, Figure 1) belonging to the genera *Asellariales* and *Eccrinales* remain non-culturable (Lichtwardt *et al.*, 2003). Trichomycetes grow in the gut of insects, crustaceans and millipedes that often breed in discrete and disjunct lentic habitats (Lichtwardt and Williams, 1999; Roa *et al.*, 2009). The roles and importance of Trichomycetes in pelagic food webs are not known at all. Horn and Lichtwardt (1981) have demonstrated, experimentally, that the Trichomycetes *Smittium culisetae* in the hindgut of the aquatic mosquito larvae *Aedes aegypti* could serve as a source of essential elements for insect growth, primarily of sterols and B-vitamins (Lichtwardt *et al.*, 2003). Similar to the case of fungi that can improve the palatability of leaves for macroinvertebrates in wetlands and running waters (Suberkropp *et al.*, 1983), mutualistic Trichomycete fungi may be able to improve the assimilation of recalcitrant compounds directly in the gut of their hosts. In some cases, the distinction between parasitism and mutualism is not easy. For example, the growth and survival of the cyanobacteria *Anabaena circinalis* are enhanced by the chytrids order of *Rhizophydiale*, with mutual benefits for both parasite and host (Gleason and Macarthur, 2008). Overall, more information about symbiotic associations between fungi and hosts are needed for a better understanding of the functional roles of fungi in pelagic ecosystems.

Fungi as nutritional resources in food webs. In soil, Fungi are known to be an important food resource for diverse predators including multi-celled eukaryotes (e.g. termites) and protistan or amoebozoan microorganisms (Ruess and Jussenhop, 2005), and we believe the same is true in pelagic ecosystems. Fungal tissue is composed of chitin, about 14% N, and reproductive structures containing high P concentrations (Ruess and Jussenhop, 2005). Fungal production is partitioned between mycelium and spores, both of which can be ingested by zooplankton. Fungal spores encountered in pelagic areas range in size from 2 µm for chytrids (zoospores) to > 200 µm for hyphomycetes (conidiospores) (Figure 3). It has been clearly demonstrated that parasitic yeast (e.g. *Metschnikowia bicuspidate)* or chytrids (particularly zoospores) can be ingested by *Daphnia* (Kagami *et al.*, 2004; Lampert and Sommer, 2007). The diet of zooplankton also includes detritus and it is not clear whether zooplankton ingestion of detritus is a means to collect dead organic matter or attached living microorganisms such as fungi. Indeed, fungi that live on detritus may have a higher nutritional content (i.e. than free-living forms) and thus be preferred by grazers, a situation already demonstrated for prokaryotic prey (Lampert and Sommer, 2007). Although current knowledge about nutritional resources represented by fungi is sketchy, these microorganisms may represent an important energy link, both within the pelagic food web and between the water bodies and the catchment area. However, the importance of these energetic pathways of nutrient cycling remains to be studied.

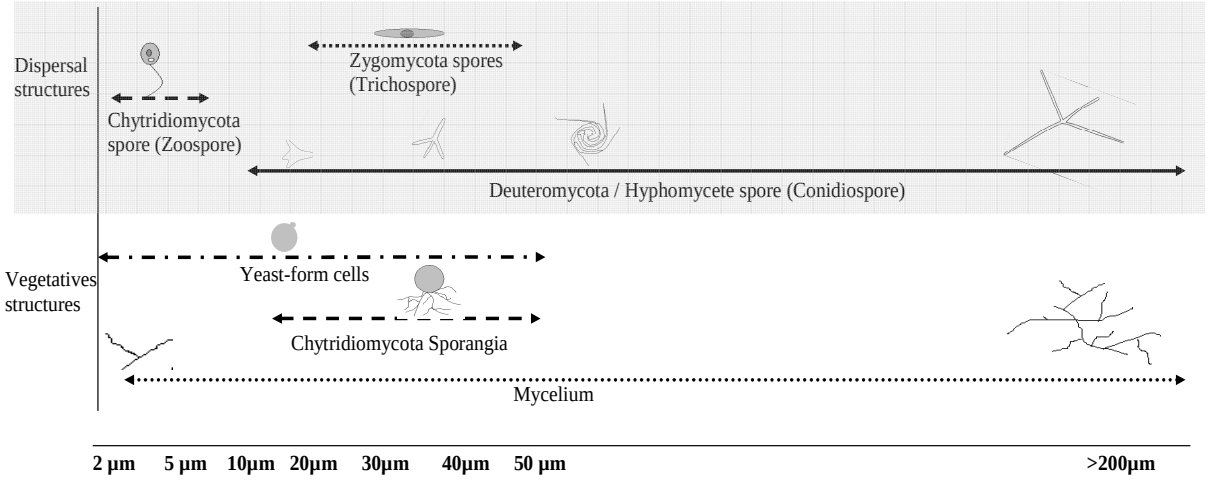

Figure 3: Dispersal and vegetative life stages and size range of fungal structures encountered in aquatic habitats. Drawings were inspired from Ingold (1975).

Fungus-like microorganisms: Oomycetes and Myxomycota

In aquatic environments, some microorganisms (i.e. fungus-like) previously classified in the kingdom Fungi are recognized today as phylogenetically different from Eumycota (i.e. true fungi). These microorganisms include two major groups, the Oomycetes and Myxomycota (Figure 1), which

exhibit the same trophic strategies compared to true-fungi. The data on their occurrence in typical pelagic areas are few, particularly for Myxomycota. One example is a subgroup called Prostostelid, in which organisms have been recently detected, for the first time, in pelagic habitats (Spiegel *et al.*, 2004; Lindley *et al.*, 2007; Tesmer and Schnittler, 2009). In natural ecosystems, it is likely that the mycelium-like stage of Prostostelid grow saprophytically, whereas their dispersive amoeboid cells feed phagotrophically on other microorganisms like bacteria and yeasts (Blackwell and Spatafora, 2004). This is an important ecological potential because Prostostelid species can switch their trophic strategy during their life cycle, i.e. from saprotrophy to phagotrophy.

Oomycota, also called water moulds, are omnipresent in aquatic habitats all over the world but most of the aquatic forms seem to thrive better in fresh- than in marine waters (Sigee, 2005; Shaerer *et al.*, 2007). It is well known that many aquatic Oomycota species are saprophytes, playing a major role in the degradation and recycling of nutrients in well-aerated streams, rivers, ponds, lakes, and shallow waters near to the shoreline as well (Sigee, 2005). Their contribution to nutrient cycling in pelagic waters has never been quantified (Shearer *et al.*, 2007). These microorganisms represent a large fraction of the overlooked fungus-like diversity in aquatic ecosystems, with about 138 saprolegnialean species reported from various aquatic habitats all over the world (Shaerer *et al.*, 2007). Oomycota also contains parasitic species, infecting a large variety of hosts (algae, rotifers, crustaceans, nematodes, mosquito larvae, fish), and may play an important role in host population control (Müller *et al.*, 1999; Shearer *et al.*, 2007). During their life cycle, they produce spores with two flagella, which probably have been misidentified as bacterivorous flagellates in previous studies (Lefèvre *et al.*, 2007). From their phenotypic and functional traits, Oomycota are thus very close to chytrids and may share similar functions in the pelagic food web. The question of niche partitioning and competition between these two groups of organisms remains fully open, as does the overall ecological significance of both true fungi and fungus-like microorganisms in pelagic food webs and related biogeochemical processes.

Conclusion

Fungi occupy a variety of ecological niches and act as important decomposers of organic matter and virulent parasites in aquatic ecosystems. They display great diversity in pelagic systems but detailed knowledge of their ecological significance is lacking due to methodological limitations. As parasites, their major impact on phytoplankton may represent a strong forcing factor that controls autotrophic species, thereby affecting carbon transfer from primary producers to higher trophic levels. As decomposers, fungi may increase the release of labile substrates (e.g. for heterotrophic prokaryotes) from more refractory colloidal, detrital or living particles, thereby lessening energy loss from sinking and increasing the retention time of organic matter in the water column. It is likely that fungi and fungus-like microbes are at the centre of multiple interactions in the food web, including

neglected energetic pathways (saprophytism, parasitism, symbiosis, and maybe even phagotrophy). Many different species or phylotypes share similar functions. This highlights interesting ecological questions (e.g. functional redundancy, competition, niche partitioning), and the answers will undoubtedly improve our overall understanding of the structure and functional properties of aquatic ecosystems. We conclude that considering fungi and fungus-like microorganisms in aquatic food webs would dramatically change our conceptual knowledge of the structure and functioning of pelagic systems, although the transition between saprophytism and parasitism and between saprophytism and predation are not always obvious. This is fundamental for the case study of pelagic ecosystems where heterotrophy forms the root of microbial loop and microbial food web dynamics. Establishing this is one of the future challenges for aquatic ecology and environmental scientists.

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OBJECTIFS DE LA THESE ET PRESENTATION DES ETUDES

L'état de l'art établit montre que la prise en compte des champignons présente des implications écologiques significatives pour le fonctionnement des réseaux trophiques microbiens aquatiques. En effet, la prise en considération du saprophytisme et du parasitisme de ces organismes peut, d'une part, améliorer nos connaissances sur les successions planctoniques. D'autre part, cette prise en considération permettrait d'affiner notre connaissance des flux de matière et d'énergie qui transitent par les écosystèmes aquatiques, puisque les champignons peuvent solubiliser la matière organique particulaire et mettre à disposition du zooplancton des éléments nutritifs essentiels pour sa croissance. A nos yeux, il est évident qu'un compartiment biologique entier, porteur de « nouvelles » propriétés métaboliques et trophiques, a été négligé en écologie microbienne aquatique, principalement en raison de difficultés méthodologiques liées à l'étude des Eumycètes dont une majeure partie a été, au cours des études antérieures, simplement confondue avec les protistes phagotrophes (Jobard *et al.*, *in press*; Gachon *et al.*, *in press* ; Rasconi *et al.*, *in press* ; Sime-Ngando *et al.*, *in press*).

Dans ce travail de thèse, nous étudions la diversité, la structure génétique, et l'importance quantitative saisonnière de populations fongiques eumycètes se développant dans les écosystèmes pélagiques lacustres. Ces études ont permis la mise sur pied d'une base de données moléculaires conséquente, qui nous a, par ailleurs, permis de mettre au point des outils pour aborder l'écologie quantitative et fonctionnelle des champignons pélagiques.

Les différentes études menées sont présentées sous forme de manuscrits écrits en anglais, publiés ou en préparation, regroupés au sein de différents chapitres. Suite à la partie introductive (**Chapitre I)**, le **Chapitre II** de la thèse présente une étude moléculaire des populations fongiques présentes dans trois écosystèmes lacustres du Massif Central Français (Pavin, Aydat, Vassivière). Cette étude a été menée afin d'évaluer la diversité et les fonctions putatives associées aux champignons pélagiques. Dans une première partie, une étude globale de la diversité phylogénétique de la communauté fongique est présentée. Une approche de clonage/séquençage du gène codant pour l'ARN ribosomal 18S et pour l'espace intergénique transcrit (*Intergenic Transcript Spacer*, ITS), a été appliquée. Dans une deuxième partie, cette approche de clonage/séquençage et une approche à « haut débit », le pyroséquençage, sont comparées, à partir d'échantillons prélevés dans deux lacs (Pavin, Aydat). De plus, la distribution spatiale de la biodiversité a été abordée dans cette deuxième partie, afin de prendre en compte l'interférence des apports terrigènes dans la dynamique des peuplements fongiques.

Dans le **Chapitre III**, les résultats sur la structure génétique saisonnière des communautés fongiques des trois écosystèmes pilotes sont présentés. Ces résultats proviennent d'une analyse du polymorphisme des longueurs des fragments de restrictions terminaux (*Terminal Restriction Fragment Length Polymorphism*, t-RFLP). Cette étude est complétée par des données quantitatives concernant les trois divisions majeures de champignons (Ascomycota, Basidiomycota et Chytridiomycota) se

développant dans les trois lacs, obtenues par PCR quantitative en temps réel (qPCR) à partir d'amorces de la littérature.

Les Chapitres II et III nous ont permis d'obtenir des séquences que nous avons confrontées aux espèces connues et séquences inconnues répertoriées dans les bases de données, ce qui a permis d'entrevoir les fonctions potentielles associées aux Eumycètes dans les lacs pilotes. De plus, ces travaux ont permis d'établir une base de données de séquences conséquente, ce qui nous a servi de tremplin pour l'élaboration d'outils moléculaires applicables en milieux naturels pour l'étude, notamment quantitative, de groupes de champignons pélagiques lacustres d'intérêt. Ce travail est présenté dans le **Chapitre IV**. Il concerne le développement d'amorces utilisables en qPCR et en Hybridation Fluorescente *in situ* (*Fluorescent In Situ Hybridization*, FISH), deux techniques quantitatives qui se sont révélées adéquates pour l'étude des champignons dans les écosystèmes pélagiques. En l'absence de cultures en laboratoire, une sonde oligonucléotidique ciblant les chytrides a été testée par une approche originale dite du 'Clone-FISH', que nous appliquons, pour la première fois, à la cellule eucaryote. Il s'agit de la mise au point de clones bactériens (*E. coli*) génétiquement modifiés par insertion du gène eucaryote d'intérêt, et ensuite utilisés pour l'optimisation des conditions d'hybridation.

Il est à noter que les illustrations supplémentaires (i.e. Supplementary materials, Tables S, ou Figure S) sont présentées à la fin des manuscrits, suite aux références bibliographiques, selon le modèle standard des revues scientifiques.

Enfin, en plus des différents manuscrits qui font le corps de ce mémoire, nous présentons, en annexes, les publications émanant de collaborations diverses. Ces collaborations scientifiques se sont déroulées dans le cadre de la participation à des expériences internes au laboratoire d'accueil, le LMGE, mais aussi dans le cadre du Master II effectué à l'Institut National de Recherche Agronomique de Dijon. Elles ont trait à l'écologie microbienne, notamment des champignons.

CHAPITRE II

Diversité phylogénétique des Eumycètes dans les milieux pélagiques

Première partie

Molecular characterization of fungal diversity and the associated putative functions in three contrasting freshwater pelagic ecosystems

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Molecular characterization of fungal diversity and the associated putative functions in three contrasting freshwater pelagic ecosystems

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Preliminary, *MS in preparation*

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Short Title: Fungal diversity in pelagic freshwater ecosystems

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Abstract

This study presents an original rDNA PCR survey of pelagic freshwater fungal communities and was designed to unveil the diversity of microscopic fungi in three contrasting lake ecosystems (lakes Pavin, Aydat and Vassivière) located in the French Massif Central. Three clone libraries were constructed from samples collected in the euphotic layers of the lakes, during the spring period of the year 2007. Phylogenetic analysis clustered our sequences into 3 divisions belonging to the Kingdom Eumycota: Ascomycota (40% of the total sequences), Basidiomycota (10%), and Chytridiomycota (50%), when combining the data from the three lakes. These results confirm the importance of Chytridiomycota in the lake pelagial where they are known as parasites, primarily of phytoplankton. Novel Chytridiomycota clade previously recovered in Lake Pavin in 2005 were strengthened by novel sequences recovered in other lakes, which tends to confirm the omnipresence of these fungi in freshwaters. We also unveiled novel fungal lineages belonging to the divisions Ascomycota and Basidiomycota, detected in the 3 sites, excepted for the eutrophic Lake Aydat where Basidiomycota sequences were not detected. Asco- and Basidiomycota are putatively associated with saprophytism in natural ecosystems. Furthermore, using cloning sequencing to analyse fungal diversity has allowed us to reveal the morphological diversity associated to the recovered sequences. These sequences were affiliated to species known to display 3 distinct morphologies: mycelial, yeast-forming, and zoosporic fungi. This suggests that fungi in pelagic ecosystems have the capacity to colonise various ecological niches, and thrive as attached organisms on both living and dead particulate organic matter, and as free-living zoospores as well, known as excellent food sources for zooplankton. Overall, this study provides insight into the diversity and the associated putative functions of fungi, and is the first study on the molecular analysis of the whole fungi in pelagic freshwater ecosystems.

Keywords: Fungi, Eumycota, Microbial diversity, Pelagic ecosystems

Introduction

Fungi are ubiquitous in the environment where they are highly diverse, with a numerical biodiversity estimated at 0.7 to 1.5 millions of taxa on the Earth (Hawksworth 2001, Schmitt and Mueller, 2004), although only 74,000 to 120,000 species has been identified so far (Hawksworth, 2001). In addition, fungi develop diverse life forms inhabiting aquatic and terrestrial environments where they represent one of the major ecological players in biomes, extending from tropical to polar zones (Hawksworth and Mueller, 2005), including the most extreme conditions of life such as deep sea hydrothermal vents (López-Garcia *et al.*, 2006; Burgaud *et al.*, 2009; Le Calvez *et al.*, 2009), hypersaline and anoxic ecosystems (Alexander *et al.*, 2009; Stock *et al.*, 2009). In aquatic ecosystems, fungi are potentially important in various processes, e.g. organic matter decomposition (saprophytism), top-down control of microbial communities, primarily of phytoplankton (parasitism), nutrition of zooplankton which can preferentially feed on free-living fungal zoospores (reviewed in Jobard *et al.*, 2010; Rasconi *et al.*, *in press*).

Fungi have been studied since 1942, particularly in freshwaters, with the discovering of hyphomycetes that often accumulate in running waters (Ingold, 1942), followed by the first works on Chytridiomycetes in freshwater lakes (Sparrow, 1960). The occurrence of fungi in aquatic ecosystems is thus not new but their diversity and functional importance in ecological processes are still as yet about totally ignored, primarily in the pelagic realm. Fungi have been well studied in stream litters (Fabre, 1998; Bärlocher, 2005; Bärlocher *et al.*, 2006; Duarte *et al.*, 2006; Sampaio *et al.*, 2007; Jørgensen and Stepanauskas, 2009), associated with substrates in marine environments (Hagler and Mendonça-Hagler, 1981; Gadanho and Sampaio, 2004; Damare and Raghukumar, 2008; Wang *et al.*, 2008; Gao *et al.*, 2008, 2009), and more recently in benthic ecosystems (Bass *et al.*, 2007; Bhadury *et al.*, 2009; Burgaud *et al.*, 2009). In general, the diversity of fungi in the world aquatic ecosystems is largely derived from those organisms associated to substrata in lotic systems, mangroves and wetlands, where they act as the main decomposers of leaves, wood, detritus and other recalcitrant organic particles (reviewed in Jobard *et al.*, 2010; Wurzbacher *et al.*, 2010). In typical pelagic ecosystems, as known primarily from freshwaters, fungi were detected relatively recently, more or less accidentally, during investigations of the molecular diversity of small eukaryotes (Chen *et al.*, 2008; Diez *et al.*, 2001; Lefèvre *et al.*, 2007, 2008; Lefranc *et al.*, 2005; Lepère *et al.*, 2006, 2007, 2008; Massana *et al.*, 2004; Romari and Vaulot, 2004).

Based on relatively few studies, the role of fungi as phytoplankton parasites in pelagic ecosystems is now about well accepted (Gleason *et al.*, 2008; Ibeling *et al.*, 2004; Kagami *et al.*, 2007a; Rasconi *et al.*, 2009; Sigee, 2005), although the effects of parasitism on food web dynamics and the related biogeochemical cycling (Niquil *et al.*, *in press*) remain scarce (see review by Rasconi *et al.*, in press). In contrast, the other trophic modes of fungi, primarily saprophytism and mutualism, have been totally neglected in the pelagic ecosystems (Jobard *et al.*, 2010; Wurzbacher *et al.*, 2010).

This can partly be explained by the major detection, in the diversity studies, of members of the fungal division Chytriodiomycota (i.e. chytrids), known as typical parasites of phytoplankton (Gromov *et al.*, 1999a, b; Takano *et al.*, 2008; Hoffman *et al.*, 2008). Other fungi detected in lakes, e.g. yeast, are considered associated with allochthonous inputs from saprophytic associations with terrestrial organic matters (Kurtzman and fell, 2004; Lefèvre *et al.*, 2007). This is why investigations of the global diversity of fungal communities thriving in pelagic ecosystems are necessary, as a first step towards understanding their ecological potentials in these environments (Jobard *et al.*, 2010).

In this context, the major bottlenecks are mainly from technical difficulties related to the exploration of the diversity and the quantitative and functional ecology of fungi at the complex natural community level. Much of what is known about aquatic fungi is based on the few cultivable specimens using microscopic techniques, which selectively biases our knowledge of fungal diversity towards a small fraction of natural communities. Indeed, these techniques can only allow the detection of specimens with particular morphological features and often large in size (Jobard *et al.*, 2010). Fungi are known to display different life stages, including unicellular vegetative growing forms such as for yeast (Kutty and Philip, 2008), forms with prospective mycelium with numerous different dispersal forms (Ingold, 1975), or forms with reduced mycelium (i.e. rhizoïd) such as the vegetative chytrid sporanges. The latter also are known to produce flagellated dispersal forms, i.e. propagules (reviewed in Sime-Ngando *et al., in press*), which confer a typical pelagic lifestyle to chytrids (Sparrow, 1960). These complex characteristics involve the use of different culture media or microscopic visualisation techniques. The development and application of molecular techniques that allow direct detection of cultured and uncultured fungal taxa from environmental samples is thus warranted. The microbial diversity can now be assessed from nucleic acids extracted directly from natural environments, for the analysis of conserved genes such as the eukaryotic 18S rDNA. Clone library construction and phylogenetic affiliation of the retrieved sequences provide substantial information on the species present, and the associated putative functions when comparing with the closest known relatives (Lefèvre *et al.*, 2008).

To our knowledge, investigation of the overall diversity of fungal diversity in pelagic ecosystems is lacking in the context of aquatic microbial ecology. This was the aim of the present study conducted in three freshwater ecosystems whose trophic statuses varied but were located in the same geographical region. All the 4 divisions (i.e. Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota) known as true-fungi, which are grouped together in the kingdom of Eumycota, were investigated without a priori in the whole plankton fraction, for evidence of their global molecular diversity and the related putative functions. Although time-consuming, cloning and sequencing approach allows accurate diversity investigation. A limiting factor is the choice of primers to target microorganisms of interest in complex natural environments. In contrast to previous investigations in which universal eukaryotic primers are used to target free-living fungi in the small (often < 5 µm) planktonic size classes (Chen *et al.*, 2008; Diez *et al.*, 2001; Lefèvre *et al.*, 2007, 2008; Lefranc *et al.*,

2005; Lepère *et al.*, 2006, 2007, 2008; Massana *et al.*, 2004; Romari and Vaulot, 2004), we used primers NS1 and ITS4 defined as fungus-specific (White *et al.*, 1990).

Materials and methods

Study sites and sampling. Samples were collected in 3 freshwater lakes, which differed in trophic status and were located in the French Massif Central (Table 1). Lake Pavin (45°29'41'' N, 002°53'12'' E, alt. 1197 m) is an oligo-mesotrophic deep volcanic mountain lake (Zmax = 98 m), characterized by small surface (44 ha) and small drainage basin (50 ha) areas. Lake Vassivière (45°48'48''N, 001°51'15''E, alt. 650 m, Zmax = 34 m) is a large (976 ha) brown-colored humic and mesotrophic lake, moderately acidic, with a large catchment area (23300 ha) surrounding by bait bogs. Lake Aydat (45°39'48'' N, 002°59'04'' E, alt. 825 m) is a small eutrophic lake (Zmax = 15.5 m, surface area = 60.3 ha), with a large catchment area (3000 ha). Samples were collected far from the banks at the point of maximum depth area, during one occasion for each lake, in April for Lake Pavin and in May for Lakes Aydat and Vassivière.

Table 1: Main characteristics of the different lakes sampled.

	Sampling lakes		
	Pavin	Vassivière	Aydat
Trophic state	Oligo-mesotrophic	Mesotrophic	Eutrophic
Altitude (m)	1197	650	825
Area (ha)	44	976	60.3
Catchment area (ha)	50	23300	3000
Maximum depth (m)	98	34	15.5
Volume (m^3)	$22.98x10^6$	$106x10^6$	$4.14x10^6$
Sampling date	2007/04/04	2007/05/03	2007/05/10
Euphotic layer sampled	0-20m	0-4.5m	0-4.5m
Temperature (°C)	5.96	14.02	11.64
DO (mg/l)	10.89	5.67	10.06
pH	6.57	6.12	8.21
Secchi (m)	3	2.9	1.7

For sampling, 20 litres of the whole euphotic layers of the lake (estimated from Secchi depths) were sampled manually using a flexible plastic tube (diameter 4 cm) provided by a rope connecting

the ballasted bottom of the tube with a surface manipulator. The euphotic layers were from the surface to 20 m deep for Lake Pavin, and from the surface to 4.5 m deep for Lakes Aydat and Vassivière. Collected samples were prefiltered on site through 150 µm pore-size filters (to remove most of the metazoan zooplankton), transferred into clean plastic carboys previously rinsed thoroughly with lake water, and transported rapidly to the laboratory for immediate processing. In the laboratory, planktonic microorganisms were collected on 0.6 µm pore-size polycarbonate filters (47 mm diameter) until saturation, using a vacuum pump (pressure < 100 mbar). 300 ml of water from Lake Pavin were necessary to saturate filter and 150 ml for Aydat and Vassivière. Filters were then stored at -80°C until DNA extraction. Water temperature, dissolved oxygen concentration and pH were measured with a multiparameter probe (Amel 345®, Milan, Italy).

Nucleic acid extraction. Total environmental DNA was extracted using the NucleoSpin® Plant DNA extraction Kit (Macherey-Nagel, Düren, Germany), adapted to fungal material in different steps. Filters with planktonic microorganisms were incubated overnight at 30°C with 500µl of a buffer containing 400 Unit of lyticase enzyme (Sigma, NSW, Australia), 0.1 M sorbitol, 100mM Tris–HCl, 100mM EDTA and 14mM β-mercaptoethanol, pH 8.0. in order to digest the fungal chitin wall (Karakousis *et al*., 2006). In a second step, proteinase K (0.1 $mg.ml^{-1}$) and Sodium Dodecyl Sulfate (SDS, 1% final concentration) were added to the buffer and filters were incubated during 1 hour at 37°C for protein digestion and the release of DNA. From here on, all subsequent DNA extraction steps were as recommended by manufacturer's instructions, except for DNA elution which was performed in 2 fold 50 µl of elution buffer instead of 100 µl at one time to improve final DNA concentration. Three filters per sampling date were treated independently to obtain 3 replicats of extracted DNA.

PCR for cloning and sequencing. The complete fungal 18S rRNA gene and Internal Transcript Spacers (ITS) were amplified from environmental genomic DNA extracts using primers NS1 and ITS4, which were designed to target the 4 divisions of true-fungi, i.e. Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (White *et al.*, 1990). The PCR mixture (50 µl) contained 50 ng of environmental DNA as template, 200 µM of each dNTP, 1.5 mM of $MgCl_2$, 0.2 µM of each primer, 2.5 units of Biotaq DNA polymerase (Bioline) and the PCR buffer supplied with the enzyme. Reactions were carried out in an automated thermocycler (PTC 200 MJ Research) with the following cycle: an initial incubation (3 min at 95°C) followed by 35 identical cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec, and extension at 74°C for 4 min, and a final 10 min extension at 74°C. To minimize PCR bias and obtain enough material for cloning, a minimum of 3 individual reactions per extraction replicats were realized. Five µl of PCR products were checked for amplification efficiency and fragments size by electrophoresis in 1% agarose gels, stained with ethidium bromide.

All the PCR products from the separate amplification reactions of each DNA extractions were pooled together, prior to clone library construction, by precipitation at -20°C overnight using 2 volumes of ethanol 100% and 1/10^{e} of salt sodium acetate (3 M, pH5.2). This step allowed to clean the product and to increase DNA concentrations. Purified PCR products were check for quantity and quality by visualisation under UV light on gel after electrophoresis in 1% agarose gels, stained with ethidium bromide.

rDNA genetic library construction. Aliquots of PCR products were cloned using TOPO-TA cloning kit (Invitrogen) following the manufacturer's recommendations. Putative positive colonies were picked and stored for analysis. The presence of the rDNA insert in the colonies was checked by PCR amplification with the M13 universal primers (M13rev (-29) and M13uni (-21)). PCR amplification products of the correct size were digested by the restriction enzyme *Hae*III (New England Biolabs, Hitchin, UK) for 12h at 37°C to obtain restriction fragment length polymorphism (RFLP) profil for each positive clone. The digested products were separated by electrophoresis at 100V for 4 h in a 2.5% agarose gel and visualized under UV light after staining with ethidium bromide. Clones displaying the same RFLP patterns were grouped together and considered to belong to the same Optional Taxonomic Unit (OTU). One representant clone of each OTU was selected for subsequent sequencing. Plasmid containing the insert of interest was extracted with NucleoSpin® plasmid extraction kit (Macherey Nagel), following manufacturer's recommendations.

Sequence analysis. In order to analyse fungal diversity by phylogenetic affiliation, whole 18S rRNA gene of one representative of each OTU was sequenced from plasmid products in an ABI PRISM using BigDye Terminator V3.1 kit and primer NS1 and 1520R (5'-CTGCAGGTTCACCTAC-3'), following manufacturer's recommendations. All sequences obtained were matched against their nearest neighbour using BLAST (Altschul *et al.*, 1990) for a first approximate of phylogenetic affiliation, and in order to select representative taxa for phylogenetic tree construction. The sequences were screened for potential chimeric structures using Bellerophon server (*http://foo.maths.uq.edu.au/~huber/bellerophon.pl*) and aligned with representatives of each match retrieved from the online database, using the automatic alignment tool of the ARB package (*http://www/arb-home.de/*). The resulting alignments were checked and corrected manually in the view of the secondary structure of the rRNA molecule, and areas of ambiguous alignment were excluded using Bioedit software (*http://www.mbio.ncsu.edu/BioEdit/bioedit.html*). Phylogenetic trees highlighted the main groups for which sequence alignments were analysed both by Neighbor Joining method using the PHYLIP package, and by the Bayesian method using MrBayes3 software (*http://morphbank.ebc.uu.se/mrbayes/authors.php*) (Ronquist and Huelsenbeck, 2003). Because the topologies of the phylogenetic trees generated by the two methods were fairly similar, only the tree generated by the Bayesian method is shown. Nucleotide sequences obtained in our study have been

deposited in GenBank under accession numbers XXXXX (to be done before submitting the final manuscript).

Results

Overview of the retrieved diversity. From 3 to 9 PCRs were necessary depending on the sample to collect enough PCR products for subsequent analysis. Indeed, for Lake Vassivière whose waters contain humic substances, amplifications were often inhibited, reducing amplification efficiency, and more PCR reactions were necessary. Because of the amplification of the total ITS and the size variability of this zone within fungi (Ranjard *et al.*, 2001), the size of the insert varied between PCR products. The 3 libraries constructed comprised totals of 94, 94, and 188 clones for Lakes Pavin, Aydat, and Vassivière, among which 43, 37 and 46 OTUs were identified based on the RFLP profiles, respectively. One representative of each of these OTUs was sequenced and blasts were conducted to select representative sequences for phylogenetic tree construction. Sequences sharing each other more than 99% identity were grouped into OTUs. Due to the presence of large amount of non-specific environmental DNA in our samples and the weak specificity of probes used, a sizeable part of our clones belonged to non targeted microorganisms (i.e Chlorophyceae algae). These sequences were excluded from the phylogenetic analysis. It was more difficult to obtain fungal sequences in Lake Vassivière than in the other lakes, and we have finally obtained 20, 16 and 15 fungal sequences from Pavin, Aydat and Vassivière respectively (Figure 1).

Phylogenetic diversity of fungi. Trees constructed clustered fungal sequences together with those selected into 3 major divisions (Ascomycota, Basidiomycota, and Chytridiomycota) (Figure 1). Chytridiomycota and Ascomycota dominated the retrieved sequences in all lakes, averaging 50% and 40% of the total numbers of sequences, respectively, but with differences between lakes (Table S1). Chytridiomycota represented 26%, 84% and 47% of sequences retrieved in Lakes Pavin, Aydat and Vassivière, whereas Ascomycota represented 62%, 16% and 33%, respectively (Figure 2A). Basidiomycota sequences were only retrieved in Lakes Pavin and Vassivière, where they represented 12 and 20% of the total fungal diversity, respectively (Figure 2A).

Chytridiomycota (Chytrids) sequences were highly diverse with 24 sequences matching with 6 clades (Figure 1). Twenty-one chytrid sequences, including 4 in Lake Pavin (Pa2007F6, Pa2007A2, Pa2007G2, Pa2007G8), 1 in Lake Vassivière (Va2007BF11), and 5 in Lake Aydat (Ay2007B1, Ay2007H2, Ay2007B11, Ay2007E5, Ay2007B12), matched with Rhizophydiales sequences previously detected in lake (Lefèvre *et al.*, 2008) and soil (Freeman *et al.*, 2009) ecosystems.

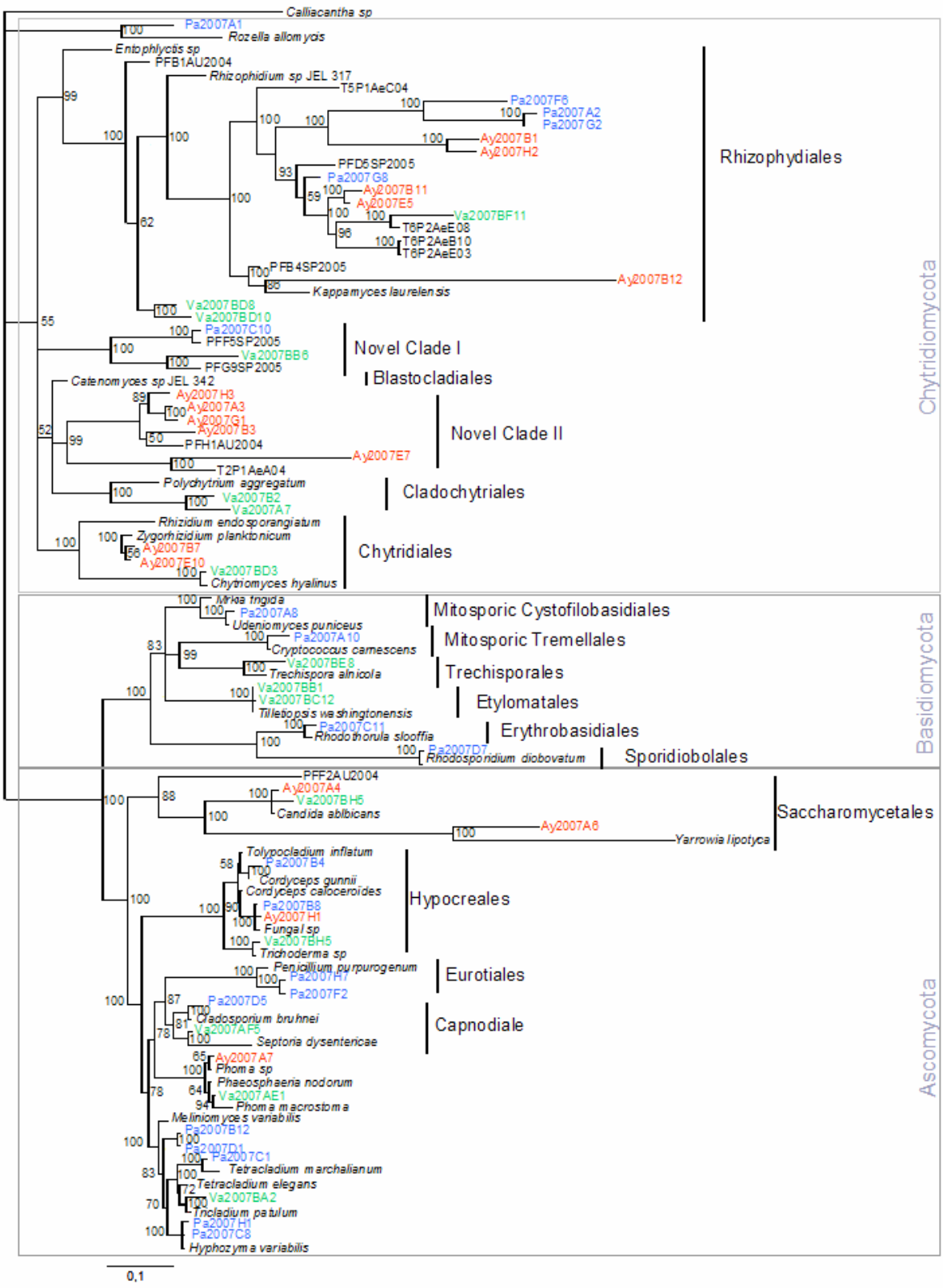

Figure 1: Bayesian phylogenetic tree of 18S rRNA gene obtained from Lakes Pavin, Vassivière and Aydat. Tree was inferred from an alignment of 98 sequences. The origine of phylotypes are indicated by the 2 first letters of the name Pa, Ay, and Va for Lakes Pavin, Aydat and Vassivière, respectively. The numbers at the nodes correspond to Bayesian Posterior Probalilities given as percentages.

In addition, 2 sequences in Lake Aydat (Ay2007B7, AY2007E10) and 3 in Lake Vassivière (Va2007B2, Va2007A7, Va2007BD3) were closed to the orders of Chytridiales and Cladochytriales. These sequences were phylogenetically related to the genera *Rhizophydium* and *Zygorhizidium* or to the species *Chytriomyces hyalinus* and *Polychytrium aggregatum*.

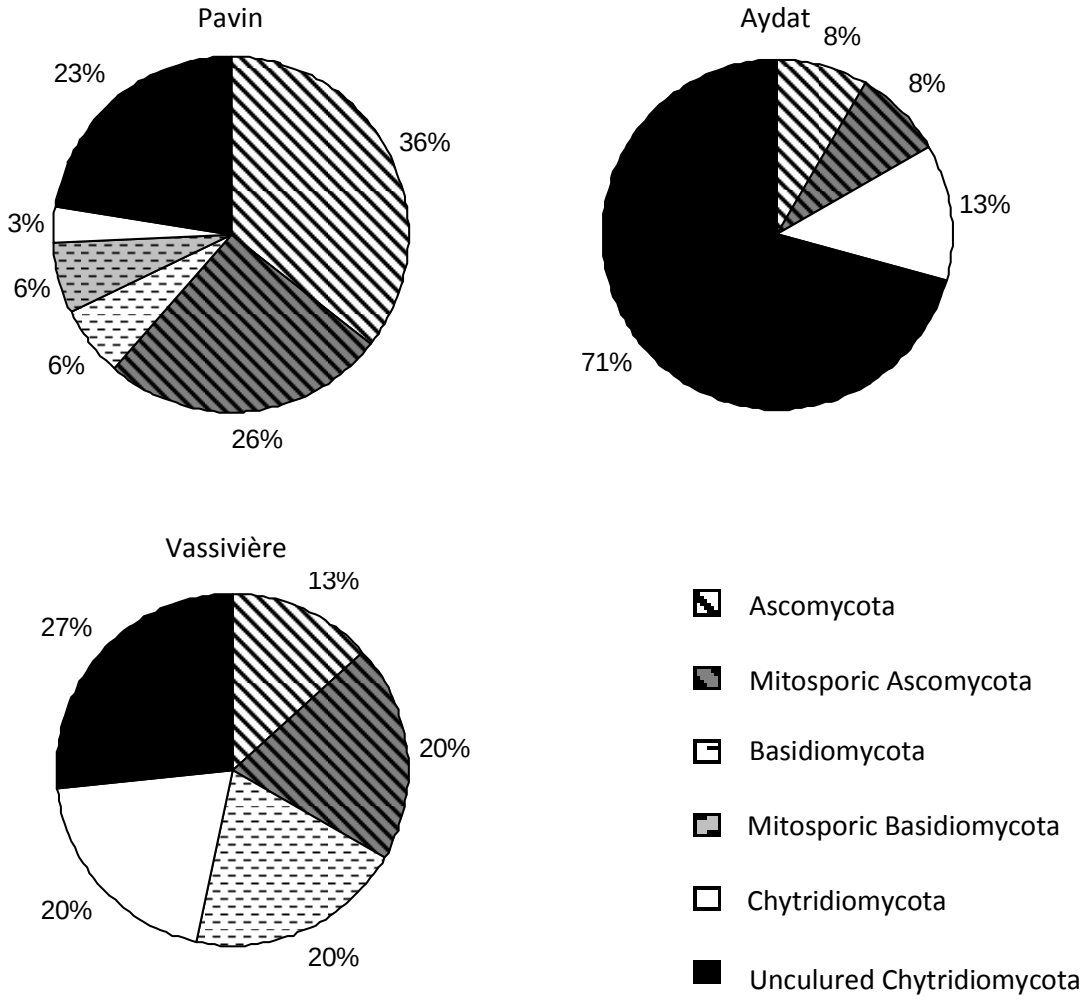

Figure 2A: Relative abundance of OTUs within the phylogenetic groups in the 3 libraries from Lakes Pavin, Vassivière and Aydat. Grouping are based on BLAST search and on a preliminary distance eukaryotic tree. The numerical abundances represented the percentage of total OTUs for each taxon.

A total of 7 sequences affiliated to Basidiomycota were recovered in Lakes Pavin (Pa2007A8, Pa2007A10, Pa2007C11¸ Pa2007D1) and Vassivière (Va2007BE8, Va2007BB1, Va2007BC12). All these sequences were strongly affiliated to yeast species excepted for 1 sequence retrieved in Vassivière (i.e. Va2007BE8) (Figure 1). The latter sequence was close to the microbial fungus *Trechispora alnicola*, a pathogenic fungus of the plant *Poa pratensis* but also known as saprophytic fungus (Wilkinson, 1987).

Ascomycota sequences were mainly represented by Hypocreales, Saccharomycetales, Eurotiales, Capnodiales and mitosporic fungi (Table S1 and Figure 1). Saccharomycetales were detected only in Lakes Aydat and Vassiviere. This latter order contains fungal species in the form of yeast and the 3 sequences recovered (i.e. Ay2007A4, Ay2007A6, and Va2007BH5) were closely related to the species *Candida albicans* and *Yarrowia lipotyca* (Figure 1). Amongst the order of Hypocreales, several sequences, closely affiliated to 2 species of *Cordyceps* (*C. Gunnii* and *C. Caloceroïdes*), were detected in Lakes Pavin and Aydat (Table S1 and Figure 1). To our knowledge, this is the first time that this fungal genus is detected in pelagic ecosystems. The other taxa (Eurotiales, Capnodiales) were represented by few sequences in Pavin and Vassivière. The latter Ascomycota taxon grouped together 8 sequences closed to mitosporic Ascomycota species and retrieved from the 3 lakes.

Finally, a large proportion of sequences belonged to uncultured fungi was recovered (Table S1 and Figure 2A). The number of these sequences was higher in Lake Aydat (17) than in Lakes Pavin (7) and Vassivière (4). Most of these sequences were indeed affiliated to environmental sequences PFH1AU2004, PFF5SP2005, PFG9SP2005 and T2P1AeA04 detected in previous surveys (Figure 1), where they were considered members of the Chytridiomycota division but were not clearly affiliated to known species (Lefèvre *et al.*, 2007, 2008; Freeman *et al.*, 2009). Moreover these novel Chytridiomycota clades (namely “novel clade I”, “novel clade II”) defined by sequences PFF5SP2005 and PFH1AU2004 respectively, and previously recovered in Lake Pavin in 2005 and 2004 (Lefèvre *et al.*, 2007, 2008), were strengthened by novel sequences recovered in Lakes Pavin and Vassivière for “novel clade I”, and in Lake Aydat for “novel clade II”.

Inference of morphological diversity. Under the assumption that the retrieved sequences belong to species that harbour the same morphology as their closest known relatives, cloning-sequencing method can also be used to infer the morphological features of environmental uncultured microorganisms. This could inform on the potential ecological niches of fungal sequences retrieved. Based on this assumption, fungal sequences detected in Lakes Pavin, Aydat, and Vassivière were close to known fungal species with three major putative morphological forms (myceliale-form, yeast-form, and zoosporic-form). Among all these forms, zoosporic and mycelial forming ‘species’ dominated in Pavin and Vassivière, whereas zoosporic morphologies dominated in Aydat. Indeed, the number of sequences which were close to zoosporic fungi increased from Pavin to Aydat, contrasting with the other forms (Figure 2B).

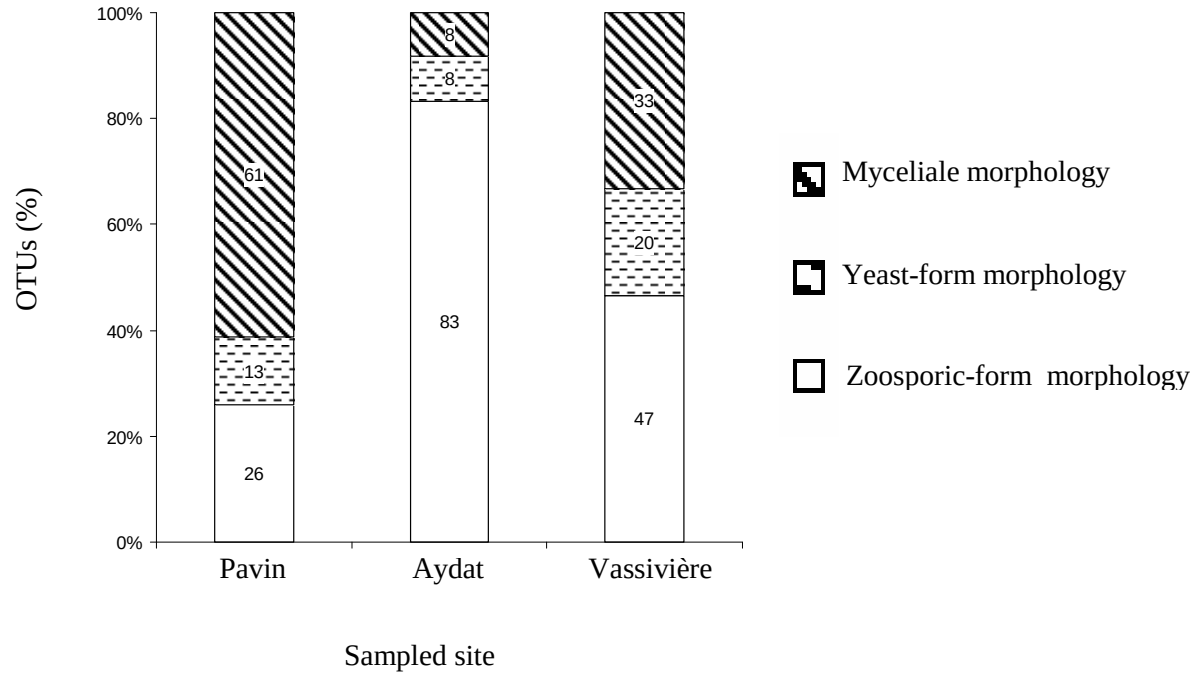

Figure 2B: Relative abundances of putative fungal morphologies in total OTUs retrieved from the pelagic areas of Lakes Pavin, Aydat and Vassivière during spring 2007. For each lake, the relative abundances of OTUs correspond to different morphological features displayed. The numerical abundances of these OTUs are included in the bars.

Discussion

Methodological considerations. For the first time, a molecular biology based strategy targeting small subunit rRNA genes was used to characterize the fungal diversity at the complex pelagic community level of three freshwater lake ecosystems. Previous studies favor the whole eukaryotic diversity investigations in pelagic ecosystems (Chen *et al.*, 2008; Diez *et al.*, 2001; Lefèvre *et al.*, 2007, 2008; Lefranc *et al.*, 2005; Lepère *et al.*, 2006, 2007, 2008; Massana *et al.*, 2004; Romari and Vaulot, 2004), or the fungal diversity in benthic marine ecosystems (Burgaud *et al.*, 2009, Bhadury *et al.*, 2009; Bass *et al.*, 2007), demonstrating that community DNA extraction, PCR, cloning, RFLP analysis and sequencing are good strategies in investigating fungal community composition and diversity (Landeweert *et al.*, 2003; O'Brien *et al.*, 2005). The cultural methods are tedious, time-consuming and target only part of the total fungi. Moreover, it is hard to cultivate individual species like Chytridiomycota species, which possess a limited vegetative growth and are known to growth slowly on culture medium. We used 18S rDNA genes which have been recognized as powerful markers for identifying fungi and resolving taxonomic position at different levels (Bruns *et al.*, 1991). Indeed, 18S rDNA sequences have highly conserved regions separated by more variable sequences which allow efficient amplification by PCR and postamplification separation and detection (Nikolcheva *et al.*, 2003). Primers used were defined to target all true fungal divisions of interest

(White *et al.*, 1990), on the assumption that all these divisions thrive in pelagic freshwater ecosystems (Jobard *et al.*, 2010). Using molecular method was well-adapted for our objectives and although not specific to fungi, the primers in our study used allowed detection of various fungi in the 3 lakes investigated.

Fungal diversity. During our investigation, we detected various fungal sequences in Ascomycota, Basidiomycota, and Chytridiomycota divisions whereas sequences belonged to the ancient phylum Zygomycota (Hibbett *et al.*, 2007) were not retrieved in any of these lakes. This corroborates with the current knowledge on Zygomycota in freshwater habitats, mainly restricted to the group of Trichomycetes which are obligate symbionts in the gut of arthropods (Lichtwardt *et al.*, 2003). The pelagic niche for Zygomycota is most likely the gut of zooplankton, most of which were discarded in our sampling strategy when prefiltering through 150 µm pore-size nylon filter. In addition, the cryptic presence of Zygomycota in the gut of zooplankton was also another physical barrier for an efficient extraction of their DNA, when present in our samples. According to Lichtwardt *et al.* (2003), trichomycetes can be collected in virtually all regions of the world and represent a major source of undiscovered fungal diversity (Hawksworth and Rossman, 1997), but they typically remain undetected until their hosts are dissected.

Fungal diversity was harder to investigate in Lake Vassivière than in the 2 others lakes because sampling effort was higher (188 clones retrieved) but fungal sequences obtained were weak (15) in Vassivière. Yet, this lake was the one with the largest catchment area and thus likely receives more allochthonous inputs (compared to the other lakes investigated), which is favourable to the import of soil fungi. This finding contrasts with previous hypothesis that most of the fungi in pelagic ecosystems come from terrestrial inputs from catchment area (Lefèvre *et al.*, 2007; Kurtzman and Fell, 2004), which may only be partially true, at least in specific environments. In addition, we retrieve very few sequences from typical lotic fungal species (see below). One of the reasons for the low fungal diversity in Vassivière is likely the enhanced presence of humic substances that provide the brown-colored waters. This is inhibitory for light penetration and the relative importance of autotrophic microorganisms, compared to heterotrophs (Carrias, 1996). Clearly, the inhibition of primary producers in colored humic lakes such as Vassivière could limit the availability of substrates such as dead and living organic particles which are excellent resources for most of the fungi known as saprophytes an/or parasites (Jobard *et al.*, 2010). This can affect both the population dynamics and the diversity of fungi.

Sequences of fungi recovered during our surveys were slightly different from species colonising leaves in streams, recovered with traditional and molecular approaches in previous surveys (Bärlocher *et al.*, 2006; Nikolcheva *et al.*, 2003; Seena *et al.*, 2008). Our sampling strategy was different compared to these surveys which collected attached fungi on substrates, and was designed to detect the dominant species in the pelagial of the investigated lakes, including those potentially imported from

rivers or from the littoral zone. Except for 2 genera, currently reported in streams, *Tetracladium* and *Tricladium*, (Ingold, 1975; Nikolcheva and Bärlocher, 2002, 2005), no other typical lotic fungal species were detected during our study. Fungal species colonising pelagic ecosystems thus seem to be different from those colonising other aquatic ecosystems like rivers. Moreover, most clades detected in our surveys were represented by sequences retrieved from at least 2 different lakes. Despite the difference between our sampling sites, the recovered fungal communities were similar, indicating that the species of fungi present in pelagic ecosystems are adapted to the pelagic realm. These observations strengthen our previous rejection of the hypothesis that fungal species in pelagic ecosystems are mainly contaminations from allochthonous inputs (Jobard *et al.*, 2010).

Among our sequences, we found that Chytridiomycota and Ascomycota phyla were dominant compared to Basidiomycota which were, in addition, represented only in 2 (Vassivière and Pavin) of the 3 lakes. This is a major difference between freshwaters where chytrids are important members of the diversity of small eukaryotes (Lefèvre *et al.*, 2007, 2008; Lefranc *et al.*, 2005; Lepère *et al.*, 2006, 2008), and marine waters where fungal detection is very common but restricted to Basidiomycota and Ascomycota (Alexander *et al.*, 2009; Gao *et al.*, 2009), primarily to yeasts as the most common category (Kutty and Philip, 2008). Recently, a survey of fungal diversity in the Hawaian (USA) pelagic coastal waters revealed a majority of Basidiomycota phyla (Gao *et al.*, 2009). However, it is important to mention that most of the eukaryotic diversity surveys in freshwater lakes concerns a fraction of the plankton (often smaller than 20 µm) (Lefèvre *et al.*, 2007, 2008; Lefranc *et al.*, 2006; Lepère *et al.*, 2006, 2008), and most of the Ascomycota and Basidiomycota species cannot be sampled this way, due to their large size (Jobard *et al.*, 2010). Our survey was done with the total community of microbial fungi (from 0.6 to 150 µm size). Although our data still corroborate the fact that chytrids were important members of fungi, we also found a sizeable number of Ascomycota and Basidiomycota sequences, compared to the previous studies in Lakes Pavin and Aydat. In these investigations, none or less than 10% of sequences belonged to clades others than Chytridiomycota (Lefranc *et al.*, 2005; Lefèvre *et al.*, 2007, 2008), whereas we found 74% and 16% of sequences affiliated to Ascomycota and/or Basidiomycota in Lakes Pavin and Aydat, respectively.

Chytridiomycota. The most diverse fungal division recovered in the 3 environments investigated was that of Chytridiomycota. Our sequences mostly fell within the Rhizophydiale order. These sequences were phylogenetically related to the genera *Rhizophydium* and *Zygorhizidium* well known as host-specific parasites of phytoplankton, such as diatoms, in freshwater lakes (Bruning *et al.*, 1992; Gromov *et al.*, 1999 a, b; Holfeld, 2000; Kudoh and Takahashi, 1990; Rasconi *et al.*, 2009; Van Donk and Brunning, 1992). They are known to reduce phytoplankton blooms and to play a crucial role in phytoplankton successions and, as a consequence, in freshwater food-web dynamics (Kagami *et al.*, 2004, 2007a ; Rasconi *et al.*, 2009). The higher diversity of chytrids was detected in the eutrophic Lake Aydat, likely because of the higher phytoplanktonic host abundance compared to the other lakes

investigated (Rasconi, 2010). Moreover, novel Chytridiomycota clades previously recovered in Lake Pavin in 2004 and 2005 were strengthened by novel sequences recovered in other lake (Lake Vassivière), confirming the presence of these fungi in various ecosystems but also the putative importance of unknown functions associated with these novel clades.

In the other hand, we have detected a sequence affiliated to *Rozella allomycis*. This genus is known as an hyperparasite of other fungal species, such as *Zygorhizidium*, *Allomyces*, (*R. allomycis*) and *Rhizophlyctis* (*R. Rhizophlyctii)* (James *et al.*, 2006; Canter and Lund, 1969; Held, 1972). Sequences affiliated to *Zygorhizidium* sp have been detected in Lake Aydat while sequences of *Rhizophlytis sp* has also already been detected during previous surveys in Lake Pavin (Lefèvre *et al.*, 2007, 2008). Fungal parasitism in freshwater systems is certainly more complex that previously though, with the occurrence of fungal hyperparasites. This may result in a complex trophic network with several stages of fungal parasites and hyperparasites. In addition, detection of parasitic fungal species in different freshwater lakes is in accordance with current ecological investigations implying parasites, and primarily fungal parasites, as a particular component of pelagic food webs (Lafferty *et al.*, 2006, 2008; Lefèvre *et al.*, 2008; Lepère *et al.*, 2008; Rasconi *et al.*, 2009).

Sequences affiliated to *Chytriomyces hyalinus* and *Polychytrium aggregatum* were detected only in Lake Vassivière, which was in accordance with James and colleagues (2006) who described *Polychytrium aggregatum* as colonising acidic lakes and bogs. These species are mainly saprophytic, growing on the exuvia of may flies, fragments of chitin, pollens or snakeskin in freshwaters, and on submerged leaves in soil or boggy areas (Schoenlein-Crusius and Milanez, 1998; Sigee, 2005; James *et al.*, 2006). Previous observations have already visualized fungi and particularly *Polychytrium aggregatum* and *Chytriomyces hyalinus* on dead zooplankton carcasses (Czeczuga *et al.*, 2002; Tang *et al.*, 2006). These chytrids certainly act in the decomposition of refractory polymers such as chitin or cellulose that represent a great pool of recalcitrant organic matter originated from zooplankton and phytoplankton dead microorganisms in pelagic areas. The humic conditions of Lake Vassivière are certainly a good environment for the development of these fungi.

Basidiomycota. Basidiomycota fungi were the division with the minimum of sequences recovered in Pavin and Vassivière. In Aydat, no sequence was encountered probably because Basidiomycota species seem to prefer low nutrient and acidic environments (López-Archilla *et al.*, 2004; Gadanho *et al.*, 2006; Russo *et al.*, 2008) such as Lakes Pavin (oligomesotrophic) or Vassivière (mesotrophic lake) which displays slightly acidic waters. Amongst Basidiomycota, sequences affiliated to *Rhodosporidium* genus were recovered during our investigation and was also found in Lake Pavin in previous surveys but during autumn (Lefèvre *et al.*, 2007), strengthening the hypothesis that fungi encountered in lakes are present several times during a year and not only randomly. *Rhodosporidium* are yeast form fungi and 3 other sequences were affiliated to basidimycota yeast. The occurrences of yeast in freshwater pelagic ecosystems are obvious. Yeasts have been previously described as mainly

actor of the decomposition of leaf litter in streams (Sampaio *et al.*, 2007) but waterborne yeasts are often known in marine and extreme environments (Hagler and Mendonça-Hagler, 1981; Gadanho and Sampaio, 2004; De Garcia *et al.*, 2007; Butinar *et al.*, 2005; Bass *et al.*, 2007; Gao *et al.*, 2009).

Ascomycota. In the environment, Ascomycota and mitosporic Ascomycota can form symbiotic, parasitic or saprophytic associations. In aquatic ecosystems, they are often associated with marine macroalgae (Kohlmeyer and Demoulin, 1981) or stream litter (Nikolcheva *et al.*, 2003; Nikolcheva and Bärlocher, 2004). In our study, one of the most surprising results was the abundance and the diversity of sequences belonging to a novel clade closed to *Cordyceps* sp detected in 2 lakes (Pavin and Aydat). Species of *Cordyceps* are mainly known as parasites, including pathogens of arthropods such as the genera *Cordyceps*, *Hypocrella*, and *Torrubiella*, or parasites of other fungi (Sasaki *et al.*, 2008; Sung *et al.*, 2007). Recently, sequences of *Cordyceps* have been recovered in aquatic environment in conjunction with marine nematodes leading the authors to assume that *Cordyceps* species are associated with the nematode tissue or are part of their gut contents (Bhadury *et al.*, 2009). The detection of sequences closed to *Cordyceps* in 2 of our lakes suggests that these fungi should be present in pelagic ecosystems where they could act as parasites of animals. Moreover, according to Bhadury and colleagues (2009), *Verticillium* sp, which is a fungal species close to *Cordyceps* sp (Hypocreales, Hypocreomycetidae), has been reported from *Daphnia.* Few investigations on fungal parasitism on zooplankton are available, most of which being focused on chytrids parasites, primarily of phytoplankton (Johnson *et al.*, 2006, 2009). Planktonic infections by Ascomycota or by Basidiomycota are thus obvious, and the related research perspectives are new for ecological studies in aquatic sciences.

Morphological diversity. Sequences of fungi detected in Lakes Pavin, Aydat and Vassivière displayed 3 putative morphological forms; myceliale-form, yeast-form and zoosporic-form. Chytridiomycota belong to fungi called zoosporic fungi because they exhibit during their life cycle a dispersal stage with a flagellated stage (zoospore). Zoospores have small size (2-5 µm) and may be important in freshwater aquatic ecosystems as nutritional resources for zooplankton species such as *Daphnia* sp. (Kagami *et al.*, 2007b). As chytrids are known to be parasite of various phytoplankton species (Rasconi *et al.*, 2009), their implication in carbon transfer from primary producers to zooplankton and higher trophic levels are important (Kagami *et al.*, 2007a, b; Lefèvre *et al.*, 2008).

Sequences affiliated to species with yeast-form mainly belong to Basidiomycota but some sequences were closed to Ascomycota yeast-form species (*C. Albicans and Y. Lipotyca*). Presence of yeasts in pelagic ecosystems leads to assume that they could be in competition with heterotrophic bacteria for organic matter mineralization. Indeed, yeasts may colonise the same niches as bacteria, as it has been previously known in sediment aggregates (Damare and Raghukumar, 2008; Raghukumar 2006). Nevertheless, yeast fungi size is larger than bacteria and so may be not grazed by bacterivorous

flagellates but can be ingested by zooplankton species such as *Daphnia* species (Lampert and Sommer, 2007). The presence of yeast form fungi in pelagic area may reduce the importance of carbon transfer from organic matter to bacterivorous flagellates through bacteria. This may increase carbon transfer from organic matter to zooplankton, thereby reducing the importance or microbial loop in some environments.

Detection of sequences close to fungal species that form mycelial vegetative (filamentous fungi) during part of their life was sizeable. It was not the first time and we wonder if the presence of these fungi in pelagic ecosystems were only random. The presence of mycelial fungi in pelagic areas may be cryptic in macroaggregates as it has been recently reported (Raghukumar, 2006; Damare and Raghukumar, 2008; Jobard *et al.*, 2010). In the other hand, sequences retrieved from our environments and closed to fungal species that display mycelial growth and yeast-form may come from catchment area associated with allochthonous inputs and saprophytic associations with plant- or animal-derived organic compounds (Kurtzman and Fell, 2004). Nevertheless, these sequences were important in Lake Pavin, which is surprising because Lake Pavin receives few allochthonous inputs (absence of inflow river, small catchment surface area).

Conclusions

During our study, we unveiled fungal diversity matching with 3 large divisions (Ascomycota, Basidiomycota and Chytridiomycota). Most of the sequences retrieved in the 3 lakes were affiliated to known fungal species and allowed us to enhance our knowledge about fungi in pelagic ecosystems. The main putative functions appeared to be parasitism and saprophytism but more investigations are needed to enhance or not this observation, since a sizeable part of true fungi community was probably discarded (Zygomycota division). Fungal community in pelagic ecosystems seem to be characteristic of these systems, with few allochthonous contaminations as previously thought. Moreover, sequences recovered were affiliated to fungi which display 3 different main morphologies. This unique specificity in life forms makes fungi competitive since they can putatively develop in various niches in pelagic ecosystems. Filamentous fungi have been well studied on aquatic woody litter in rivers or in marine ecosystems like mangrove forests. While it was not surprisingly to detect zoosporic and yeast in pelagic areas, detection of fungi displaying filamentous morphology raises various questions. Indeed, their presence in pelagic ecosystems of small size species like yeast or zoosporic fungi has been well accepted (Raghukumar *et al.*, 2006) but filamentous fungi are totally neglected. Hypotheses to explain the presence of filamentous fungi in pelagic are various. They could, for example, really be active and colonize detritus (Tang *et al.*, 2006; Jobard *et al.*, 2010), and produce spores or conidia which can also serve as nutritive resources for zooplankton.

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Supplemental materials

Table S1: Number of clones belonging to each OTU in clones libraries from Lakes Pavin, Aydat and Vassivière. BLAST were conducted to obtain the closest relative (Accession number) and the % of similarity of the representative clones sequenced. The number of sequence sharing more than 99% identity and grouped together into the same OTU is given for each lake.

OTU id	Taxon		Closest relative (accession no.)	Percentage of similarity (%)	No of clones in library of Lake :		
	Phylum	Order			Pav	Ayd	Vas
Pa2007B8	Ascomycota	Hypocreales	*Cordyceps caloceroides* (AY245654)	99	7		
Ay2007H1			*Cordyceps caloceroides* (AY245654)	99		1	
Pa2007B4			*Cordyceps gunnii (DQ838790)*	99	1		
Va2007BH5			*Trichoderma sp. (FJ026619)*	99			1
Pa2007D5		Capnodiales	*Cladosporium bruhnei* (AY251096)	99	1		
Va2007AF5			*Septoria dysentericae* (GU214699)	99			1
Pa2007F2		Eurotiales	*Penicillium purpurogenum* (AF245268)	98	2		
Pa2007H7			*Penicillium purpurogenum* (AF245268)	98			
Ay2007A6		Saccharomycetales	*Yarrowia sp* (FJ153124)	95		1	
Pa2007C1		mitosporic Ascomycota	*Tetracladium marchalianum* (AF388580)	98	1		
Pa2007C8			*Hyphozyma variabilis (AJ496240)*	99	3		
Pa2007B12			*Tetrachaetum elegans* (AY357280)	98	2		
Pa2007H1			*Hyphozyma variabilis (AJ496240)*	99	1		
Ay2007A7			*Phoma sp* (AB252869)	99		1	
Va2007AE1			*Phoma macrostoma (AB454217)*	99			1
Pa2007D1			*Tetrachaetum elegans (AY357280)*	99	1		
Va2007BA2			*Tetrachaetum elegans (AY357280)*	99			1
Ay2007A4		mitosporic Saccharomycetales	*Candida albicans* (M60302)	99		1	
Va2007BH5			*Candida albicans (M60302)*	99			1
Pa2007C11	Basidiomycota	Erythrobasidiales	*Rhodotorula slooffiae (AB126653)*	99	1		
Pa2007D7		Sporidiobolales	*Rhodosporidium diobovatum* (AB073271)	99	1		
Va2007BB1		Entylomatales	*Tilletiopsis washingtonensis* (AJ271382)	98			1
Va2007BC12			*Tilletiopsis washingtonensis* (AJ271382)	98			1
Va2007BE8		Trechisporales	*Trechispora alnicola* (AY657012)	98			1
Pa2007A10		mitosporic Tremellales	*Cryptococcus carnescens* (AB085798)	98	1		
Pa2007A8		mitosporic Cystofilobasidiales	*Udeniomyces puniceus* (DQ836006)	99	1		
Pa2007A1	Chytridiomycota	Spizellomycetales	*Rozella allomycis* (AY635838.1)	94	1		
Ay2007E10		Chytridiales	*Zygorhizidium planktonicum* (FJ799984)	99		2	
Ay2007B7			*Zygorhizidium planktonicum* (FJ799984)	99		1	
Va2007BD3		Chytridiales	*Chytriomyces hyalinus* (DQ536487)	99			1
Va2007AB2		Cladochytriales	*Polychytrium aggregatum* (AY601711)	94			1
Va2007BA7			*Polychytrium aggregatum* (AY601711)	94			1

Pa2007A2	Uncultured Chytridiomycota	clone T5P1AeC04 (GQ995431)	91	1		
Pa2007C10		clone PFF5SP2005 (EU162641)	99	2		
Pa2007F6		clone PFB4SP2005 (EU162636)	91	1		
Pa2007G2		clone T5P1AeC04 (GQ995431)	94	1		
Pa2007G8		clone PFD5SP2005 (EU162640)	98	2		
Ay2007B1		clone PFB4SP2005 (EU162636.1)	92		4	
Ay2007B11		clone T5P2AeB10 (GQ995435)	98		1	
Ay2007B12		clone T6P2AeE03 (GQ995436)	91		2	
Ay2007E5		PFD5SP2005 EU162640.1	97		1	
Ay2007E7		clone T2P1AeA04 (GQ995409)	94		1	
Ay2007H2		clone PFB4SP2005 (EU162636)	92		1	
Va2007BB6		clone PFG9SP2005 (EU162638)	97			1
Va2007BF11		clone T6P2AeE08 (GQ995426)	97			1
Ay2007A3		clone PFH1AU2004 (DQ244009)	96		2	
Ay2007B3		clone PFH1AU2004 (DQ244009)	96		2	
Ay2007H3		clone PFH1AU2004 (DQ244009)	97		1	
Va2007BD8		PFB1AU2004 (DQ244005)	96			1
Ay2007G1		clone PFH1AU2004 (DQ244009)	96		2	
Va2007BD10		PFB1AU2004 (DQ244005)	96			1

Deuxième partie

Diversity and spatial variability of freshwater fungal communities studied by rDNA cloning/sequencing and pyrosequencing

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Diversity and spatial variability of freshwater fungal communities studied by rDNA cloning/sequencing and pyrosequencing

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Preliminary, *MS near submission*

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Short Title: Fungal diversity in freshwater lakes

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Abstract

The diversity and spatial variability of eukaryotes and particularly of fungi were studied in two French aquatic ecosystems: the oligomesotrophic Lake Pavin, and the eutrophic Lake Aydat. Water samples were collected along a transect from the banks to the centre of each lake, and the biodiversity analyzed using two approaches: the classical approach consisting of cloning/sequencing of the 18S, ITS1, 5.8S, ITS2 and partial 28S region using fungus designed primers, and the 454-pyrosequencing of 18S rRNA hypervariable V2, V3, and V5 regions using two primer sets (one universal for eukaryotes, and one for fungi). The fungus primers were specifically designed to amplify the major phyla of fungi. The classical approach yielded 146 (Pavin) and 143 (Aydat) sequences, corresponding to 46 and 63 operational taxonomic units (OTUs), respectively. Lake Pavin showed a higher diversity of fungi compared to Lake Aydat. Fungi represented half of the OTUs identified in Lake Pavin, and 30% in Lake Aydat. The Ascomycota (and particularly the group of *Saccharomycetales*) were mostly located in the coastal areas of the lakes, whereas the Chytridiomycota were found throughout Lake Pavin and mostly in the central pelagic area of Lake Aydat. The 454-pyrosequence approach yielded 42,064 (Pavin) and 61,371 (Aydat) reads, allowing to qualitatively and quantitatively determine species diversity. Depending on the set of primer used, from 12 to 15% and 9 to 19% reads, in the Lakes Pavin and Aydat respectively, were assigned to fungi. Chytridiomycota was the major group of fungi in the two lakes, with the OTU sequences PA2009E6 (in Lake Pavin) and AY2009C4 (in Lake Aydat), corresponding to new clades, being dominant. The chytrid species corresponding to OTU AY2009C4 displayed an above 33 fold overrepresentation in the centre compared to the coastal areas of Lake Aydat. Besides fungi, both approaches revealed other major eukaryote groups (Katablepharidophyta, Cryptophyta, Alveolata, Choanoflagellida, Stramenopile, and Viridiplantae), with the highest diversity in the central areas of lakes. One of the major findings of our study was that the two lakes displayed contrasting spatial distribution (a homogenous one in Lake Pavin, and a heterogeneous one in Lake Aydat) that was related to their peculiarities. This study demonstrates (i) the importance of using two complementary methods to improve the diversity of microbial eukaryotes, including both rare and abundant species, and (ii) provides evidence, for the first time, that most of the fungi unveiled, primarily chytrids, together with the related putative functions discussed, could not strictly originate from allochthonous contaminations. These are crucial steps for the understanding of the dynamics and ecology of pelagic systems. In this context, matching sequences to organisms represents one of the major challenges over the years to come.

Keywords: Fungi, Microbial diversity, Spatial diversity, Aquatic ecosystems, Cloning/sequencing, 454-Pyrosequencing

Introduction

Fungi represent one of the last frontiers of the undiscovered biodiversity and trophic compartment that challenge the pelagic microbial ecology today. They have a crucial role in ecosystem functioning or are crucial for the maintenance of ecological balance since they influence many environmental processes such as nutrient cycling in the food web. From studies in soil systems, fungi are known to be vital in recycling nutrients through the metabolism of complex organic materials. In pelagic ecosystems it does not seem unreasonable to postulate that their activity may also be of importance in many ecosystem processes (Treseder, 2005; Watling, 2005). However, our ecological knowledge of fungi in pelagic ecosystems is scant. The Kingdom Fungi was previously subdivided into four main phyla : Basidiomycota, Ascomycota, Zygomycota and Chytridiomycota (Alexopoulos *et al.*, 1996). But the two latter groups do not emerge as monophyletic groups in recent phylogenetic analyses (James *et al.*, 2000; James *et al.*, 2006b). Today, seven phyla are recognized in the phylogenetic classification of Fungi (Hibbett *et al.*, 2007). The Chytridiomycota is retained in a restricted sense, with Neocallimastigomycota and Blastocladiomycota representing segregate phyla of flagellated Fungi. Taxa traditionally placed in Zygomycota are distributed among Glomeromycota and several subphyla *incertae sedis*, including Mucoromycotina, Entomophtoromycotina, Kickxellomycotina and Zoopagomycotina (Hibbett *et al.*, 2007). The number of fungi present on earth was estimated to 1.5 million species, from which only 74,000 to 120,000 have so far been identified (Hawksworth, 2001). This suggests a large underestimation of the diversity of this eukaryotic group, because of methodological constraints, but also partly because of the undersampling of pelagic environments, which represent the largest reservoir for life on earth.

In pelagic systems, several culturing assays allowed the isolation of ascomycetous or basidiomycetous yeasts. Freshwater Ascomycetes occur in both lentic (Hyde and Goh, 1998a; Hyde and Goh, 1999) and lotic habitats (Hyde and Goh, 1998b; Hyde and Goh, 1998c; Hyde and Goh, 1999) and more than 500 species have been reported so far (Shearer, 2001; Shearer *et al.*, 2007). Although ascomycetous fungi, together with bacteria, are the most efficient decomposers of organic matter, their functional importance in lakes remains unclear. Basidiomycetous yeasts were retrieved in marine habitats, particularly in deep waters (Munn, 2004), and from high-altitude cold lakes (Libkind *et al.*, 2009). Basidiomycota are often adapted to extremely cold environments, including ice, snow, and sea ice (Jobard *et al.*, 2010).

Chytridiomycota, i.e. chytrids, are considered as typical pelagic organisms. This contrasts with the other phyla of Fungi that are often thought to be present in pelagic systems mainly as allochthonous terrestrial inputs (Wong *et al.*, 1998). There are about 800 species of chytrids (Powell, 1993); many of them have been characterized as parasites of freshwater algae (Gromov *et al.*, 1999a; Gromov *et al.*, 1999b; Ibelings *et al.*, 2004). One chytrid species (*Batrachochytrium dendrobatidis*) was also described as the causative agent of die-offs and population declines of amphibian species

(Berger *et al.*, 1998). Previous studies have unveiled a large reservoir of unsuspected fungal diversity in lakes (Holfeld, 1998; Hyde and Goh, 1998a; Richards *et al.*, 2005; Van Donk and Ringelberg, 1983), primarily of chytrids (Lefèvre *et al.*, 2007; Lefèvre *et al.*, 2008), in which many species are clearly adapted to aquatic life, as their propagules (i.e. uniflagellated zoospores) have specialized aquatic dispersal abilities. Pelagic freshwater species of chytrids mostly fall within the Rhizophydiales order, that contains many host-specific parasitic fungi of various phytoplankton species primarily diatoms. These organisms are known to often reduce phytoplankton blooms and to play a crucial role in phytoplankton succession (Kagami *et al.*, 2004). Several studies have demonstrated epidemic occurrences of chytrids infecting phytoplankton in lakes (Holfeld, 1998; Kudoh, 1990; Van Donk and Ringelberg, 1983).

In the oligomesotrophic Lake Pavin (France), the occurrence of these parasitic fungi was confirmed from direct microscopic observation of their sporangia (i.e. the infective stage) fixed on various classes of phytoplankton, including diatoms, chlorophytes, and cyanobacteria (Rasconi *et al.*, 2009). Chytrids can be characterized by their zoospores that typically have a single, posteriorly-directed flagellum (James *et al.*, 2000), corresponding to one of the major forms observed directly via epifluorescence microscopy. However, because chytrid life forms are not distinguishable from some small flagellated protists, they may have been miscounted as bacterivorous flagellates in previous studies (Carrias *et al.*, 1996; Carrias and Amblard, 1998). Carrias *et al.* (1996) reported that, the dominant small unidentified heterotrophic flagellates present in Lake Pavin during a seasonal study, were not able to ingest bacteria. Large grazing-resistant diatoms, primarily *Aulacoseira italica* and *Asterionella formosa*, are important components of Lake Pavin, where they typically account for 50 to 98% of the total phytoplankton biomass production during the spring bloom in the whole water column (Amblard and Bourdier, 1990). These diatoms are well known as preferential hosts for chytrids in Lake Pavin. Similarly, filamentous cyanobacterial blooms, often dominated by the highly chytrid-sensible species *Anabaena flosaquae*, are characteristics of autumn in the eutrophic Lake Aydat, France (Rasconi *et al.*, 2009). The activity of fungi is suspected to be an important factor in Lake Pavin, Lake Aydat, and other pelagic inland and coastal ecosystems, which regulates phytoplankton community structure and species successions. It has also been shown that the small flagellated zoospores produced by parasitic chytrids are efficiently grazed by zooplankton such as *Daphnia* (Kagami *et al.*, 2004). Above considerations suggest that chytrids are important microbial players in pelagic ecosystems, where they are involved both in the release of dissolved organic matter from parasitic epidemics or saprotrophy, and in the transfer of matter and energy to higher trophic levels (Lefèvre *et al.*, 2008).

However, still little is known about the diversity of fungi in pelagic ecosystems. Most of the related studies are relatively recent (Richards *et al.*, 2005), including those in Lakes Aydat and Pavin (Lefèvre *et al.*, 2007; Lefèvre *et al.*, 2008; Lefranc *et al.*, 2005). The latter studies were performed initially with the main aim to examine the phylogenetic composition of picoeukaryotes (i.e. < 5 µm),

and ended up with the unveiling of an important diversity of fungi. For example, in Lakes Aydat and Pavin, studies on the diversity of small heterotrophic eukaryotes based on small subunit (SSU) rRNA gene sequences have shown that Alveolata, Cercozoa, Stramenopile and Fungi represented the major part of the total diversity (Lefèvre *et al.*, 2007; Lefèvre *et al.*, 2008). However, the molecular approach used in these studies, i.e. PCR amplification and cloning of the entire SSU rDNA gene followed by analysis of the positive clones by restriction fragment length polymorphism (RFLP), and sequencing of the selected clones or operational taxonomic units (OTUs), represents both a costly and tedious method, providing only a limited analysis of the diversity associated with the number of clones analyzed. Therefore, we have initiated a complementary approach represented by the pyrosequencing of hypervariable regions of the SSU rRNA gene from selected samples. This method, already successfully used for the exploration of deep sea community composition (Huse *et al.*, 2008; Sogin *et al.*, 2006), is cost effective and offers around three orders of magnitude larger SSU rRNA sequencing, compared to classical approaches. In addition, it eliminates cloning bias and, allows the maximizing of the number of organisms sampled in a run, while minimizing the chimera formation.

The aim of this work was: (i) to complete earlier studies on the diversity of microbial eukaryotes, focusing on fungi, in the whole plankton fraction (size class 0.6-150 µm); (ii) to determine the spatial variability in the diversity of pelagic fungi; and (iii) to understand the effects of allochthonous inland inputs with samples collected along transects from the banks to the centre of Lakes Pavin and Aydat (French Massif Central). To our knowledge, this study is the first exploration of the spatial dynamics in the diversity of pelagic fungi using a combination of two complementary approaches. The complete sequencing of the SSU rDNA gene, considered as the classical approach, attributed to each sequence a precise taxonomical position and identified potential new clades, based on the use of fungus designed primers. The second approach, consisting of pyrosequencing a large number of SSU rRNA hypervariable tags, provided a general overview of the eukaryote and fungus diversity, with the use of a universal eukaryote and a fungus designed set of primers. This second method, referred to as the 454-pyrosequencing approach, unveiled rare fungal species, and gave quantitative information about the OTUs identified by the classical approach. In this study, we have unveiled all the major and minor typical pelagic fungus species and their within-lake internal variability pattern, which can be considered as the first step towards the understanding of population dynamics and functional role of fungi in pelagic food webs.

Materials and methods

Sampling procedure and DNA extraction

Lake Pavin (45°30'N, 2°53"W) is a meromictic, dimictic, oligo-mesotrophic lake situated in the Massif Central of France. It is a deep volcanic mountain lake (Z_{max} = 92 m), characterized by a

permanently anoxic monimolimnion from 60 m depth downwards. This site offers a unique environment with low human influences, characterized by a small surface area (44 ha), about equal to the drainage basin area (50 ha), with no river inflow (Figure 1). Lake Aydat (45°39'N, 2°59"W) is a small (Z_{max} = 15m, surface area = 60 ha) dimictic, eutrophic lake, also located in the Massif Central of France. It was formed when a lava flow dammed the small river Veyre. Compared to the surface of the lake, the catchment area (2.5 x 10^3 ha) is very large and contains intensive agricultural lands (Figure 1).

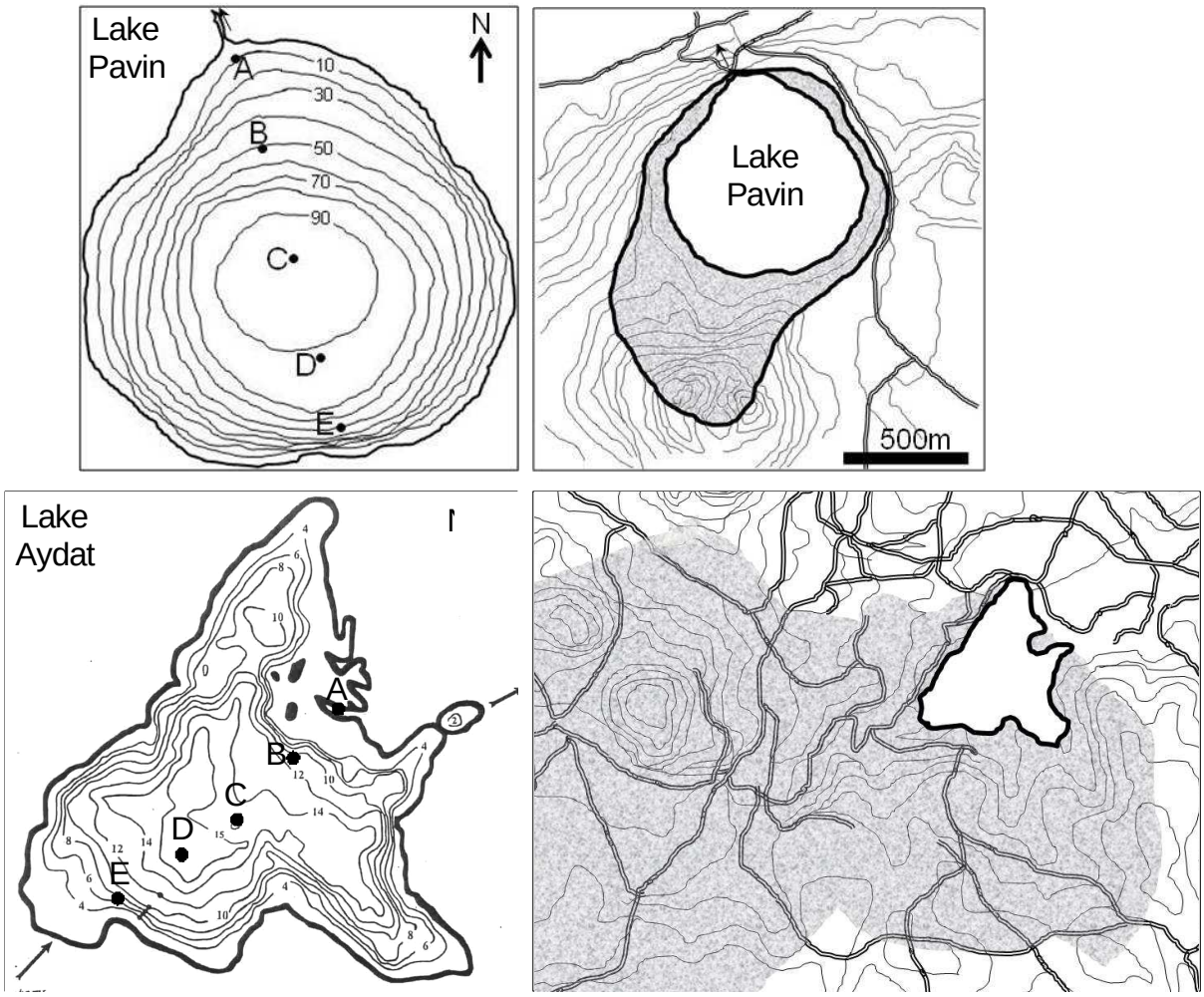


Figure 1: Bathymetric maps (left) and water catchment area (right) from Lake Pavin and Lake Aydat. The bathymetric maps of Lake Pavin (from Delbecque, 1898) and Lake Aydat (from Rabette and Lair, 1999) displayed the location of the five sampling stations: two littoral (A and E), one central (C) and two intermediaries (B and D). On the bathymetric maps, the arrows show the flux of water and the numbers indicate the depth in meter. The two water catchment areas of Lake Pavin (from Lair, 1978) and Lake Aydat (this study) showed the larger size of Lake Aydat drainage basin (grey shade) compared to Lake Pavin. Arrows indicate the flux of water, lines show the ground topography and roads are indicated by double lines.

For each of the two lakes, five samples were collected along a transect across the lake, including two littoral (points A and E), two intermediary (points B and D), and one central sites (point C) located in the deepest area of the lake (Figure 1). For Lake Pavin, station A is located near the lake

outlet, while on the other side of the lake, station E is influenced by the catchment effects (Figure 1). For Lake Aydat, the stations A and B were located near the Veyre River outlet and the within-lake islands, while station E was located next to the lake 'estuary' (Figure 1). Samples were collected during the end of the thermal stratification period, in July 16th 2008 for Lake Pavin and in July 21st 2008 for Lake Aydat. In Lake Pavin, the temperature of surface waters fluctuated between 17.5 and 17.8^{o}C, and pH values were at 8.33, 8.30, 8.27, 7.94, and 8.28 for the sampling points A, B, C, D, and E, respectively. In Lake Aydat, the temperature fluctuated between 19.6 and 19.5^{o}C and pH values were at 9.70, 9.74, 9.81, 9.85 and 9.83 for sampling points A, B, C, D, and E, respectively.

The whole euphotic water column (determined from Secchi disk measurements, $Z_{eu} = 1.7 \times Z_s$, (Reynolds, 1984)) was collected at each sampling point using a flexible plastic tube (diameter 4 cm) provided by a rope connecting the ballasted bottom of the tube with a surface manipulator. The euphotic depths were at 4.5 m and 20 m in Aydat and Pavin, respectively. Samples were immediately prefiltered trough 150 µm pore size nylon filter (i.e. to eliminate larger metazoans), poured into clean recipients previously washed with the lake water, and returned back to the laboratory for immediate processing. Subsamples of 150 and 300 ml from Aydat and Pavin, respectively, were filtered onto 0.6 µm pore-size polycarbonate filters (47 mm diameter) using a vacuum pump (pressure < 100 mbar) to collect all planktonic microorganisms. The filters were stored frozen at -80°C until DNA extraction.

DNA from each sampling point was extracted using the kit NucleoSpin® Plant DNA extraction Kit (Macherey-Nagel, Düren, Germany) adapted for fungal material. The initial step aimed at digesting fungal chitin wall. Filters were then incubated overnight at 30°C with 400 Units of lyticase enzyme (Sigma, NSW, Australia) in a 500 µl sorbitol based buffer (Karakousis *et al.*, 2006). Then sodium dodecyl sulfate (final concentration 1%) and proteinase K (final concentration, 0.1 mg.ml^{-1}) were added and incubated one hour at 37°C. All subsequent DNA extraction steps were conducted following the manufacturer's instructions.

The classical approach: PCR, cloning/sequencing, and phylogenetic analyses

The primers sense NS1 (GTAGTCATATGCTTGTCTC) and antisense ITS4 (TTCCTCCGCTTATTGATATGC) known to preferentially amplify fungal DNA (White *et al.*, 1990), were used to amplify the complete 18S rDNA, ITS1 (Internal Transcribed Spacer region), 5.8S rDNA, ITS2, and partial 28S rDNA region (Supplementary Figure S1). Only the 18S rDNA gene sequences were used in the phylogenetic analyses. Polymerase chain reactions were carried out in 50 µl volume according to standard conditions for *Taq* DNA polymerase (Bioline) with 50 ng of environmental DNA as template. After the denaturation step at 95 °C for 3 min, 34 cycles of amplification were performed with a GeneAmp PCR System Apparatus (Applied Biosystems) as follows: 30 s at 95 °C, 30 s at 59 °C, and 3 min at 72 °C. The reaction was completed with an extension step at 72 °C for 10 min. The products were separated on agarose gel electrophoresis in order to confirm the presence of

bands of the expected size (around 2,600 bp). PCR products were precipitated by sodium acetate/ethanol and titrated for cloning. For each sampling point, a genetic library was constructed. An aliquot of PCR product was cloned using the TOPO-TA cloning kit (Invitrogen) following the manufacturer's recommendations. The presence of the insert in the colonies was checked by PCR amplification using M13 forward and reverse universal primers. PCR products of the correct size were analyzed by restriction fragment length polymorphism (RFLP) using the restriction enzyme *Hae*III (Invitrogen). Clones showing the same RFLP patterns were grouped into a single OTU and one representative of each OTUs was sequenced from minipreparations of plasmid DNA using the Nucleospin kit (Macherey-Nagel). A total of 146 and 143 SSU rDNA gene sequences for Lake Pavin and Lake Aydat, respectively, were obtained and aligned using Clustalw (Thompson *et al.*, 1994). Sequences sharing each other more than 99% identity were grouped into OTUs. A total of 46 OTUs in Lake Pavin and 63 OTUs in Lake Aydat were identified. One representative sequence of each OTU was submitted to a BLAST search (Altschul *et al.*, 1990) on the non redundant nucleotide database (NCBI) for an approximate phylogenetic affiliation and in order to select representative taxa for tree constructions. The resulting alignments including representative OTU sequences and reference sequences were corrected manually and regions of ambiguous alignment were removed using the Bioedit software (*http://www.mbio.ncsu.edu/BioEdit/bioedit.html*). Phylogenetic trees were built both using the Neighbor Joining (NJ) method from the PHYLIP package (*http://evolution.genetics.washington.edu/phylip.html*) and the Bayesian method from MrBayes3 software (*http://mrbayes.csit.fsu.edu/index.php*) (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Topologies of the phylogenetic trees generated by the two methods were similar; therefore, only the tree generated by the Neighbor Joining (NJ) method will be shown with bootstrap values obtained from 1000 replicates.

The 454-pyrosequencing approach

DNAs extracted from the sampling points A and C of Lake Pavin, and C and E of Lake Aydat were used in a 454-pyrosequencing approach. Two set of primers were used in this study (Supplementary Figure S1). The first set of primers, 18S-82F (GAAACTGCGAATGGCTC) and Ek-516r (GGAGGGCAAGTCTGGT) (Casamayor *et al.*, 2002; Lepère *et al.*, 2006; López-Garcia *et al.*, 2003), was designed to amplify a 480 bp region containing the complete V2 and V3 domains of all eukaryote SSU rDNA gene. We will further refer to this universal set of primers as the eukaryote primers. The second set, composed of primers nu-SSU-0817-5' (TTAGCATGGAATAATRRAATAGGA) and nu-SSU-1196-3' (TCTGGACCTGGTGAGTTTCC), was previously designed by Borneman and Hartin (2000) to preferentially amplify an around 400 bp region containing the V4 (partial) and V5 (complete) variable domains of the SSU rDNA gene from all four major phyla of fungi. We will refer to this set of primers as the fungus primers. A 10 bp tag

specific to each sample, a 4 bp TCAG key, and a 26 bp adapter for the GsFLX technology, were added to the sequences of the primers. The pyrosequencing project was performed by the Genoscreen company (Institut Pasteur de Lille, France). The library and the 454 titanium (Roche) pyrosequencing run were prepared following the manufacturer's recommendations. A total of 103,435 sequences with 53,856 and 49,579 sequences from the eukaryote and fungus primers, respectively, were obtained. The reads were classified according to the tag sequence corresponding to each of the four samples of interest. With the eukaryote primers, 12,133 and 11,386 sequences from the sampling stations A and C in Lake Pavin, and 15,259 and 15,078 sequences from the sampling stations C and E in Lake Aydat, were respectively obtained. Using the fungus primers, a total of 8,018 and 10,527 sequences from the sampling stations A and C in Lake Pavin, and 16,672 and 14,362 sequences from the sampling stations C and E in Lake Aydat, were respectively obtained. Primers, tag and key fragments were subsequently removed before analyzing the sequences. Globally, around two third of the reads showed a length above 200 bp and one third of the reads had a length above 400 bp.

For identification, the resulting sequences were compared to the Silva SSU rRNA database (*http://www.arb-silva.de/*) release 102 (updated on February 15, 2010) comprising 1,246,462 18S SSU rRNA sequences (including 134,351 eukaryotic sequences) using the BLASTN software (Altschul *et al.*, 1990). BLAST results (with 10^{-5} E-value threshold) were visualized using the metagenomic software MEGAN (Huson *et al.*, 2007). This software allows exploring the taxonomic content of the samples based on the NCBI taxonomy using the option "import BLASTN". The program uses several thresholds to generate sequence-taxon matches. The « min-score » filter, corresponding to a bit score cut-off value, was set at 35. The « top-percent » filter used to retain hits, whose scores lie within a given percentage of the highest bit score, was set at ten. The « min-support core » filter, used to set a threshold for the minimum number of sequences that must be assigned to a taxon was set to three. These stringent parameters result in the "safe" assignment of many sequences to lower branch (with less precision) of the taxonomic tree. Distribution of the sequences was schematically represented by trees and pie diagrams.

***Comparison between the classical and the* 454-pyrosequencing approaches**

The pyrosequences from the SSU hypervariable regions were compared to the full-length 18S rDNA gene sequences obtained with the classical approach. This comparison allowed the identification of the dominant species, the determination of the within-lake spatial variation of species, and an evaluation of the correlation and complementarities between the two approaches. The 18S rDNA gene pyrosequences were submitted to a Blast analysis (with 10^{-23} E-value threshold for Blastn) against databases containing the full-length 18S rDNA gene sequences from each lakes obtained by the classical approach. This analysis was done independently for both lakes. Several criteria were applied to select relevant blast results, including a minimum query sequence of 60 bp and a minimum of 99% similarity over 80% of the sequence length with match starting, at least, from the third

nucleotide of the query sequence. Blast results were manually checked in order to remove duplicates, i.e. a 454-pyrosequence read that was matching several times with the same full-length 18S rDNA gene sequence (i.e. from the classical approach) on different sites. However, 18S rDNA gene pyrosequences could still match with several full-length 18S rDNA gene sequences from the classical approach, when their lengths are too short for a precise identification.

Rarefaction curves

The quality of the sampling effort was assessed through the calculation of rarefaction curves, i.e. the number of operational taxonomic units (OTUs) versus the number of clones (Hughes *et al.*, 2001). Rarefaction curves for sequences obtained from the classical and the 454-pyrosequencing approaches were done independently but following the same procedure. For the classical approach, all the obtained 18S rDNA gene sequences were considered for analysis. For the 454-pyrosequencing approach, only sequences with a length over 400 bp were considered in order to include in the analysis the entire variable V2 (obtained with the eukaryote primers) and V5 (obtained with the fungus primers) regions of the 18S rDNA gene sequence. With the eukaryote primers, a total of 3,786 and 3,684 sequences for stations A and C in Lake Pavin, and 4,182 and 5,141 sequences for stations C and E in Lake Aydat, were respectively analyzed. With the fungus primers, a total of 2,948 and 3,691 sequences for stations A and C in Lake Pavin, and 5,935 and 5,444 sequences for stations C and E in Lake Aydat, respectively, were analyzed. The sequences considered for both approaches were aligned using the program MUSCLE (Edgar, 2004) (with parameters –diags and –maxiters 2) and were manually corrected using the Bioedit software to remove ambiguous terminal region of the alignment. The resulting alignment was used as input for the Mothur program (Schloss *et al.*, 2009), with a cut-off value set to 0.01 and 0.03 (i.e. OTUs with differences that do not exceed 1 and 3%) for the analysis.

Accession numbers

Nucleotide sequences obtained from the complete 18S, ITS1, 5.8S, ITS2, and partial 28S sequences were deposited in Genbank under the accession numbers HQ191282-HQ191427 for Lake Pavin samples and HQ219333- HQ219474 for Lake Aydat samples. The 454-pyrosequences were deposited in Genbank-SRA under the accession numbers XXXXXX (to be done before submitting the final manuscript).

Results

Overview of the retrieved diversity

The “classical approach” yielded a total of 146 (Lake Pavin) and 143 (Lake Aydat) complete SSU rDNA gene sequences, considered an overview of the biodiversity in the pilot lakes at the sampling

time. After clustering sequences sharing more than 99% identity, the total number of OTUs increased from 46 OTUs in Lake Pavin (Figure 2) to 63 OTUs in Lake Aydat (Figure 3).

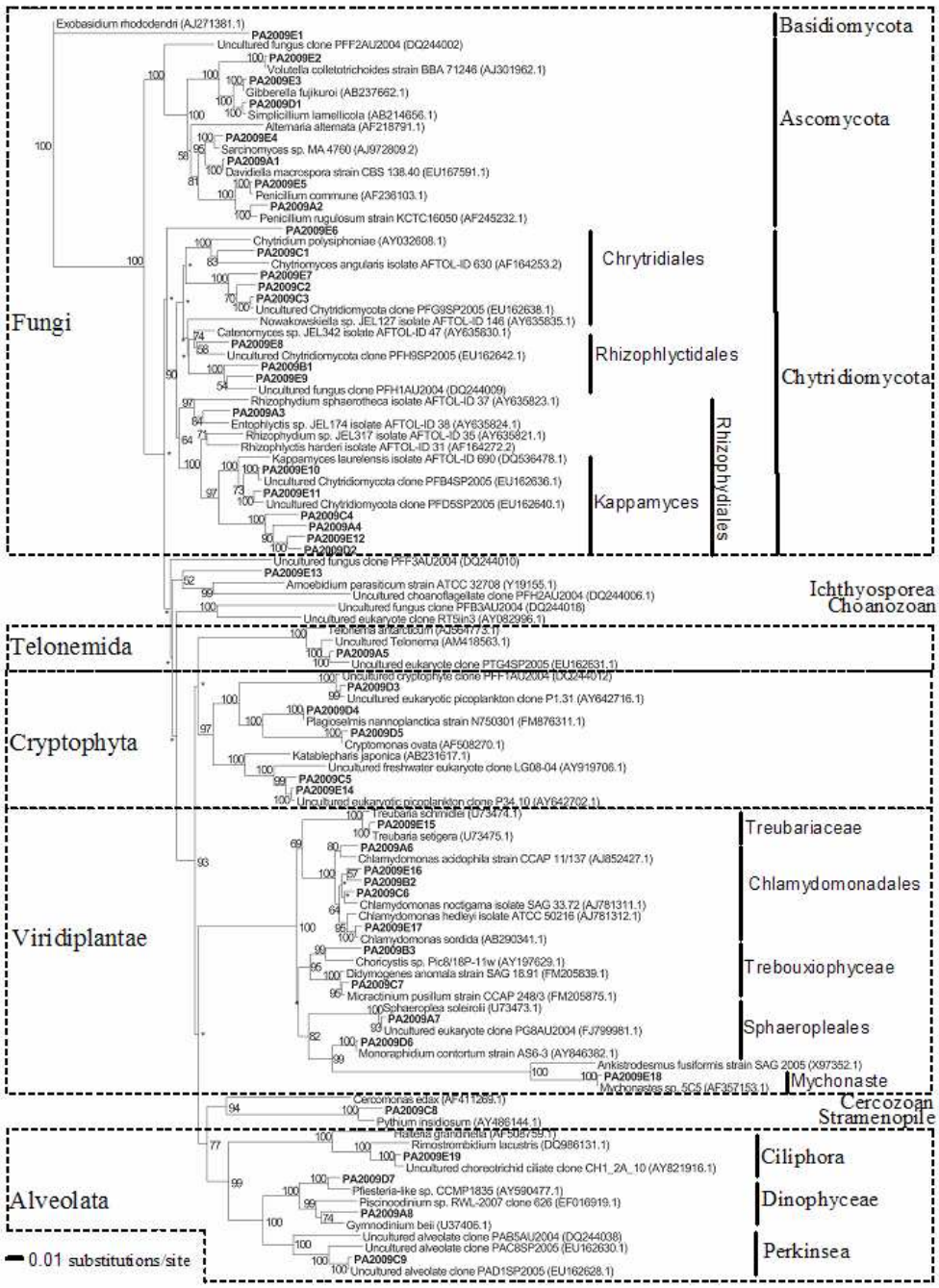


Figure 2: Phylogenetic tree based on 18S rRNA gene sequences obtained from the Lake Pavin. The unrooted phylogenetic tree displayed 46 operational taxonomic units (OTUs) inferred from 146 18S rRNA gene sequences sharing less than 99% sequence identity. Representative from each OTU were submitted to a BLAST search (Altschul *et al.*, 1990) to determine the closest homologous 18S rRNA gene sequences from the non redundant nucleotide database (NCBI). The OTUs and reference sequences were aligned using Clustalw (Thompson *et al.*, 1994), and the construction of the phylogenetic tree was done using the Neighbor Joining (NJ) method from the PHYLIP package (*http://evolution.genetics.washington.edu/phylip.html*). Numbers indicate bootstrap values generated from 1000 replicates (an asterisk indicates bootstrap value below 50). The boxes (dash lines) showed the different phyla, where each taxonomical subdivisions are delimitated by vertical lines.

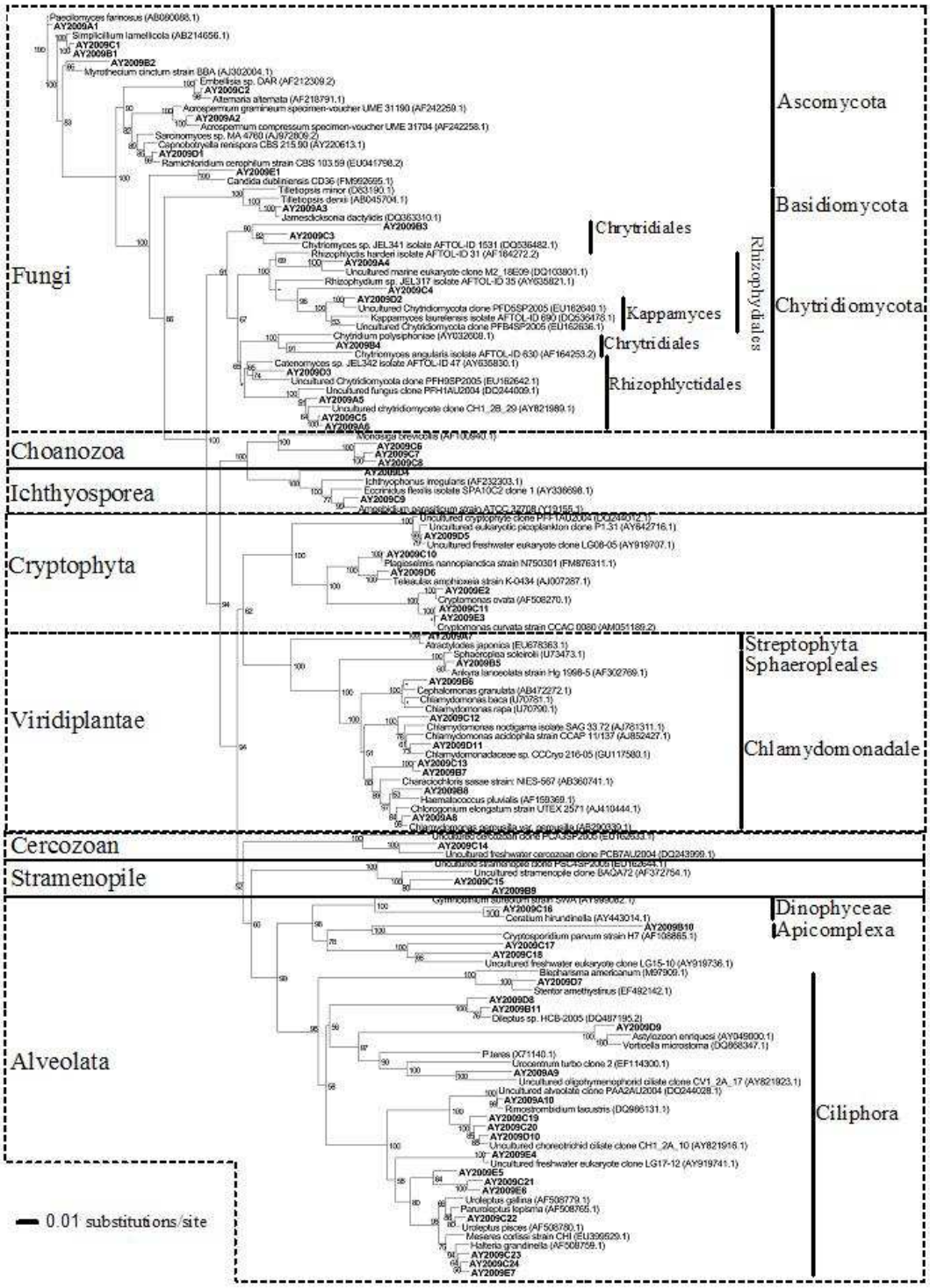


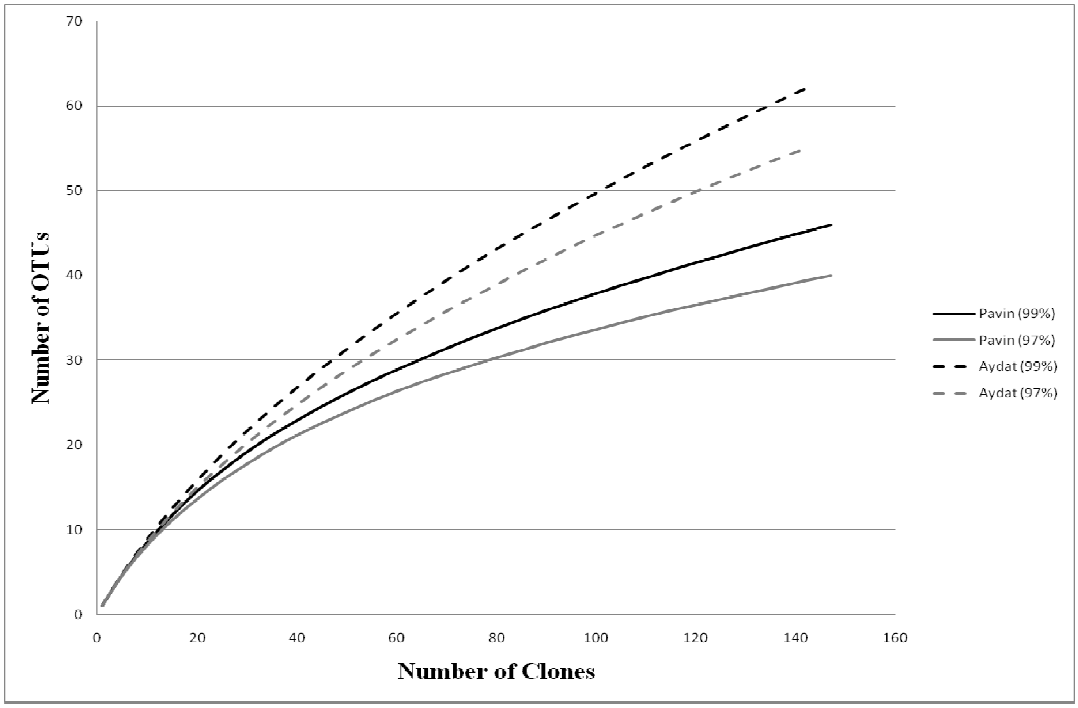
Figure 3: Phylogenetic tree based on 18S rRNA gene sequences obtained from the Lake Aydat.
The unrooted phylogenetic tree, similarly to Figure 2, displayed 63 OTUs inferred from 143 rDNA gene sequences.

In Lake Pavin, half of the OTUs identified belonged to fungi with 15, 7, and 1 representatives (i.e. OTUs) from the Chrytridiomycota, Ascomycota, and Basidiomycota phyla, respectively (Figure 2, Supplementary Table S1a). The other represented taxonomical groups comprised SSU rDNA gene OTUs from 11 Viridiplantae, 5 Cryptophyta, 4 Alveolata, 1 Telonemida, 1 Ichtyosporea, and 1 Stramenopile (Figure 2). In Lake Aydat, one third of the OTUs belonged to fungi, with lower number

of representatives compared to Lake Pavin, including 10 Chytridiomycota, 8 Ascomycota, and 1 Basidiomycota (Figure 3, and Supplementary Table S1b). The diversity of chytrids was 50% lower in Lake Aydat compared to Lake Pavin. Similar to the findings in Lake Pavin, other eukaryotic groups were found in Aydat, with SSU rDNA gene OTUs corresponding to 21 Alveolata, 9 Viridiplantae, 6 Cryptophyta, 2 Ichtyospora, and 2 Stramenopile (Figure 3, and Supplementary Table S1b). In addition, three Choanoflagellida and one Cercozoan OTUs were only retrieved from Lake Aydat (Figure 3, and Supplementary Table S1b). Within all the identified OTUs, only five were common to both lakes: two Cryptophyta, one Alveolata, and two fungi (one Ascomycota and one Chytridiomycota).

For both lakes, the rarefaction curves with >97 and >99% levels of sequence similarities did not reach saturation (Figure 4a), indicating biodiversity underestimation using the classical approach. This result justifies the implementation of a complementary 454-pyrosequencing approach, to improve the exploration of the hidden biodiversity, including rare species. With this approach, a total of 23,519 and 18,545 sequences from Lake Pavin, and of 30,337 and 31,034 sequences from Lake Aydat, using eukaryote and fungus primers, were respectively obtained. For both lakes and for all sampling stations, the rarefaction curves continued to rise almost linearly with >99% level of sequence similarity (data not shown), but approaching a plateau when applying >97%, the apparent diversity being higher when using the fungus set of primers (Figure 4b).

a)



b)

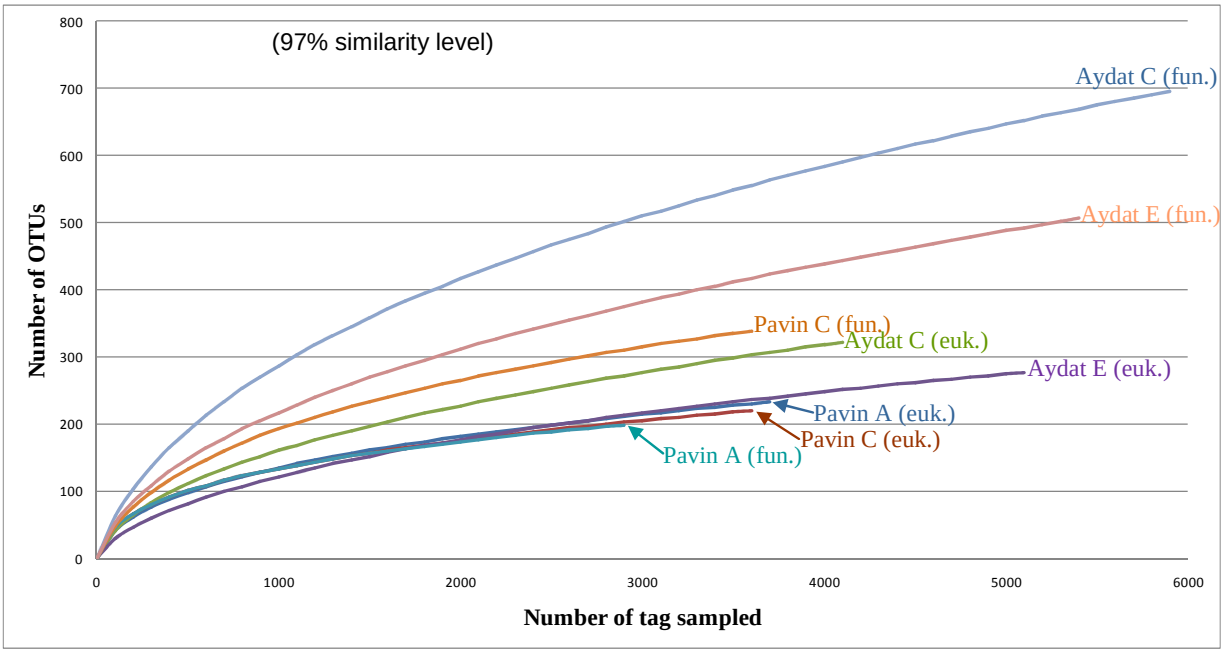
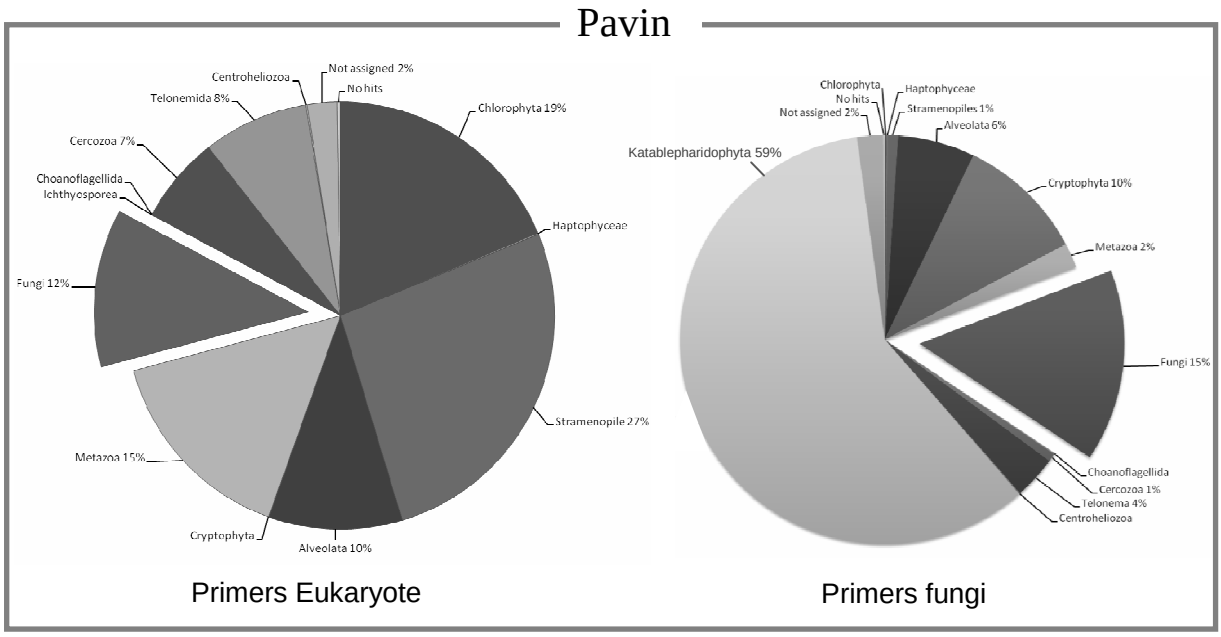
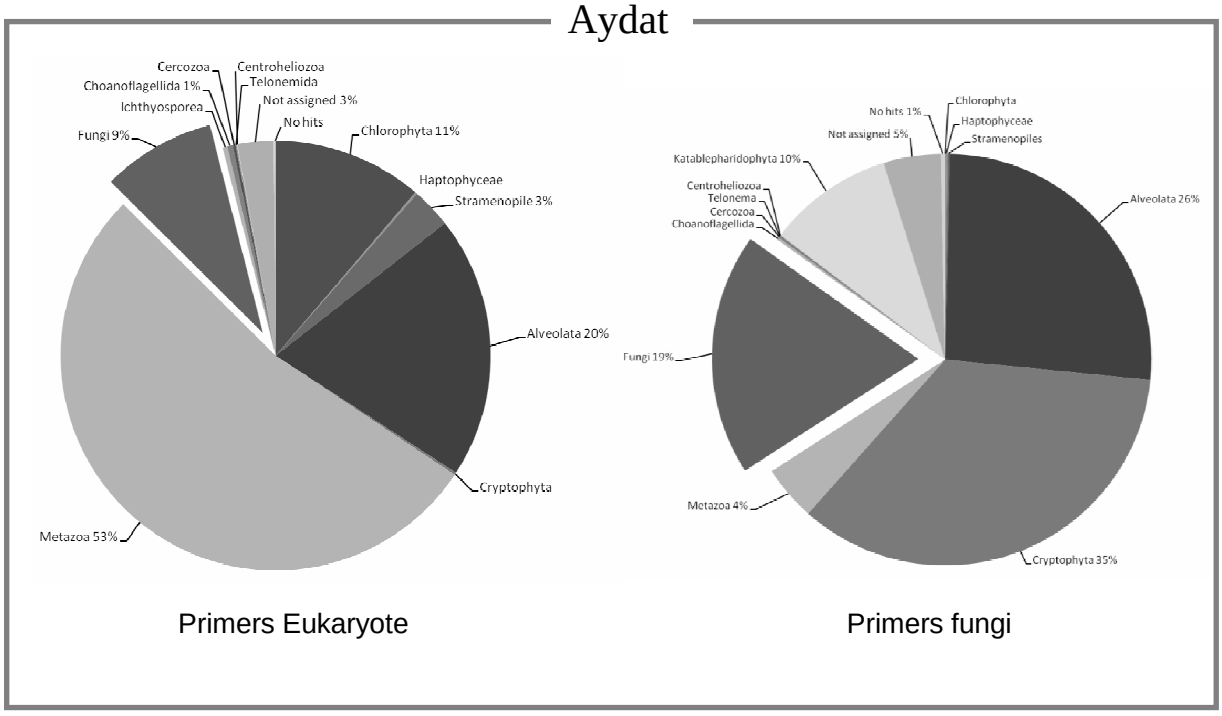


Figure 4: Rarefaction curves
Rarefaction curves, representing the numbers of OTUs versus the number of clones, were plotted from sequences obtained with the classical (a) and the 454-pyrosequence (b) approaches. The curves were calculated using the program Mothur (Schloss *et al.*, 2009), with a cutoff value set to 0.01 and 0.03 (OTUs with differences that do not exceed 1 and 3%) for the analysis.
(a) For the classical approach, all the 18S rRNA gene sequences obtained from Lake Pavin (full lines) and Lake Aydat (dash lines) were grouped into OTUs at a level of sequence similarity ≥99% (black lines) and ≥97% (grey lines).
(b) The 454-pyrosequence reads obtained from lakes, sampling stations, and using the two sets of primers (eukaryote (euk.) and fungus (fun.)), were curated (see Material and methods) and grouped into OTUs at a level of sequence similarity ≥97%.

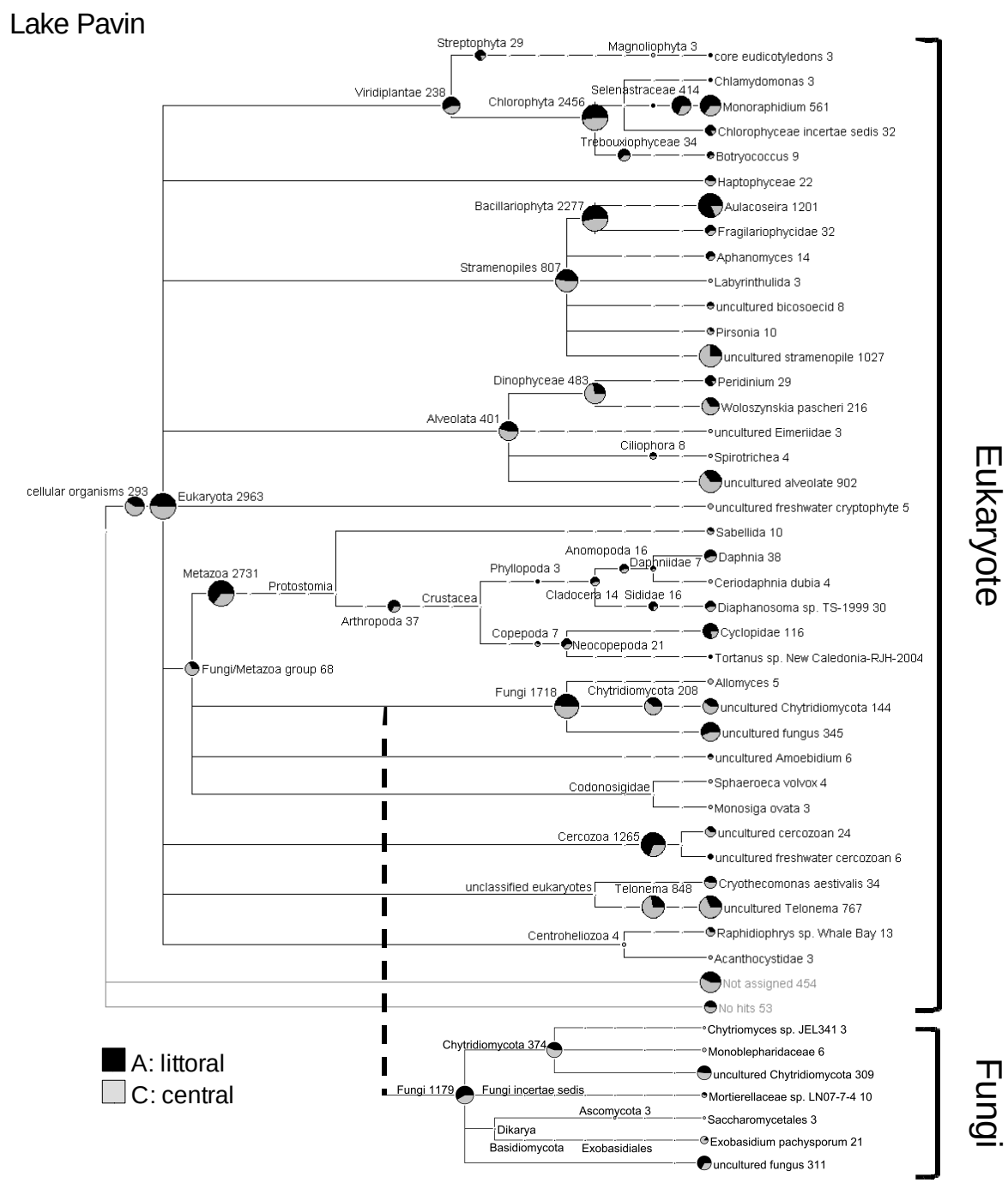
With the 454-pyrosequencing approach, an important diversity of fungi was observed in the two investigated environments, but also of many other eukaryotes with the two sets of primer used (eukaryote and fungi). When considering the data obtained with the eukaryote primers, the most represented groups were Fungi (representing 12 and 9% of the 454-pyrosequence reads in Pavin and Aydat, respectively), Metazoa (15 and 53%), Stramenopile (27 and 3%), Alveolata (10 and 20%), and Chlorophyta (19 and 11%) (Figure 5). The Cercozoa and Telonemida were also among the major groups in Lake Pavin, accounting for 7 and 8% of the sequences, respectively (Figures 5 and 6). When considering the data obtained with the Fungus primers, enrichment in fungal sequences (15 and 19% of the 454-pyrosequence reads in Pavin and Aydat, respectively) was observed. With these Fungus primers, only a few metazoan and Viridiplantae sequences were retrieved, while the Katablepharidophyta (60% and 10% of the 454-pyrosequence reads in Pavin and Aydat, respectively) and Cryptophyta (10 and 36%) were among the dominant groups (Figure 5). Finally, whatever the primer considered, it was not possible to assign a taxonomic group for 1 to 5% of the sequences from the different samples because of suspicious or ambiguous assignment of the SSU rDNA gene fragments in the database, while less than 1% of the sequences had no hit in the Silva SSU database.





<u>Figure 5</u>: Proportion of taxonomic groups identified in Lake Pavin and Lake Aydat using 454-pyrosequencing of 18S rRNA gene hypervariable region.

The reads obtained from pyrosequencing of 18S rRNA hypervariable region were subject to BlastN (Altschul *et al.*, 1990) search against the Silva SSU rRNA database (*http://www.arb-silva.de/*) to assign a taxonomic group. The pie diagram displayed the proportion of reads, obtained from both lakes sampling stations, and using the two primers sets (eukaryote and fungus), belonging to a particular phylum. "No hit" correspond to reads having no homologous sequence in the Silva database (threshold $E=10^{-5}$). "Not assign" correspond to reads having a match in the Silva database but without a precise taxonomic phylum assignment. Reads matching eukaryotes, corresponding to 21.2% of the reads in Lake Pavin and 47.5% of the reads in Lake Aydat, without further precision were not include in the diagram.



<u>Figure 6</u>: Taxonomic assignment of the 454-pyrosequences 18S rRNA reads obtained from Lake Pavin.

The 18S rRNA hypervariable tag pyrosequences obtained from Lake Pavin were analyzed using the software MEGAN, after BlastN (Altschul *et al.*, 1990) search against the Silva SSU rRNA database. The MEGAN software (Huson *et al.*, 2007) plots on a schematic phylogenetic tree the number of 454-pyrosequence reads matching a particular taxonomical group. Each taxonomic nod is represented by a pie diagram, with littoral sample in black and central samples in grey color, which size is proportional to the number of assigned reads (given by the numbers). The tree displayed the entire taxonomic groups identified from the assignment of 454-pyrosequence reads obtained with eukaryote primers, while only the fungal Kingdom is given (on the bottom) for reads obtained using fungus primers.

The 454-pyrosequencing method was used to identify dominant species and determine their spatial distribution using data obtained from eukaryote primers for the overall biodiversity, and from fungal primers for the diversity of fungi. In the following sections, we describe the taxonomical groups and species unveiled by the complementary 'classical' and pyrosequencing approaches and we compare the diversity observed in the two pilot lakes, with focus on Fungi.

The diversity of Fungi

Chytridiomycota. In both lakes, the identified chytrids belonged to the Chytridiales, Rhizophydiales and Rhizophlyctidales orders. The chytrid sequences found here confirmed the high diversity of Chytridiomycota-affiliated sequences reported in previous studies (Lefèvre *et al.*, 2007; Lefèvre *et al.*, 2008) (Figures 2 and 3).

Members of Rhizophydiales, which occur in aquatic ecosystems primary as phytoplankton parasites (James *et al.*, 2006a; Letcher *et al.*, 2006), were the most represented fungi in Lake Pavin with seven OTUs (Figure 2). Only one Rhizophydiales species was common to both lakes, as suggested by the 99.58% identity between the OTUs PA2009E11 (Pavin) and AY2009D2 (Aydat) sequences (Figures 2 and 3). These SSU rDNA gene sequences were closely related to PFD5SP2005 sequence previously identified in Lake Pavin and considered as being part of a new clade (Lefèvre *et al.*, 2008). These sequences were grouped with *Kappamyces laurelensis* in the *Kappamyces* subclade (Letcher and Powell, 2005). In Lake Pavin, six OTUs were clustered within the *Kappamyces* group (Figure 2). Interestingly, four of them (PA2009C4, PA2009A4, PA2009E12 and PA2009D2) formed a new sister clade of the main *Kappamyces* group (Figure 2) which was not found in the pool of sequences obtained from the Lake Aydat.

In both lakes, one OTU (PA2009C1 in Lake Pavin and AY2009B4 in Lake Aydat) belonged to the *Chytridium angularis* clade (James *et al.*, 2006b). This clade includes *Chytridium polysiphoniae*, a parasite of the marine brown alga *Pylaiella littoralis*, and *C. angularis* that grow on pollen and heat treated *Oedogonium* (a green algae) baits (Longcore, 1992). Interestingly, in Lake Pavin, three new sequences clustered together to form a sister clade of the *Chytridium angularis* clade (Figure 2). In Lake Aydat, AY2009C3 and AY2009B3 sequences form an early divergent novel clade within the Chytridiomycota (Figure 3).

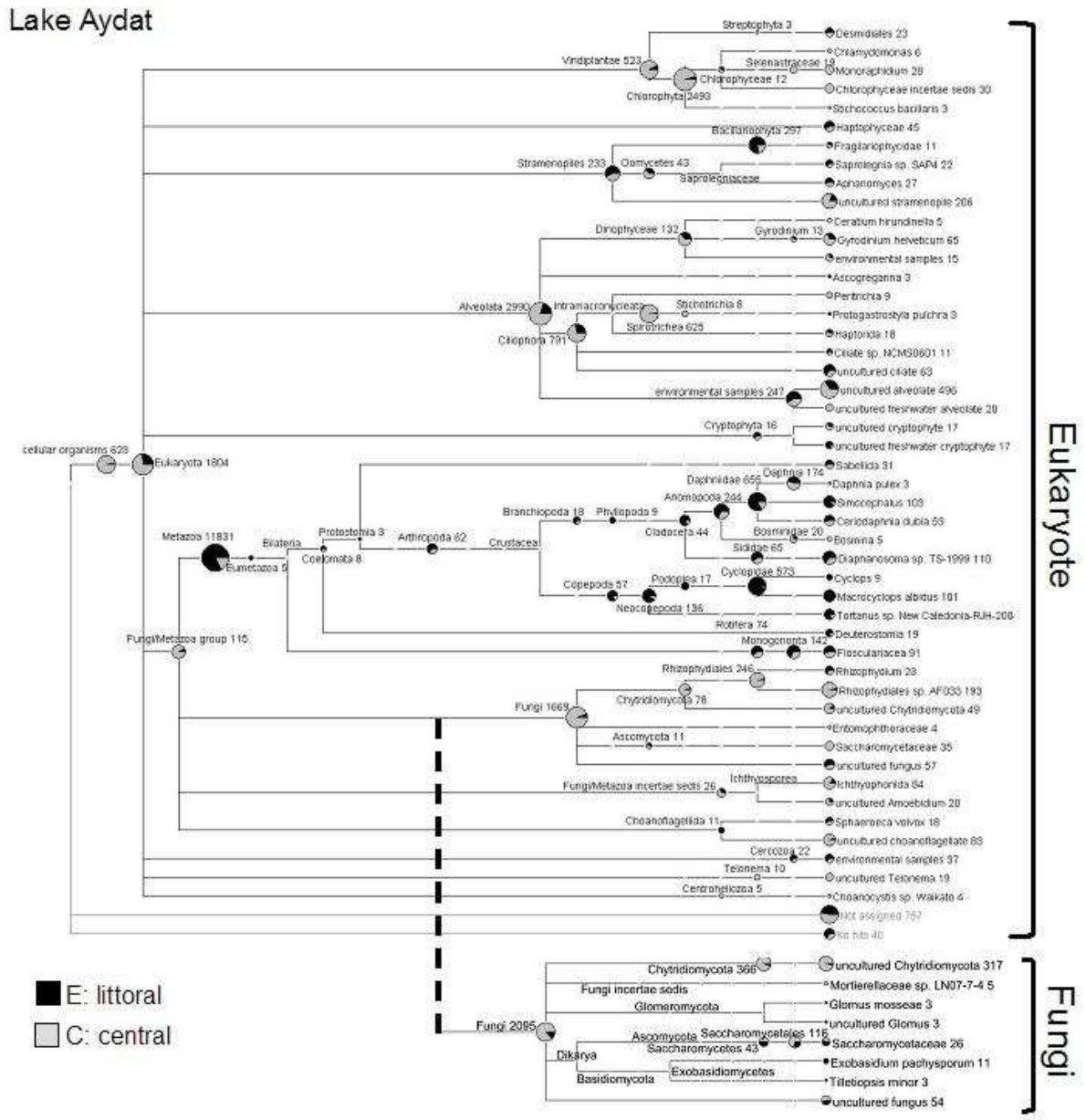
Concerning the order Rhizophlyctidales, one OTU (PA2009E8 in Lake Pavin and AY2009D3 in Lake Aydat) belonged to the *Rhizophlyctis* clade described by James *et al.* (2006b). Indeed, in this latter study, *Catenomyces persinius*, a cellulose decomposer found in aquatic habitats, currently included in the order Blastocladiales (phylum Blastocladiomycota), was assigned to the *Rhizophlyctis* clade (including *Rhizophlyctis rosea*), a sister clade to the Spizellomycetales. Based on molecular monophyly and zoospore ultrastructure, this *Rhizophlyctis* clade was recently designated as a new

order, the *Rhizophlyctidales* (Letcher *et al.*, 2008). To note, in each lake, three OTUs constituted a novel clade, which appear to be the sister group of the *Rhizophlyctis* clade (Figures 2 and 3).

The proportion of each fungal OTU (classical approach) was quantified according to the number of matching 454-pyrosequence reads (Supplementary Tables S1a and S1b). The two approaches consistently displayed the same dominant species. In Lake Pavin, a new clade PA2009E6, out-grouping from the main Chytridiomycota phylum (Figure 2), was dominant according to 454-pyrosequence data obtained with fungus primers. This OTU represented more than 40% of the 454-pyrosequence reads matching a fungal OTU identified from the classical approach (Supplementary Table S1a). In this lake, pyrosequence data obtained from both primer sets (eukaryotes and fungi) consistently displayed PA2009E8, PA2009E9, PA2009E10 and PA2009E11 as the dominant chytrids' OTUs (Supplementary Table S1a). In lake Aydat, the OTU AY2009C4 was consistently found highly dominant among the pyrosequence reads obtained from both sets of primers. This OTU represents 80% of the pyrosequence reads matching a fungal OTU identified from the classical approach (Supplementary Table S1b), and corresponds to a novel species having only 90.3% similarity with *Rhizophydium* sp. JEL317, its closest relative.

When combining pyrosequence data obtained with the two set of primers, additional sequences were retrieved compared to the sole use of the classical approach. Some *Chytriomyces* sp. JEL341 and representatives of the Monoblepharidaceae family were found in Lake Pavin, while several Rhizophydiales sp. AF033 sequences were retrieved in Lake Aydat (Figures 6 and 7).

Other fungi. One Ascomycota species was common to both lakes and identified as OTUs PA2009D1 (Pavin) and AY2009B1 (Aydat). They indeed shared 99.4 % identity, and were similar to *Simplicillium lamellicola*, a pathogen of another fungus, *Agaricus bisporus* (Basidiomycota) (Spatafora *et al.*, 2007). This pathogen was the dominant Ascomycota in both lakes (Supplementary Tables S1a and S1b). The other Ascomycota identified in the two lakes represented minor species according to the low number of corresponding 454-pyrosequence reads. In addition, pyrosequencing yielded Saccharomycetales as the sole group of Ascomycota in both lakes (Figures 6 and 7). Only one sequence per lake, retrieved from the SSU rDNA clone libraries, clustered within the Basidiomycota group, i.e. *Exobasidium rhododendri* in Lake Pavin and *Jamesdicksonia dactylidis* in Lake Aydat. Both of them are putative plant pathogens. Compared to the classical SSU rDNA approach, 454-pyrosequencing revealed the presence of *Exobasidium pachysporum* in both lakes and of three sequences affiliated to *Tilletiopsis minor* in Lake Aydat. In both lakes, the Basidiomycota sequences retrieved by the two approaches belonged to the class of Exobasidiomycetidae. The other fungus sequences, only identified through pyrosequencing, included some Glomeromycota (previously known as Zygomycota), such as *Mortierellaceae* sp. LN07-7-4 in the two lakes; and *Glomus mosseae* and representatives of the *Entomophthoraceae* family in Lake Aydat (Figures 6 and 7).



<u>Figure 7</u>: Taxonomic assignment of the 454-pyrosequences 18S rRNA reads obtained from Lake Aydat.
Similarly to <u>Figure 6</u>, the 454-pyrosequence 18S rRNA assigned reads were displayed on a schematic phylogenetic tree. Pie diagrams, at each taxonomic nod, indicate the proportion of reads obtained from the central (grey color) and littoral (black color) sampling stations of Lake Aydat.

The diversity of other eukaryotes

Alveolata. In clone libraries from Lake Pavin, two Alveolata OTUs belonged to the Dinophyceae, one to the Perkinsea, and one to the Ciliophora group (<u>Figure 2</u>). In Lake Aydat, one third of the identified OTUs from the clone libraries corresponded to Alveolata, from which 17 belong to the

Ciliophora, one to the dinoflagellates, and one to the Apicomplexa group (Figure 3). One species of Ciliophora was common to both lakes, i.e. the OTUs PA2009E19 (Pavin) and AY2009D10 (Aydat) sharing 99.7% sequence identity. On their respective phylogenetic trees, these two OTUs form new clades, supported by bootstrap values of 100%. These clades clustered two additional OTUs in Lake Aydat (AY2009C19, AY2009C20), with *Rimostrombidium lacustris* as a sister group. In Lake Aydat, another new clade of Ciliophora clustering three OTUs (AY2009E5, AY2009C21 and AY2009E6) was supported by a bootstrap value of 80%. The Apicomplexa group was only found in Lake Aydat with one OTU (AY2009B10) having *Cryptosporidium parvum* as closest relative, i.e. a human protozoan parasite that can cause an acute short-term infection and become severe for immunocompromised individuals. Considering the phylogenetic distance between AY2009B10 and *C. parvum*, it was unlikely that Lake Aydat contained this parasite. Therefore, this sequence could represent a new clade or a *Cryptosporidium*-like organism. Consistent with the observations made from the classical approach, pyrosequence data displayed a larger diversity of Alveolata in Lake Aydat, compared to Lake Pavin (Figures 6 and 7), which contributed to the apparent overall higher eukaryote diversity in Lake Aydat.

Cryptophyta. Two Cryptophyta species, identified by the classical approach, were found in both lakes. The first one, corresponding to OTUs PA2009D3 (Pavin) and AY2009D5 (Aydat) sharing 99.6% sequence identity, clustered together with a sequence isolated from Lake Georges (LG08-05) (Richards *et al.*, 2005), and a sequence (P1.31) previously obtained from Lake Pavin (Lefranc, 2005). The second, corresponding to OTUs PA2009D5 (Pavin) and AY2009E2 (Aydat) sharing 99.4% sequence identity, was closely related to *Cryptomonas ovata*. In both lakes, two distinct OTUs (having less than 99% identity) were related to *Plagioselmis nannoplanctica* strain N750301. In Lake Pavin, two OTUs (PA2009C5 and PA2009E14) clustered together in a clade with sequence P34-10 previously isolated from Lake Pavin (Lefranc *et al.*, 2005) and sequence LG08-04 from Lake Georges (Richards *et al.*, 2005). This clade, named LG-E in the phylogenetic tree based on sequences obtained from the Lake Georges (Richards *et al.*, 2005), showed branches that were consistently attracted to either the cryptomonads or to the glaucocystophytes. In fact, this branch derived from the *Katablepharis japonica* group for which SSU rDNA gene sequences were not available at that time, and belongs to the katablepharids, a distant sister group from the Cryptophyta (Okamoto and Inouye, 2005). These two OTUs (PA2009C5 and PA2009E14) and their corresponding katablepharids group, were matching the highest number of pyrosequence reads obtained when using fungus primers, but were poorly detected when using eukaryote primers (Figure 5, and Supplementary Table S1a). Similarly in Lake Aydat, the OTUs AY2009E3 and AY2009C11, related to *Cryptomonas curvata* strain CCAC 0080, and their corresponding Cryptophyta group, were displaying the highest number of matching with pyrosequence reads obtained from fungus primers, but were also poorly detected using eukaryote primers (Supplementary Table S1b).

Viridiplantae. This group of organisms was well represented in the clone libraries, accounting for 37% and 21% of the total numbers of sequences in Lakes Pavin and Aydat, respectively. Representatives of the Sphaeropleales and Chlamydomonadales were found in both lakes, with the addition of the Treubariaceae, Trebouxiophyceae, and Mychonaste groups in Lake Pavin (Figures 2 and 3). Two distinct new clades within the Chlamydomonadale group were identified in the two lakes, including the OTUs PA2009E16 and PA2009B2 in Lake Pavin (Figure 4) and AY2009C13 and AY2009B7 in Lake Aydat (Figure 5). Reads corresponding to Viridiplantae 18S rRNA genes were only retrieved from 454-pyrosequencing when using eukaryote primers.

Minor groups. Stramenopiles were poorly identified in both lakes' clone libraries (Figures 2 and 3), but abundantly in Lake Pavin according to the 454-pyrosequence data (Figure 5). Through the classical approach, the Telonemida group was only identified in Lake Pavin, with the OTU PA2009A5. The 454-pyrosequencing approach confirmed the abundance of Telonemida in Lake Pavin, and their rarity in Lake Aydat. On the opposite, Choanozoa sequences were only retrieved in Lake Aydat, both with the classical approach (i.e. OTUs, AY2009C6, AY2009C7 and AY2009C8, clustering together with *Monosiga ovata* and more distantly with *Monosiga brevicollis*) (Figure 3), and with the 454-pyrosequencing approach (Figure 7). Finally, in addition to species identified with the classical approach, 454-pyrosequencing using eukaryote primers allowed the retrieval of SSU rDNA sequences from numerous Arthropoda (n = 312 in Lake Pavin and 2,502 in Lake Aydat), Rhizaria (n = 1329 and 59), a few Haptophyceae (n = 22 and 45), and Centroheliozoa (n = 20 and 9) (Figures 6 and 7). Overall, the pyrosequencing of 18S rRNA hypervariable regions confirmed the higher eukaryote diversity in Lake Aydat, compared to Lake Pavin (Figures 6 and 7).

Within-lake spatial variability

Lake Pavin. Both the classical and the pyrosequencing approaches showed a relatively homogenous spatial distribution of eukaryotes in Lake Pavin (Figures 6, and Supplementary Table S1a). The classical approach yielded 23 OTUs in the centre of the lake and 15, 18, 18 and 19 OTUs for stations A, B, D and E, respectively (Figure 2, and Supplementary Table S1a). The 454-pyrosequence data, independently from the set of primer used (eukaryote or fungi), displayed a similar number of reads corresponding to the main groups of species retrieved at the two sampling stations (Figure 6). The number of pyrosequence reads corresponding to fungi slightly decreased from the coastal station A to the central station C, due to a higher number of reads related to uncultured fungi (i.e. having not clear taxonomical assignment) on the littoral zone (Figure 6).

Chytridiomycota was the most identified group of fungi among the pyrosequence reads obtained using fungus primers. Chytrid sequences accounted for 25.8% (coastal station A, n = 313) and 37.7% (centre

station C, n = 379) of all the fungal reads. A similar result was observed when using eukaryote primers, but with about two times less chytrid reads retrieved from 454-pyrosequencing. The dominant chytrids OTUs were equally found located on the coastal and central areas of the lake (Supplementary Table S1a). The Ascomycota were consistently more frequently retrieved from the coastal station by both approaches (classical and 454-pyrosequencing) (Figure 6, and Supplementary Table S1a). The dominant Ascomycota (OTU PA2009D1) was almost exclusively located in the coastal station (Supplementary Table S1a). Finally, the Basidiomycota OTU (PA2009E1) retrieved with the classical approach was mostly found at the littoral station of Lake Pavin (Figure 2, and Supplementary Table S1a). This contrasts with the general within-lake distribution of the other Basidiomycota, which were mostly retrieved in the central (n = 18) compared to the littoral (n = 3) stations of the lake according to 454-pyrosequence data (Figure 6).

The spatial patterns for other eukaryote species could also be inferred using data from pyrosequencing. In this respect, most of the eukaryotic groups displayed a homogeneous localization between the coastal and central sampling stations. Some differences were observed for Cercozoa which were mostly sampled in the littoral waters while, in contrast, Telonemida were preferentially sampled in the pelagic central waters of Lake Pavin. Finally, the Cryptophyta group, only unveiled when using fungus primers, was three-fold more abundant in the centre (n=1135) compared to the littoral (n=347) areas of the lake, according to the 454-pyrosequencing.

Lake Aydat. Both methods (classical and 454-pyrosequencing) showed a more heterogeneous spatial distribution of species in Lake Aydat (Figure 7, and Supplementary Table S1b), compared to Lake Pavin. The classical approach yielded 24 OTUs in the central area, and 19, 19, 22 and 20 OTUs at stations A, B, D, and E, respectively (Figure 3, and Supplementary Table S1b). Among the eukaryotic groups, fungi displayed a decreasing proportion from station A (45%), B (36%), C (22%), D (25%) to station E (12%) relative to the total number of sequences retrieved by the classical approach. Fungi displayed a clear spatial pattern from station E to A, following the up-downstream flow of the river. This pattern was confirmed by pyrosequencing, independently of the set of primers used (Figure 7). Fungi represented around 15% of all the pyrosequence reads at the central station C, but only 1 to 3% at the littoral station E (Figure 7).

Chytrids spatial distribution, inferred from 454-pyrosequencing and confirmed by the use of both primer sets (eukaryote and fungi), naturally showed a preferential localization in the centre pelagial of Lake Aydat. The classical approach showed that the OTU AY2009C4, representative of a novel clade within the Rhizophydiales order, was particularly abundant in the centre of the lake. When searching corresponding reads for AY2009C4 in the 454-pyrosequence data base obtained with fungus primers, it appeared that matching reads were 33 times more abundant in samples taken from the central station C (n = 701) compared to the coastal station E (n = 21) (Supplementary Table S1b). This was confirmed by the pyrosequence data obtained from eukaryote primers for the OTU AY2009C4 which

was 40 times more abundant in the central than in littoral stations. Two others OTUs, AY2009B3 and AY2009C3, clustering together with *Chytriomyces* sp. JEL341, were 2 to 3-fold more represented in the central than in the littoral areas of the lake, according to pyrosequence data and independently of the set of the primers used (Supplementary Table S1b).

The classical approach showed that the number of OTUs and of sequences composing OTUs belonging to Ascomycota decreased from the coastal sampling stations A and B (located close to the within-lake islands) to the rest of the lake (Supplementary Table S1b). This gradient was confirmed by the pyrosequence data, with an overall higher number of Saccharomycetales at the station C (n=115) compared to station E (n=70) (Figure 7). A low number of Ascomycota OTUs, identified by the classical method (Figure 5), were matching pyrosequence reads at station E (Supplementary Table S1b), thus reinforcing our hypothesis of a decreasing gradient from stations A to E for Ascomycota in Lake Aydat. Basidiomycota sequences were mainly retrieved in the coastal areas of this lake, by the two approaches. The AY2009A3 OTU, closely related to *Jamesdicksonia dactylidis*, was only retrieved by the classical method at station A (Supplementary Table S1b). The other Basidiomycota, *Exobasidium pachysporum* (n = 11) and *Tilletiopsis minor* (n = 3), were exclusively retrieved by 454-pyrosequencing at the sampling station E. These results are in contrast with those obtained in Lake Pavin, where sequences corresponding to Basidiomycota were mainly found in the centre of the lake. Others groups of fungi were identified solely by pyrosequencing, among which Glomeromycota were exclusively located in the coastal area (n = 6), while *Mortierellaceae* sp. LN07-7-4 (n = 5) was exclusively found in the centre of the lake (Figure 7).

Besides fungi, the spatial pattern for other eukaryotes was mainly inferred from the pyrosequence data obtained with eukaryote primers. The major groups of species displaying a clear spatial distribution included Viridiplantae and Alveolata which were preferentially localized in the centre of the lake, while Stramenopile and Arthropoda were mainly located in the coastal area. The minor taxonomic groups, Choanoflagellida, Centroheliozoa and Telonemida, were mostly or exclusively found in the centre of Lake Aydat (Figure 7, and Supplementary Tables S1). Finally, the kathablepharids group, only detected with the fungus primers, was almost exclusively found in the centre (n = 1559) compared to the littoral (n = 47) areas of the lake. The heterogeneous spatial distribution of the kathablepharid group in Lake Aydat contrasts with its homogenous distribution in Lake Pavin. In contrast to the observation made in Lake Pavin, Cryptophyta were homogeneously located in the central (n = 3476) and in the peripheric (n = 2219) areas of Lake Aydat. Although, two Crytophyta OTUs (AY2009C11 and AY2009E3) clustering with *Cryptomonas curvata* strain CCAC 0080, were two-fold more represented in the coastal than in the central areas of Lake Aydat (Supplementary Table S1b).

Discussion

General and Methodological considerations

This study's aim was to identify the diversity of microbial eukaryotes and their spatial distribution, within two freshwater environments, Lakes Pavin and Aydat, Massif Central, France. These two lakes differ by their surface, altitude, depth, trophic status, geological origin, water flux (river, drainage basin), and anthropogenic activities (agricultural, and industrial impact). Considering all these differences, a homogenous distribution of species was expected in Lake Pavin where typical pelagic organisms are dominant. Conversely, we anticipated Lake Aydat would reveal a heterogeneous spatial variation of species, due to its exposition to enhanced allochthonous materials from terrestrial inputs. Few studies have investigated the diversity of picoeukaryotes in these two lakes (Lefèvre *et al.*, 2007; Lefèvre *et al.*, 2008; Lefranc *et al.*, 2005), and highlighted the importance and ecological significance of chytrids (Lefèvre *et al.*, 2008). Our work intended to complete these studies by identifying new fungi, and describe their quantitative importance and spatial distribution patterns. This goal was achieved through two complementary methods to beneficiate from their respective advantages: a qualitative taxonomic identification of each species, and of new clades, by the "classical cloning/sequencing approach", and the corresponding dominant and rare species by the pyrosequencing of the 18S rRNA gene hypervariable regions. In addition, the large number of reads obtained with the 454-pyrosequencing was a powerful tool to consistently establish the distribution of species between the coastal areas vs. the central pelagial ones. While one set of primers for fungi was used for the classical approach (White *et al.*, 1990), two sets were utilized for pyrosequencing, i.e. an universal eukaryote set of primers (Casamayor *et al.*, 2002; Lepère *et al.*, 2006; López-Garcia *et al.*, 2003), and a set of fungus primers (Borneman and Hartin, 2000). Although these primers were not specific for the 18S rDNA genes of the sole fungi, they allowed a significant fungal enrichment of our data base (i.e. compared to eukaryote primers), up to 2.6 fold for total fungi and 3.5 fold for chytrids. The fungal primers did not amplify 18S rDNA genes from the abundant groups of Arthropoda and Viridiplantae, leading to the enrichment in 18S rDNA gene sequences from other eukaryotes, primarily from fungi, i.e. compared to the eukaryote primers. The concurrent use of fungal primers was therefore justified. In addition, the fungal primers were more accurate in the taxonomic identification of fungi compared to universal eukaryote primers (Figures 6 and 7), and were therefore well adapted for the identification and quantification of fungi, particularly of chytrids. Nevertheless, designing primers specific for pelagic fungus rRNA genes is one of the major bottlenecks that need to be resolved for future studies. In this regard, the ITS1, 5.8S and ITS2 sequences that were generated with the classical approach used in this study but not used in the phylologenetic studies presented in this work, will be very helpful to design new tools to follow the dynamics of particular fungal populations in natural waters.

The diversity in Lake Aydat was greater than that observed in Lake Pavin, which is likely to be linked to the differences in the trophic status of theses lakes. It is interesting to observe that both lakes shared only fives species in common (two Cryptophyta, one Alveolata, one Ascomycota, and one Chytridiomycota), highlighting the difference between the two lakes and providing evidence that aspects of eukaryote microbial diversity are specific to certain aquatic environments. The rarefaction curves constructed from the 146 sequences in Lake Pavin and 142 sequences in Lake Aydat displayed a linear pattern, indicating a still under-sampling of the total diversity. Consistently with this result, the same groups of organisms were identified, and confirmed the existence of new clades known from previous studies (Lefèvre *et al.*, 2007; Lefèvre *et al.*, 2008), in addition to the unveiling of many new rDNA gene sequences that were not previously retrieved. Finally, this low throughput approach allows the retrieval of SSU rDNA sequences from species that were probably the dominant ones in the lakes. In contrast, the high throughput 454-pyrosequencing of 18S rRNA gene hypervariable regions was initiated in order to increase the sampling effort of rDNA gene sequences in the target lakes and to unveil a much extended eukaryote diversity, with a focus on fungi. Rarefaction curves computed from pyrosequence data displayed linear pattern when applying 99% sequence identity threshold, while it almost reached saturation when applying 97% identity threshold, which is better adapted for deep pyrosequencing of rRNA genes (Kunin *et al.*, 2010). These patterns of rarefaction curves suggest a good sampling of the lakes and, accordingly, the eukaryote primers retrieved more sequences than the fungus primers. The variability within different regions of the SSU rRNA molecule and the length of the amplicon have a great effect on the apparent species richness, as it was shown when tracking the microbial diversity of the termite hindgut (Engelbrektson *et al.*, 2010). Species evenness and richness should not be directly compared between different regions of the rRNA molecules (Engelbrektson *et al.*, 2010). In addition, richness should also be temperate by some technological limits (alignments of large sets of sequences, pyrosequencing errors, statistical tools…) (Kunin *et al.*, 2010) and by the unknown variability of the multiple copy of rDNA operon from the same organism, as it was demonstrated for bacteria belonging to the genus *Vibrio* (Moreno *et al.*, 2002).

Pyrosequencing of rRNA gene hypervariable region reveal much greater eukaryote diversity in the studied lakes than in the previously studies (Lefèvre *et al.*, 2007; Lefèvre *et al.*, 2008; Lefranc *et al.*, 2005). The reason is that only the classical approach was used in these studies, unveiling dominant but not minor species. Rare species, corresponding to species that are maintained at a low number (arbitrarily chosen below 0.05 to 1% of the total number of pyrosequence reads, Huse *et al.*, 2008; Sogin *et al.*, 2006), may play an important role in lakes by becoming dominant in response to environmental changes, in addition to represent a nearly inexhaustible source of genomic innovation (Sogin *et al.*, 2006). They indeed represent an hypothetically important reservoir of ecological redundancies that can buffer the effects of dramatic environmental shifts and, perhaps, insure the maintenance of basic biogeochemical processes in natural ecosystems (Sime-Ngando and Niquil, *in press*). However, rare microbial eukaryotes are largely underexplored in aquatic systems, primarily in

freshwaters. All studies on the 'rare biosphere' are indeed restricted to marine prokaryotes, with only one theoretical attempt considering microbial eukaryotes (Caron and Countway, 2009). The improved unveiling of eukaryote diversity, such as in the present study, thus opens great perspectives for future identification of novel 'species', genes, and metabolic pathways in lakes by complete deep sequencing of complex community, similar to what was done for prokaryotes in the Sargasso Sea (Venter *et al.*, 2004).

Diversity patterns

A greater diversity of fungi and chytrids was observed in Lake Pavin compared to Lake Aydat. The classical approach showed that fungi represent half of the OTUs identified in Lake Pavin, and one third in Lake Aydat. Both approaches used confirmed that Lake Pavin contains around 20% more fungal diversity than Lake Aydat, suggesting a preference or the capacity of most fungi unveiled to adapt to a pelagic life style. In both lakes, the four phyla of the main Fungal divisions were represented in our sequences. The presence of Glomeromycota 18S rDNA gene sequences in the coastal area of Lake Aydat is consistent with their distribution in terrestrial rather than aquatic habitats and further implies a contribution of specimens in our samples from allochthonous inputs. In contrast, the low water input explained their absence in Lake Pavin. It is often difficult to compare results obtained from pyrosequencing and classical approaches, because of the use of different primers. A good correlation was observed between the two methods for the identification of dominant fungi: the highest number of pyrosequence reads corresponds to the fungus OTUs with the highest number of sequences in the classical approach (Supplementary Tables S1a and S1b). Still, the quantitative information given by pyrosequencing data should be interpreted with care, because of the inherent bias of the PCR technique and also because no information is available on the number of copies of rRNA operons per fungal cells. Yet, such comparison revealed that the OTUs identified by the classical approach corresponded to around 13.8% (Station A, Pavin), 9.3% (C, Pavin), 19.2% (C, Aydat), and 10.4% (E, Aydat) of the pyrosequence reads obtained with fungus primers. This result suggests a realistic representation of the fungal complete 18S rDNA gene sequences obtained with the classical approach. Several dominant chytrids were specific to each lake. The chytrids OTUs PA2009E6 and PA2009E8 were dominant in Lake Pavin, while the OTU AY2009C4 corresponds to the main chytrid sequence isolated from Lake Aydat. These dominant chyrids are unquestionably playing a major role in the lakes, but further characterization of these species is needed to better understand their dynamics and functional importance.

The classical approach reveals the presence of species belonging to the group of Viridiplantae in both lakes, and a large diversity of Alveolata in Lake Aydat. Among the Alveolata, one rDNA gene sequence was corresponding to a *Cryptosporidium parvum*-like organism. Additional studies and analysis are required to characterize this parasite-like organism, and to determine, if whether or not, it

is of concern to human safety. Within the Alveolata group, some Perkinsus rDNA sequences were identified, which have not been yet characterized earlier by traditional microscope observations because these organisms do not display distinctive morphological characteristics (Burreson *et al.*, 2005; Gestal *et al.*, 2006). In recent molecular studies, a growing number of *Perkinsus* have been identified from freshwater environments (Brate *et al.*, 2010), indicating that more efforts are needed to culture and characterize members of this lineage. Only one cercozoan sequence (AY2009C14) was found in Lake Aydat. This was surprising since many cercozoans were isolated from both lakes in previous studies (Lefèvre *et al.*, 2007; Lefèvre *et al.*, 2008; Lefranc *et al.*, 2005). It may be suggested that cercozoans were poorly represented in the two lakes at the time of our sampling.

The pyrosequencing of the 18S rDNA gene hypervariable regions led to the identification of many other eukaryotes. Among them, the Cryptophyta/Katablepharidophyta was the most represented group, accounting for more than 50% of the total pyrosequencing reads when using fungus primers, but was not detected with eukaryote primers. On the contrary, the groups of Viridiplantae, Stramenopile and Arthropoda, were only retrieved when using eukaryote primers. These two examples highlight the importance of using a combination of primers sets, to accurately determine environmental biodiversity. Few metazoan rDNA sequences were retrieved by the 454-pyrosequence approach. Since most metazoan could not pass through the filtration process, these sequences may come from larva, decomposed dead cells or free DNA molecules. In this study, we consider ourselves to have unveiled the most exhaustive catalog of eukaryotes, and particularly that of fungi, identified from freshwater environments, by taking advantage of the complementarities between the classical and pyrosequencing approach, and the specificity of different primers.

Spatial distribution

Pyrosequence data showed a more heterogeneous distribution of species in Lake Aydat compared to Lake Pavin (Figures 6 and 7, Supplementary Tables S1a and S1b). This observation is linked to the specific characteristics of both lakes, including the heterogeneous eutrophic status of Lake Aydat compared to the oligomesotrophic homogenous Lake Pavin. Chytrids are typical pelagic organisms and were located throughout Lake Pavin but more commonly in the central water column of Lake Aydat, suggesting an enhanced influence of terrestrial input into this lake, contrasting with the deep-crater Lake Pavin which is characterized by a low drainage basin and the absence of an influent river, establishing a typical pelagic environment in the whole water body of the lake. The Lake Aydat chytrid OTU AY2009C4, belonging to the Rhizophydiales order, was at least 33 times more represented in the centre compared to the coastal area of the lake, a spatial pattern which is apparently consistent with the typical pelagic lifestyle of chytrids. Ascomycota (and particularly the group of Saccharomycetales) and Basidiomycota are both parasites of animals and/or plants. They were mostly located on the coastal areas of the lakes. The higher number of Ascomycota/Saccharomycetes OTUs

located close to the Aydat islands may result from a higher quantity of nutrient resulting from the proximity of trees (vegetation decomposition) and the funnel shape of the area that concentrate nutrients and plant debris. This observation suggests that the retrieved Ascomycota close to the Aydat islands might correspond to saprophytic organisms. Besides fungi, the difference between the two lakes, also explains the rather homogeneous eukaryotes repartition in Lake Pavin, and their heterogenic repartition in Lake Aydat. In both lakes, particularly in Lake Aydat, the Arthropoda were mostly located in the coastal areas. This localization can be explained by the food source of these metazoans that might be more located in coastal areas. This may also explain the spatial distribution of some group of Ascomycota or Chytrids that can be parasites of these organisms. A Preferential localization of the Alveolata, Viridiplantae, Telonemida, and Centroheliozoa was observed in the centre of Lake Aydat. Pyrosequence data obtained using fungus primers revealed a preferential central localization of Cryptophyta in Lake Pavin, and for Katablepharidophyta, its sister group, in Lake Aydat. In general, in both lakes, the greatest diversity of eukaryotes was observed at the central point.

Conclusions

Very few species were shared between Lakes Pavin and Aydat, which indicates a different community composition in the same regional area. Geographical, physical and chemical factors of the biotope influence the species community structure and spatial variability. The characterization of the biodiversity is the first, but perhaps not the most important, step to understand the functioning of an ecosystem. Matching sequences to organisms represents one of the last frontiers of the undiscovered biodiversity that challenge pelagic microbial ecology today (Sime-Ngando and Niquil, *in press*). This study has to be completed by further characterization of the species, at least for the dominant ones, from their mode of nutrition (autotrophy, heterotrophy, mixotrophy), to their interactions between each other (food web, saprotrophism, parasitism, commensalism…). In order to do that, we first need to learn how to grow them in the laboratory. Chytrids play a major role in lake ecology, primarily as parasites of inedible phytoplankton, and by transferring these matter and energy (in the form of zoospores) to higher trophic levels (Lefèvre *et al.*, 2008). Sequencing the genome of chytrids, developing molecular tools (mutagenesis), and performing phenotypic analysis will lead to a major breakthrough in the understanding of the functioning of lake ecosystems. This knowledge could be integrated into mathematical models to predict ecosystem functioning. In this respect, Lake Pavin, because of its species composition homogeneity, is an ideal model for this type of study. Although the data presented in this paper are only a snapshot of the whole lake's biodiversity, the massive parallel pyrosequencing of the 18S rRNA gene hypervariable regions allows the assessment of the impact of space, time and complex environments on microbial communities. A timeline survey of the lake's biodiversity, with a regular sampling over several years is needed. This will give crucial information to understand the succession of stable and unstable repartition of eukaryote community, the dynamics,

interactions and successive blooms of species, and the changes of the eukaryote biodiversity due to climate change or anthropogenic activities.

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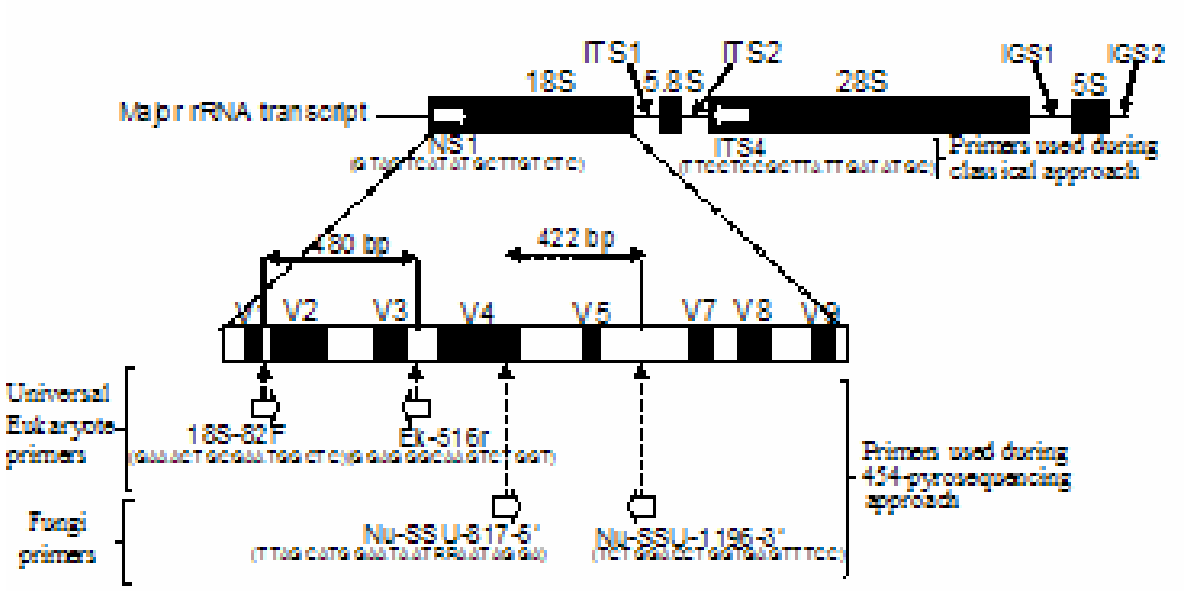
Supplemental materials



<u>Figure S1</u>: Schematic representation of rRNA transcript and localization of the primers used for the classical and the 454-pyrosequence approaches of this study.
This figure shows a schematic representation of the rRNA transcript (18S, ITS1, 5.8S, ITS2, 28S, IGS1, 5S and IGS2) with the localization of the primers and resulting amplicons that were obtained by the classical and 454-pyrosequence approaches. The hypervariable regions of the 18S rRNA gene are displayed by black boxes numbered from V1 to V9. The sequence of each primer is given between brackets.

Table S1: Number of sequence composing OTUs in the clone libraries, phylogenetic affiliation of the representative OTUs, and corresponding numbers of matching read in the 454-pyrosequence data.

These tables summarize the number of clones obtained from each OTUs from Lake Pavin (S1a) and Lake Aydat (S1b) clone libraries. For each OTU, the taxon, the closed relative with its percentage of sequence identity, the number of clones composing OTU from the classical approach and their corresponding sampling stations localization (see Figure 1), and the number and percentage of matching reads from the 454-pyrosequencing approach (both using fungi and eukaryote primers) are given.

Table S1a: Pavin

				Classical method					454-pyrosequence fungi				454-pyrosequence Eukaryote			
Taxon	**OTU**	**Closest relative (accession number)**	**Identity (%)**	**A**	**B**	**C**	**D**	**E**	**Pavin A**	**%**	**Pavin C**	**%**	**Pavin A**	**%**	**Pavin C**	**%**
Ascomycota	PA2009E2	*Volutella colletotrichoides* strain BBA 71246 (AJ301962.1)	99,8					1	0	0,0	6	2,4	0	0,0	2	0,2
	PA2009E3	*Gibberella fujikuroi* (AB237662.1)	99,7			1		1	2	0,6	0	0,0	4	0,4	1	0,1
	PA2009D1	*Simplicillium lamellicola* (AB214656.1)	99,5				1		18	5,3	2	0,8	13	1,3	6	0,5
	PA2009E4	*Sarcinomyces* sp. MA 4760 (AJ972809.2)	98,9					2	0	0,0	0	0,0	4	0,4	1	0,1
	PA2009A1	*Davidiella macrospora* strain CBS 138.40 (EU167591.1)	99,8	1					1	0,3	3	1,2	8	0,8	5	0,4
	PA2009E5	*Penicillium commune* (AF236103.1)	99,5					1	1	0,3	0	0,0	0	0,0	0	0,0
	PA2009A2	*Penicillium rugulosum* strain KCTC16050 (AF245232.1)	98,2	1					0	0,0	1	0,4	0	0,0	0	0,0
Basidiomycota	PA2009E1	*Exobasidium rhododendri* (AJ271381.1)	87,5					1	9	2,7	2	0,8	11	1,1	2	0,2
Chytridiomycota	PA2009E6	-	-	2	1	4		1	154	45,4	104	40,8	86	8,9	93	8,3
	PA2009C1	*Chytriomyces angularis* isolate AFTOL-ID 630 (AF164253.2)	93,6		1	1			1	0,3	1	0,4	0	0,0	5	0,4
	PA2009E7	Uncultured Chytridiomycota clone PFG9SP2005 (EU162638.1)	96,8					1	0	0,0	0	0,0	56	5,8	76	6,8
	PA2009C2	Uncultured Chytridiomycota clone PFG9SP2005 (EU162638.1)	97,0			1			1	0,3	0	0,0	66	6,8	93	8,3
	PA2009C3	Uncultured Chytridiomycota clone PFG9SP2005 (EU162638.1)	99,4		1	1	1		3	0,9	5	2,0	62	6,4	81	7,3
	PA2009E8	Uncultured Chytridiomycota clone PFH9SP2005 (EU162642.1)	95,4	5	2	1	2	2	72	21,2	52	20,4	47	4,8	45	4,0
	PA2009B1	Uncultured fungus clone PFH1AU2004 (DQ244009)	94,9		1				2	0,6	3	1,2	51	5,3	60	5,4
	PA2009E9	Uncultured fungus clone PFH1AU2004 (DQ244009)	96,0				1	2	20	5,9	27	10,6	91	9,4	118	10,6
	PA2009A3	*Entophlyctis* sp. JEL174 isolate AFTOL-ID 38 (AY635824.1)	96,1	1					21	6,2	22	8,6	6	0,6	5	0,4
	PA2009E10	Uncultured Chytridiomycota clone PFB4SP2005 (EU162636.1)	99,7	2	1	1		1	6	1,8	4	1,6	158	16,3	165	14,8
	PA2009E11	Uncultured Chytridiomycota clone PFD5SP2005 (EU162640.1)	98,6	2	5	5	1	3	16	4,7	15	5,9	264	27,2	287	25,7
	PA2009C4	-	-		3	1			5	1,5	4	1,6	22	2,3	36	3,2
	PA2009A4	-	-	1					0	0,0	0	0,0	13	1,3	15	1,3
	PA2009E12	-	-					1	2	0,6	1	0,4	1	0,1	2	0,2
	PA2009D2	-	-				1		5	1,5	3	1,2	7	0,7	18	1,6

Table S1a: Pavin continuation

Telonemida	PA2009A5	Uncultured eukaryote clone PTG4SP2005 (EU162631.1)	98,2	1	1		1		22		52		315		756
Ichtyosphora	PA2009E13	*Amoebidium parasiticum* strain ATCC 32708 (Y19155.1)	88,5					1	19		15		33		9
Cryptophyta / Katablepharidophyta	PA2009D3	Uncultured eukaryotic picoplankton clone P1.31 (AY642716.1)	99,1				1		4		9		0		1
	PA2009D4	*Plagioselmis nannoplanctica* strain N750301 (FM876311.1)	99,9				2		153		399		0		2
	PA2009D5	*Cryptomonas ovata* (AF508270.1)	99,6				1		57		102		0		0
	PA2009C5	Uncultured eukaryotic picoplankton clone P34.10 (AY642702.1)	98,5			1			2639		3374		6		12
	PA2009E14	Uncultured eukaryotic picoplankton clone P34.10 (AY642702.1)	99,6			1	2	1	1927		2233		6		12
Viridiplantae	PA2009E15	*Treubaria setigera* (U73475.1)	99,4	1	2	1		1	0		0		16		3
	PA2009A6	*Chlamydomonas acidophila* strain CCAP 11/137 (AJ852427.1)	97,7	2	2	2	2		0		0		54		193
	PA2009E16	*Chlamydomonas noctigama* isolate SAG 33.72 (AJ781311.1)	97,6		2	4	1	3	0		0		0		0
	PA2009B2	*Chlamydomonas noctigama* isolate SAG 33.72 (AJ781311.1)	96,8		1		1		0		0		23		108
	PA2009C6	*Chlamydomonas noctigama* isolate SAG 33.72 (AJ781311.1)	98,2			1			0		0		68		91
	PA2009E17	*Chlamydomonas sordida* (AB290341.1)	99,4	2		3		1	0		0		91		114
	PA2009B3	*Choricystis* sp. Pic8/18P-11w (AY197629.1)	95,6		1				0		0		80		67
	PA2009C7	*Micractinium pusillum* strain CCAP 248/3 (FM205875.1)	99,1			1			0		0		111		54
	PA2009A7	Uncultured eukaryote clone PG8AU2004 (FJ799981.1)	99,5	4	2	1	2		0		0		105		76
	PA2009D6	*Monoraphidium contortum* strain AS6-3 (AY846382.1)	99,8		1	1	1		0		0		553		266
	PA2009E18	*Mychonastes* sp. 5C5 (AF357153.1)	99,6	3	2	1	1	1	0		0		400		237
Stramenopiles	PA2009C8	*Pythium insidiosum* (AY486144.1)	96,7			1			0		0		3		3
Alveolata	PA2009E19	Uncultured choreotrichid ciliate clone CH1_2A_10 (AY821916.1)	98,9		1	1		2	46		52		40		38
	PA2009D7	*Pfiesteria*-like sp. CCMP1835 (AY590477.1)	97,5				1		0		0		0		0
	PA2009A8	*Gymnodinium beii* (U37406.1)	95,8	2					0		2		51		3
	PA2009C9	Uncultured alveolate clone PAD1SP2005 (EU162628.1)	99,6			1			0		0		4		22
TOTAL				30	30	36	23	27	5206		6493		2929		3183

Table S1b: Aydat

				Classical method					Metagenome fungi				Metagenome Eukaryote			
Taxon	**OTU**	**Closest relative (accession number)**	**Identity (%)**	**A**	**B**	**C**	**D**	**E**	**Aydat C**	**%**	**Aydat E**	**%**	**Aydat C**	**%**	**Aydat E**	**%**
Ascomycota	AY2009A1	*Paecilomyces farinosus* (AB080088.1)	99,6	1					1	0,1	2	1,6	5	0,4	5	4,0
	AY2009C1	*Simplicillium lamellicola* (AB214656.1)	99,4	6	1	1			10	1,2	10	8,1	13	1,0	5	4,0
	AY2009B1	*Simplicillium lamellicola* (AB214656.1)	99,4		1				2	0,2	1	0,8	6	0,5	3	2,4
	AY2009B2	*Myrothecium cinctum* strain BBA (AJ302004.1)	96,5		1				0	0,0	2	1,6	0	0,0	2	1,6
	AY2009C2	*Alternaria alternata* (AF218791.1)	99,2			1			0	0,0	0	0,0	1	0,1	0	0,0
	AY2009A2	*Acrospermum compressum* specimen-voucher UME 31704 (AF242258.1)	98,8	1					0	0,0	0	0,0	0	0,0	0	0,0
	AY2009D1	*Ramichloridium cerophilum* strain CBS 103.59 (EU041798.2)	99,3				1		0	0,0	0	0,0	2	0,2	2	1,6
	AY2009E1	*Candida dubliniensis* CD36 (FM992695.1)	95,8					1	0	0,0	0	0,0	0	0,0	0	0,0
Basidiomycota	AY2009A3	*Jamesdicksonia dactylidis* (DQ363310.1)	98,5	1					0	0,0	1	0,8	24	1,9	0	0,0
Chytridiomycota	AY2009B3	*Chytriomyces* sp. JEL341 isolate AFTOL-ID 1531 (DQ536482.1)	87,3		1				24	2,9	8	6,5	22	1,8	3	2,4
	AY2009C3	*Chytriomyces* sp. JEL341 isolate AFTOL-ID 1531 (DQ536482.1)	96,4			1			25	3,0	10	8,1	22	1,8	3	2,4
	AY2009A4	Uncultured marine eukaryote clone M2_18E09 (DQ103801.1)	97,4	1	1		2		0	0,0	1	0,8	0	0,0	0	0,0
	AY2009C4	*Rhizophydium* sp. JEL317 isolate AFTOL-ID 35 (AY635821.1)	90,3	1	1	3	2	1	701	85,3	21	16,9	1040	83,9	25	19,8
	AY2009D2	Uncultured Chytridiomycota clone PFD5SP2005 (EU162640.1)	98,5				1		4	0,5	0	0,0	24	1,9	3	2,4
	AY2009B4	*Chytriomyces angularis* isolate AFTOL-ID 630 (AF164253.2)	93,5		1				0	0,0	0	0,0	0	0,0	0	0,0
	AY2009D3	Uncultured Chytridiomycota clone PFH9SP2005 (EU162642.1)	95,6				1		15	1,8	11	8,9	17	1,4	8	6,3
	AY2009A5	Uncultured chytridiomycete clone CH1_2B_29 (AY821989.1)	98,0	1					13	1,6	17	13,7	16	1,3	8	6,3
	AY2009C5	Uncultured chytridiomycete clone CH1_2B_29 (AY821989.1)	98,0	1	1	2	1	1	15	1,8	32	25,8	24	1,9	30	23,8
	AY2009A6	Uncultured chytridiomycete clone CH1_2B_29 (AY821989.1)	97,9	1					12	1,5	8	6,5	24	1,9	29	23,0
Choanoflagellida	AY2009C6	*Monosiga brevicollis* (AF100940.1)	89,3			1			225		2		42		0	
	AY2009C7	*Monosiga brevicollis* (AF100940.1)	89,7			1	2		156		6		2		0	
	AY2009C8	*Monosiga brevicollis* (AF100940.1)	89,8			2		1	142		6		0		3	
Ichtyosphora	AY2009D4	*Ichthyophonus irregularis* (AF232303.1)	92,7				1		0		0		6		5	
	AY2009C9	*Amoebidium parasiticum* strain ATCC 32708 (Y19155.1)	98,4	1		1		2	16		16		56		7	
Cryptophyta	AY2009D5	Uncultured freshwater eukaryote clone LG08-05 (AY919707.1)	99,5				1		0		0		0		0	
	AY2009C10	*Plagioselmis nannoplanctica* strain N750301 (FM876311.1)	99,7			1		2	144		60		0		0	
	AY2009D6	*Teleaulax amphioxeia* strain K-0434 (AJ007287.1)	98,4				1		14		51		0		0	
	AY2009E2	*Cryptomonas ovata* (AF508270.1)	99,5					1	278		492		4		3	
	AY2009C11	*Cryptomonas curvata* strain CCAC 0080 (AM051189.2)	99,6		1	1	1		1247		2614		3		6	

Table S1b: Aydat continuation

	AY2009E3	*Cryptomonas curvata* strain CCAC 0080 (AM051189.2)	99,7					1	1466		3295		3		3	
Viridiplantae	AY2009A7	*Atractylodes japonica* (EU678363.1)	99,4	1					0		0		0		0	
	AY2009B5	*Ankyra lanceolata* strain Hg 1998-5 (AF302769.1)	99,1		1		1		0		0		24		5	
	AY2009B6	*Cephalomonas granulata* (AB472272.1)	98,0		1				0		0		82		4	
	AY2009C12	*Chlamydomonas noctigama* isolate SAG 33.72 (AJ781311.1)	96,5	3	3	4	4	2	0		0		963		66	
	AY2009D11	*Chlamydomonadaceae* sp. CCCryo 216-05 (GU117580.1)	98,0				2		0		0		169		4	
	AY2009C13	*Characiochloris sasae* strain: NIES-567 (AB360741.1)	96,0	2		1	1		0		0		195		16	
	AY2009B7	*Characiochloris sasae* strain: NIES-567 (AB360741.1)	92,9		1				0		0		195		16	
	AY2009B8	*Haematococcus pluvialis* (AF159369.1)	96,8		1			1	0		0		3		6	
	AY2009A8	*Chlamydomonas perpusilla* var. *perpusilla* (AB290339.1)	99,1	1					0		0		15		4	
Cercozoan	AY2009C14	Uncultured freshwater cercozoan clone PCB7AU2004 (DQ243999.1)	95,1			1			3		6		5		8	
Stramenopiles	AY2009C15	Uncultured stramenopile clone BAQA72 (AF372754.1)	92,2			1			0		0		2		5	
	AY2009B9	Uncultured stramenopile clone BAQA72 (AF372754.1)	87,7		1				0		0		14		8	
Alveolata	AY2009C16	*Ceratium hirundinella* (AY443014.1)	93,2			1			0		0		29		8	
	AY2009B10	*Cryptosporidium parvum* strain H7 (AF108865.1)	79,1		1				0		0					
	AY2009C17	Uncultured freshwater eukaryote clone LG15-10 (AY919736.1)	91,0			1		1	0		0		0		1	
	AY2009C18	Uncultured freshwater eukaryote clone LG15-10 (AY919736.1)	91,8			1		1	0		0		2		0	
	AY2009D7	*Stentor amethystinus* (EF492142.1)	96,9				1						0		0	
	AY2009D8	*Dileptus* sp. HCB-2005 (DQ487195.2)	97,5				1	1	0		0		0		0	
	AY2009B11	*Dileptus* sp. HCB-2005 (DQ487195.2)	98,9		1				0		0		0		0	
	AY2009D9	*Vorticella microstoma* (DQ868347.1)	97,3				1		0		0		0		0	
	AY2009A9	Uncultured oligohymenophorid ciliate clone CV1_2A_17 (AY821923.1)	93,4	1	1				0		1		7		13	
	AY2009A10	*Rimostrombidium lacustris* (DQ986131.1)	98,3	1					57		23		95		42	
	AY2009C19	Uncultured choreotrichid ciliate clone CH1_2A_10 (AY821916.1)	97,8			1			186		36		342		17	
	AY2009C20	Uncultured choreotrichid ciliate clone CH1_2A_10 (AY821916.1)	98,5			1		1	104		62		126		58	
	AY2009D10	Uncultured choreotrichid ciliate clone CH1_2A_10 (AY821916.1)	98,8				1		0		0		187		14	
	AY2009E4	Uncultured freshwater eukaryote clone LG17-12 (AY919741.1)	99,3					2	1		9		17		20	
	AY2009E5	-	-					1	4		6		28		18	
	AY2009C21	-	-	3	2	1	2	1	4		6		37		24	
	AY2009E6	-	-					1	0		0		167		73	

Table S1b: Aydat continuation

	AY2009C22	*Uroleptus pisces* (AF508780.1)	99,1			2			84		16		1340		13	
	AY2009C23	*Halteria grandinella* (AF508759.1)	98,4	1		2	1	1	53		44		167		70	
	AY2009C24	*Halteria grandinella* (AF508759.1)	98,0	3		3	2		53		44		304		158	
	AY2009E7	*Halteria grandinella* (AF508759.1)	98,2					1	32		30		64		21	
TOTAL				**31**	**22**	**35**	**31**	**24**	**5091**		**6949**		**5935**		**845**	

CHAPITRE III

Dynamique saisonnière de la structure génétique des Eumycètes et importance quantitative

Préambule

Dans le chapitre II, nous avions pour objectif principal l'étude de la diversité globale des Eumycètes dans trois écosystèmes pélagiques distincts, afin d'obtenir de meilleures connaissances sur les clades présents dans ces milieux. Le clonage puis le séquençage du gène codant pour l'ARN ribosomal 18S et de l'espace transcrit intergénique (Intergenic Transcrit Spacer, ITS) ont été réalisés au cours de deux saisons distinctes - au printemps (Avril/Mai 2007) et en été (Juillet 2008) - afin d'analyser la diversité mais aussi de constituer une banque de séquences conséquentes nécessaire pour développer des outils méthodologiques, comme des sondes oligonucléotidiques ciblant les clades fongiques d'intérêt. Pour répondre à ces deux problématiques, les amorces NS1 et ITS4 utilisées ont été choisies pour cibler l'ensemble des champignons (particulièrement les Chytridiomycètes) et pour obtenir les séquences 18S et ITS afin de pouvoir réaliser l'affiliation phylogénétique des séquences retrouvées mais aussi, de constituer une banque de séquences ITS.

Par ailleurs, dans la deuxième partie du chapitre II, nous nous sommes concentrés sur les deux lacs les plus riches en séquences fongiques, c'est-à-dire le lac Pavin et le lac Aydat, pour l'évaluation de la variabilité spatiale. Pour cela, l'approche classique par clonage/séquençage a été renforcée par une approche de pyroséquençage.

L'utilisation d'approches moléculaires s'est révélée bien adaptée pour cibler l'ensemble des Eumycètes présents. En effet, nous avons notamment montré la présence d'une diversité fongique non négligeable, répartie dans trois principales divisions : les Ascomycètes, les Basidiomycètes et les Chytridiomycètes. L'étude de la variabilité spatiale nous a également permis de montrer que l'influence des apports allochtones est relativement faible et dépend des écosystèmes étudiés. Cependant, ces deux études ont été ponctuelles, réalisées à des dates précises. Un autre facteur pouvant influencer la diversité dans nos lacs est l'évolution temporelle des populations. Ainsi, l'étude des changements saisonniers nous est apparue nécessaire pour compléter et renforcer les hypothèses découlant du chapitre II. Dans ce but, le chapitre III présente l'étude de la structure génétique de la communauté fongique réalisée par analyse du polymorphisme de longueur du fragment de restriction terminale (TRFLP) dans les trois lacs pilotes pendant une période de 9 mois. En parallèle, l'importance quantitative des trois principales divisions mises en évidence dans le chapitre II (i.e. Ascomycota, Basidiomycota et Chytridiomycota) a été réalisée par PCR quantitative en temps réel à l'aide d'amorces prises dans la littérature. Les résultats obtenus ont permis de préciser les périodes de l'année où des modifications significatives de la communauté sont observées ce qui donne des indications sur les préférences écologiques des champignons.

Le travail réalisé dans ce chapitre III a effectué en collaboration avec l'INRA de Dijon.

Genetic structure and quantitative dynamics of fungal communities in freshwater pelagic ecosystems

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Preliminary, *MS in preparation*

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Short Title: Fungal community dynamics in the plankton

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Abstract

Microbial community diversity and composition have critical biogeochemical roles in the functioning of aquatic ecosystems. Populations of planktonic fungi exist in pelagic freshwaters, yet their diversity and role in carbon and nutrient cycling remain largely unknown. This study present fungal community dynamic surveys, in three contrasting freshwater pelagic ecosystems, during nine months sampling period, for evidence of their ecological preferences. Fungal community composition was followed together with the quantitative importance of three main fungal divisions (Ascomycota, Basidiomycota and Chytridiomycota). Original results shown that in oligomesotrophic Lake Pavin, fungal community were complex during the sampling campaign, with 5 different periods of significant changes in the community genetic structure. On the contrary, in mesotrophic humic Lake Vassiviere and in the eutrophic Lake Aydat, fungal community composition changed only three times, with contrasting patterns in early spring and summer. These were linked to phytoplanktonic community diversity and species successions. The dynamic of fungi in Lake Pavin confirmed a peak of Chytridiomycota in spring, certainly due to the presence of diatoms parasites. Surprisingly, Basidiomycota seem to be also important in spring in Lake Pavin. In Lake Aydat, the three fungal divisions were present, with a succession from Basidiomycota in spring to Ascomycota and Chytridiomycota in autumn. Finally, in Lake Vassivière, fungi were restricted with only the detection of Chytridiomycota which peaked in August. These investigations were the first aiming to understand the fungal community seasonal composition and quantitative dynamics in their natural pelagic environments. The results revealed that fungal community changed several times during the year depending on the ecosystems, probably together with their putative functions (parasite and saprophytic).

Introduction

Fungi are exceptionally diverse with a potential of 0.7 to 1.5 million taxa on the Earth (Hawksworth, 2001; Schmit and Mueller, 2007), among which, only 74,000 to 120,000 species have so far been identified (Hawksworth, 2001). Fungi are assembled in a monophylic kingdom, called Eumycota, containing species which develop various life forms and colonize all types of ecosystems. Fungi inhabit aquatic as well as terrestrial environments, and are among the major players in biomes extending from tropical to polar zones (Hawksworth and Mueller, 2005), including the most extreme environments such as deep-sea hydrothermal (Le Calvez *et al.*, 2009), anoxic (Stock *et al.*, 2009) or hypersaline (Alexander *et al.*, 2009) ecosystems.

In freshwater ecosystems, Fungi have been well studied since the middle of the last century when Ingold's discovered tetraradiate and sigmoid conidia in foam which often accumulate in running waters (Ingold, 1942). Soon latter, an extensive description of Chytridriomycetes was provided by Sparrow (1960). Since then, aquatic fungi have been often studied in benthos (Burgaud *et al.*, 2009; Bhadury *et al.*, 2009; Bass *et al.*, 2007), streams (Bärlocher *et al.*, 2008; Duarte *et al.*, 2006; Fabre 1997; Jørgensen and Stepanauskas, 2009; Nikolcheva and Bärlocher, 2004, Sampaio *et al.*, 2007), or marine environments (Damare 2006; Damare *et al.*, 2008 ; Wang *et al.*, 2008; Gadanho and Sampaio, 2004; Gao *et al.*, 2008, 2009; Hagler and Mendonça-Hagler, 1981). In pelagic habitats, fungi may play significant role in the ecosystem functioning, primarily as parasites or saprophytes (Jobard *et al.*, 2010; Wurzbacher *et al.*, 2010). Nevertheless, previous detection of fungi in pelagic areas is scarce and we don't know, for example, how fungal populations are structured during a year in these ecosystems, where the temporal succession of species and the quantitative importance of fungus communities are about totally ignored. Such studies are needed for a better knowledge of the ecological exigencies and the functional significance of fungi in pelagic ecosystems, in relation with other biological communities and with the abiotic environment as well.

To our knowledge, no general investigation of fungal diversity and their quantitative importance in pelagic ecosystems has been conducted. The identification of fungi present in pelagic environments is a critical stage because of the great but as yet disregarded diversity among this kingdom (Shaerer *et al.*, 2006). The methodological difficulties are huge because the majority of fungal species are uncultivable. They are often cryptically present within living or non-living particles in natural environments, where various life stages exhibit distinct morphologies. In this context, molecular biology offers valid alternative approaches to study the diversity and the quantitative ecology of natural fungi, because uncultured microorganisms can be detected this way, directly from natural samples (Jobard *et al.*, *in press*; Lefèvre *et al.*, 2010). The fungal community can be targeted using specific oligonucleotide primers to amplify a DNA (often nuclear ribosomal small subunit genes) by polymerase chain reaction (PCR). Terminal restriction fragment length polymorphism (T-RFLP) analysis is a highly reproducible fingerprinting method that can be automated on a sequencer (Liu *et*

al., 1997) and allows detection of the genetic structure of natural communities at a given time, and the spatial and (or) temporal dynamics of the related diversity, using the T-RFLP profiles (Hartmann and Widmer, 2008). Although based on a polymerase chain reaction, T-RFLP technique has proved to be a suitable technique for monitoring highly diverse microbial communities for changes over time and space (Nocker *et al.*, 2007; Lukow *et al.*, 2000). This approach uses direct environmental DNA extracts, is rapid, and is currently applied to fungal communities in various environments, primarily in soil (Anderson and Cairney, 2004; Edel-Hermann *et al.*, 2004; Edel-Hermann *et al.*, 2008), lotic (Nikolcheva *et al.*, 2003; Nikolcheva and Bärlocher, 2005) or marine ecosystems (Walker and Campbell, 2010). In the present study, we used this fingerprinting method to follow the genetic structure and the seasonal dynamics of fungal communities in the euphotic layers of three freshwater lake ecosystems.

In the other hand, the quantification of fungi in natural environments provides important information on ecological preferences, since it informs on the preferential environmental conditions for fungal growth. Some methods, such as ergosterol measurements, are suitable for evaluating the biomass of higher fungi (i.e. Ascomycota and Basidiomycota) which contain ergosterol in their cellular membrane. However, Chytridiomycota, a group of typical pelagic fungi, lacks ergosterol (Gessner, 1997). A valid alternative method is real time quantitative PCR (qPC) for quantifying rRNA genes that are present in several copies in the genome, for evidence of the relative abundances of fungal communities in natural samples (Manter and Vivanco, 2007; Martin and Rygiewicz, 2005; Manerkar *et al.*, 2008; Lefèvre *et al.*, 2010; Fierer *et al.*, 2005). Furthermore, real-time PCR appears to be a robust and accurate method for the determination of fungal biomass in a mixed-template sample (Manter and Vivanco, 2007).

The objective of this study was to investigate changes in fungal community composition during a 9 months period of the year 2007. Genetic structure of total fungal community and quantitative dynamics of Ascomycota, Basidiomycota and Chytridiomycota and of total fungi were followed. Primer sets previously designed for the 3 targeted large divisions of fungi and for total fungi (Nikolcheva and Bärlocher, 2004; Borneman and Hartin, 2000; White *et al.*, 1990) were applied on our samples.

Materials and methods

Study sites and sampling. Samples were collected in 3 freshwater lakes, which differed in trophic status and were located in the French Massif Central. Lake Pavin (45°29’41’’N, 2°53’12’’E, alt. 1197 m) is an oligo-mesotrophic deep volcanic mountain lake (maximal depth= 98 m), characterized by small surface (44 ha) and small drainage basin (50 ha) areas. Lake Aydat (45°39’48’’N, 2°59’04’’E) is a small eutrophic lake (Zmax = 15.5 m, surface area = 60.3 ha), with a larger catchment area (3000 ha). Lake Vassivière (45°48’48’’N, 001°, 51’ 15’’E, alt. 650 m, Zmax = 34 m) is a large (976 ha) brown-colored humic and mesotrophic lake, moderately acidic, with a large

catchment area (23300 ha). For each of the 3 lakes, sampling point was located in a central location in the lake, at the point of maximum depth. Samples were collected monthly during 2007, from April to December. During all sampling occasions, 20 liters of the whole euphotic layers of the lake, estimated from Secchi depths (from the surface to 20 m deep down for Lake Pavin, and from the surface to 4.5 m deep down for Lake Aydat and Lake Vassivière), was sampled manually using a flexible plastic tube (diameter, 4 cm) provided by a rope connecting the ballasted bottom of the tube with a surface manipulator.

Samples were prefiltered on site through 150 µm pore-size filters, transferred into clean 20 l plastic carboys previously rinsed thoroughly with the lake water, and transported within an hour to the laboratory for immediate processing. Vertical profiles of temperature and dissolved oxygen were measured with multiparameter probe. In the laboratory, planktonic microorganisms were collected on 0.6 µm pore-size polycarbonate filters (47 mm diameter) using a vacuum pump (pressure < 100 mbar). 300 ml of water from Lake Pavin and 150 ml from Lakes Aydat and Vassivière were filtered. Tree replicate filters per sampling date were done and separately placed into 2ml tubes and stored at -80°C until DNA extraction.

Nucleic acid extraction. Total environmental DNA was extracted using the kit NucleoSpin® Plant DNA extraction Kit (Macherey-Nagel, Düren, Germany) adapted to fungal material, following different steps. An initial step was proceeded to digest fungal chitin wall. Filters with microorganisms cells were incubated with 500 µl of a buffer containing 400 Unit of lyticase enzyme (Sigma, NSW, Australia), in a sorbitol based buffer containing 0.1 M sorbitol, 100 mM Tris–HCl, 100 mM EDTA, and 14 mM β-mercaptoethanol, pH 8.0, and digested overnight at 30°C. Sodium Dodecyl Sulfate (SDS, 1% final concentration) and proteinase K (0.1 $mg.ml^{-1}$) were added during 1 hour at 37°C for protein digestion and to release DNA. All subsequent DNA extraction steps were as recommended by the manufacturer's instructions. DNA elution was performed in 2 fold 50 µl of elution buffer instead of in 100 µl at one time. The triplicates filters for each of the sampling dates were extracted independently.

Terminal Restriction length polymorphism (T-RFLP) analysis. Changes in the genetic diversity of the fungal assemblages during the sampling year were assessed by T-RFLP analysis of 18S rDNA fragments obtained after amplification with a fungal specific primer, digestion of amplicons with *Msp*I endonuclease, and fragment separation using capillary sequencer. The following steps of the T-RFLP analysis were performed as previously described (Edel-Hermann *et al.*, 2008).

PCR conditions and purification of amplicons. The fungal specific 18S primers D3-nu-SSU-0817-5' (TTAGCATGGAATAATRRAATAGGA) and nu-SSU-1536-3' (AATTGCAATGCYCTATCCCCA) (Borneman and Hartin, 2000) were used for direct amplification of about 762 bp fragments of fungal nuclear small subunit rDNA from water. The PCR mixture (25

µl) contained between 10 to 100 ng (depending on sample) of environmental DNA as template, 200 µM of each dNTP, 0.25 µM of each primer, 2 units of *Taq* DNA polymerase (Q-BIOgen, Evry, France), PCR buffer supplied with the enzyme (10 mM Tris-HCl [pH 9.0] at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 $mg.ml^{-1}$ bovine serum albumin), and 500 ng of T4 gene 32 protein (Q-BIOgen). One of the primer (i.e nu-SSU-0817-5') was labelled at the 5' end with the fluorescent dye D3 (Beckman Coulter, Fullerton, CA, USA). Reactions were performed in an automated Mastercycler (Eppendorf, Hamburg, Germany) with an initial denaturation of 3 min at 94°C, followed by 35 cycles of denaturation (1 min 94°C), primer annealing (1 min 56°C), extension (1 min 72°C), and a final extension of 10 min at 72°C. Three to 9 PCR per sample were done depending on the amplification efficiency. Aliquots of 2 µl of PCR products were checked by electrophoresis in 2 % agarose gels and stained with ethidium bromide to control the amplification and fragments size.

PCR products were purified with MinElute PCR Purification Kit (Qiagen, Courtaboeuf, France) following the manufacturer instructions, with 2 final elutions of 10 µl. Purified PCR products were quantified by comparison with known quantities of the molecular mass marker Smart ladder (Eurogentec, Seraing, Belgium) in 2% agarose gels stained with ethidium bromides. Gels were photographed under camera and staining intensity of the bands were measured with Bio-1D++ software (VilberLourmat, Marne La Vallée, France). The DNA concentrations in the PCR products were calculated using a standard curve of 10-100 ng of calf thymus DNA versus intensity.

PCR product digestion. 120 ng of PCR products were digested with 12 U restriction endonuclease *Msp*I (C'CGG) in 100 µl final volume for 3 h at 37°C in the dark. Restriction digests were precipitated with 2 µl of 2.5 $mg.ml^{-1}$ glycogen (Q-BIOgen), 10 µl of 3 M sodium acetate (pH 5.2), and 250 µl of ice-cold ethanol. The mixtures were centrifuged for 15 min at 15000 x g at 4°C, rinsed twice with 200 µl of ice-cold 70% ethanol, and resuspended in 63 µl of sample loading solution (SLS, Beckman Coulter). Samples were mixed with 1.2 µl of Size Standard-600 (Beckman Couter) and 28.8 µl of SLS and loaded onto a capillary electrophoresis sequencer CEQTM 2000XL (Beckman Coulter). Analyses were run with the Frag4-30sec-70 min method, including a denaturation of 2 min at 90°C and injection at 2000 V during 30 s and separation at 4800 V during 70 min. After electrophoresis, the length and the signal intensity of the fluorescently labelled TRFs were automatically calculated by comparison with the standard, using the CEQ 2000XL DNA analysis system version 4.3.9. Fragments under 60 bp and above 640 bp were excluded. Three electrophoretic migrations were done, given 3 similar profiles which were pooled.

For each sample, fluorescence intensity of each pic was divided by total fingerprint profil fluorescence to obtain a percentage of pic fluorescence. Fragments were separated into size classes which differed by more than 1.25 bp. The comparison of TRF size between samples was automated by assigning them to discrete categories using the program Lis (Mougel *et al.*, 2002).

Real-time PCR (qPCR) analysis of natural samples. Four sets of primers were used to estimate rDNA gene copy numbers of fungi present in our temporal series of samples from the 3 lakes. Primer Forward 5.8SR (Vilgalys and Hester, 1990**)** was used in all reactions and reverse primer was chosen following the taxon targeted. The 4 primers were named ITS4 (White *et al.*, 1990), ITS4Asco, ITS4Basidio, and ITS4Chytrid (Nikolcheva *et al.*, 2004), used to target Fungi (i.e. the total community), and Ascomycota, Basidiomycota, and Chytridiomycota divisions, respectively. PCR reactions were performed in duplicate in a 50 µl of mixture containing 1 pmole/µl of each primer, 0.2 mmol/L of each dNTP, 3 nmol/µl of $MgCl_2$, 0.5 mg/ml of bovin serum albumin (BSA), 0.5X SYBRgreen, 2.5 U of Taq DNA polymerase (Biotaq, Bioline UK. Ltd, London), and the PCR buffer supplied with the enzyme. Quantitative PCR (qPCR) reactions were carried out using a Mastercycler ep realplex detection system (Eppendorf) programmed with an enzyme activation step (95°C, 3 min), 35 cycles of 1 min of denaturation at 95 °C, an annealing-extension at 55°C for 30 sec, and at 72 °C for 1 min. Data collection was performed at 80°C for 20 sec, and a terminal extension was done at 72°C for 10 min. To confirm the absence of primer dimers, immediately following each qPCR assay, a melting curve analysis was performed by increasing the incubation temperature from 60 to 95°C for 20 min.

Three linear plasmids from cloned 18S rDNA for *Candida albicans* (Ascomycota), *Rhodothorula* sp (Basidiomycota) and *Rhizophydium* sp (Chytridiomycota) were constructed and used as standards for qPCR assays. The full ITS rRNA gene from representative strains for each genus was amplified with specific fungal primer ITS1F (Gardes and Bruns, 1993) and reverse primer depending on the fungal division of interest (see previously). Amplified fragments were cloned using the TopoTA cloning kit (Invitrogen, Cergy Pontoise, France), according to the instruction of the manufacturer. Plasmids were extracted using NucleoSpin® plasmid extraction kit (Macherey Nagel) following manufacturer's recommendations. Linearized plasmids were produced from supercoiled plasmid by digestion with the restriction endonuclease *Xba*I (New England Biolabs Beverly, MA, USA), according to the manufacturer's protocol. The concentration of genomic DNA from linear plasmids was measured using a spectrophotometer NanoDrop (NanoDrop Technologies, Inc, Wilmington, USA). Serial 10-fold dilutions of the plasmid were made, ranging from 1 to 10^9 dilution factors corresponding to 1×10^9 to 1 copy.μl^{-1}, respectively. The number of copies in the standard was calculated using the equation: Molecules.μl^{-1} = a/[3931+b]× 660×6.022.10^{23}, where a is the plasmid DNA concentration (g.μl^{-1}), 3931 the plasmid length without the cloned rDNA insert, b the ITS rDNA insert length in bp, 660 the average molecular weight of one base pair, and 6.022×10^{23} the Avogadro constant (Zhu *et al.*, 2005).

Data analysis. Principal Component Analysis (PCA) considers both the number and the intensity of TRF and provides an ordination of the communities in a two-dimensional plot. Cluster analysis was

applied to test the similarity among samples for the presence and relative abundances of fungi. The multivariate statistical analysis was applied with the XLStat software.

Results

Physico-chimical environments. The 3 lakes sampled were different in their physico-chimical parameters, primarily regarding temperature, transparency and pH (Table 1). Lake Vassivière was acidic and exhibited the lowest pH but the warmest water column, compared to Pavin and Aydat. The latter lake was the less transparent, followed by Vassivière and Pavin. The euphotic depths of the three lakes were well oxygenated during the sampling period, with oxygen concentrations comprised between about 4 and 12 $mg.l^{-1}$ (Table 1).

Table 1: Environmental parameters recovered during the sampling campaign 2007 in Lake Pavin, Aydat and Vassivière.

Sampling dates	Temp (°C)	Transparence (m)	pH	O_2 (mg/l)
Pavin				
04/04/2007	3.8	2.9	6.7	4.68
02/05/2007	13.6	3.0	8.3	10.44
12/06/2007	14.9	9.6	8.3	8.81
11/07/2007	13.7	8.5	9.02	6.31
08/08/2007	17	7.0	9.0	9.2
04/09/2007	14.4	5.0	8.45	8.45
02/10/2007	13.6	6.5	8.13	9.98
16/11/2007	4.1	5.0	9.15	12.11
12/12/2007	3.6	7.3	5.63	11.01
Mean (SD)	**11 (5.5)**	**6.1 (2.3)**	**9.1 (1.2)**	**9(1.7)**
Aydat				
13/04/2007	12	ND	8.83	11.32
10/05/2007	13.48	1.7	10.51	10.41
08/06/2007	15.73	1.8	10.51	8.59
04/07/2007	15.7	1.4	ND	5.46
21/08/2007	17.2	1.1	9.22	7.02
12/09/2007	16.2	0.8	9.32	11.38
10/10/2007	14	0.9	ND	7.54
07/11/2007	9.8	1.15	8.37	9.63
04/12/2007	5.3	1.4	7.3	9.65
Mean (SD)	**13.3 (3.8)**	**1.3 (0.4)**	**9.2 (1.1)**	**9 (2.3)**
Vassivière				
11/04/2007	21.6	2.9	6.33	5.77
03/05/2007	17.9	2.9	ND	5.7
13/06/2007	21.1	2.7	6.7	8.92
18/07/2007	19.9	2.5	7.35	8.9
02/08/2007	22.3	2.4	ND	8.74
11/10/2007	15.3	2.3	6.25	7.83
22/11/2007	7.5	1.4	6.91	10.64
19/12/2007	5	1.9	5.2	11.85
Mean (SD)	**16.3 (6.6)**	**2.3 (0.5)**	**6.5 (0.7)**	**8.5 (2.1)**

Seasonal genetic structure of fungal community. During our investigation, the numbers of ribotypes of the total fungal community (i.e. the numbers of TRF) detected were about the same in the 3 lakes, the ranges being at 9-28, 12-26, and 13-31, and the mean numbers at 19.5 ± 4.7, 18.3 ± 3.5, and 18.8 ± 5, for Lakes Pavin, Aydat, and Vassivière, respectively. However, the seasonal dynamics of

fungal ribotypes appeared different from one lake to another (Figure 1). In Lake Pavin, the number of TRF was high in spring (April-June), decreased in summer and early autumn (July-October), and then increased in late autumn (November, December). This contrasts with Lake Aydat where values increased in spring, peaked in summer (July), and decreased toward autumn. In Lake Vassivière, the absence of samples in June and September obscured the seasonal trend; the number of fungal phylotypes reached their maxima in August and December, and seemed to increase in spring toward summer, and during autumn. For all the three lakes, a consistently large number of fungal ribotypes was recorded in early winter, i.e. in December (Figure 1).

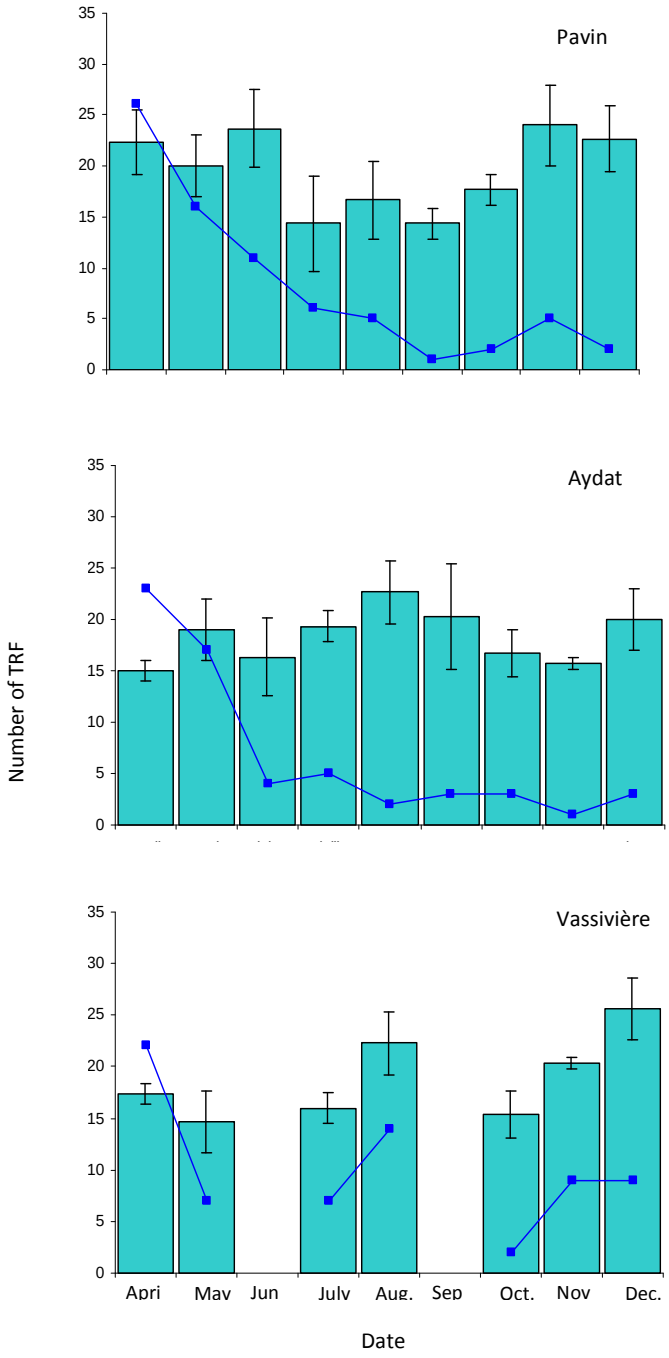

Figure 1: Seasonal dynamic of TRF number (Sticks) in the 3 Lakes Pavin, Aydat and Vassivière. The inserted curve gives the number of new ribotypes recovered per month.

The occurrence of new fungal ribotypes in our samples, i.e. ribotypes which were never recovered previously, was monitored, for evidence of the establishment of new fungal genetic populations (Figure 1). New ribotypes were retrieved in all samples for the three lakes. In Lakes Pavin and Aydat, their numbers naturally decreased with sampling effort, from April to September in Pavin, but earlier (in June) in Lake Aydat, before stabilization. In Vassivière, the discontinuity in sampling effort obscured the seasonal pattern of new ribotypes, but as in Pavin and Aydat, weak peaks were noted for one or the two last samples (i.e. November, December).

Principal Component Analysis (PCA) which considers both the number and the intensity of TRF and provides an ordination of natural communities in a two-dimensional plot revealed that fungal community responses to the sampling time were different for the 3 lakes (Figure 2). For the 3 lakes, the percentages of the variabilities accounted for by the two first axes were at 56.25%, 55.06%, and 63.44% for Lakes Pavin, Aydat and Vassivière, respectively. In Lake Pavin, different fungal communities were characteristics of 5 periods during the sampling year: April, May/June/August, July/December, September/October and November (Figure 3). For Lakes Aydat and Vassivière, the apparent variability in the fungal community composition was less marked compared to Pavin, with only 3 distinct periods where different communities were characteristics of April/May/June/December, July/August, and September/October/November for Aydat, and of April, May/July/August/October/December, and November, for Vassivière (Figure 3).

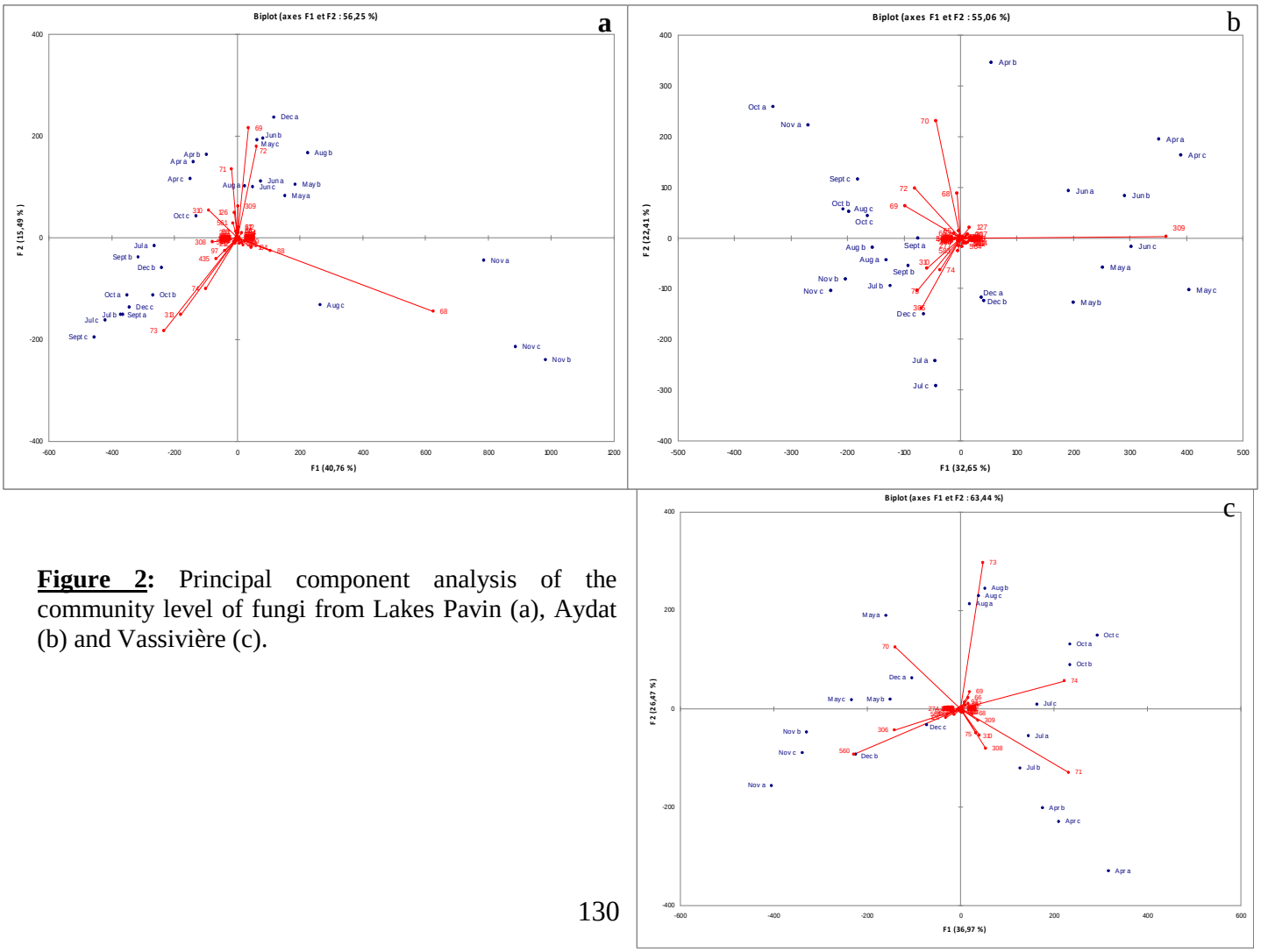

Figure 2: Principal component analysis of the community level of fungi from Lakes Pavin (a), Aydat (b) and Vassivière (c).

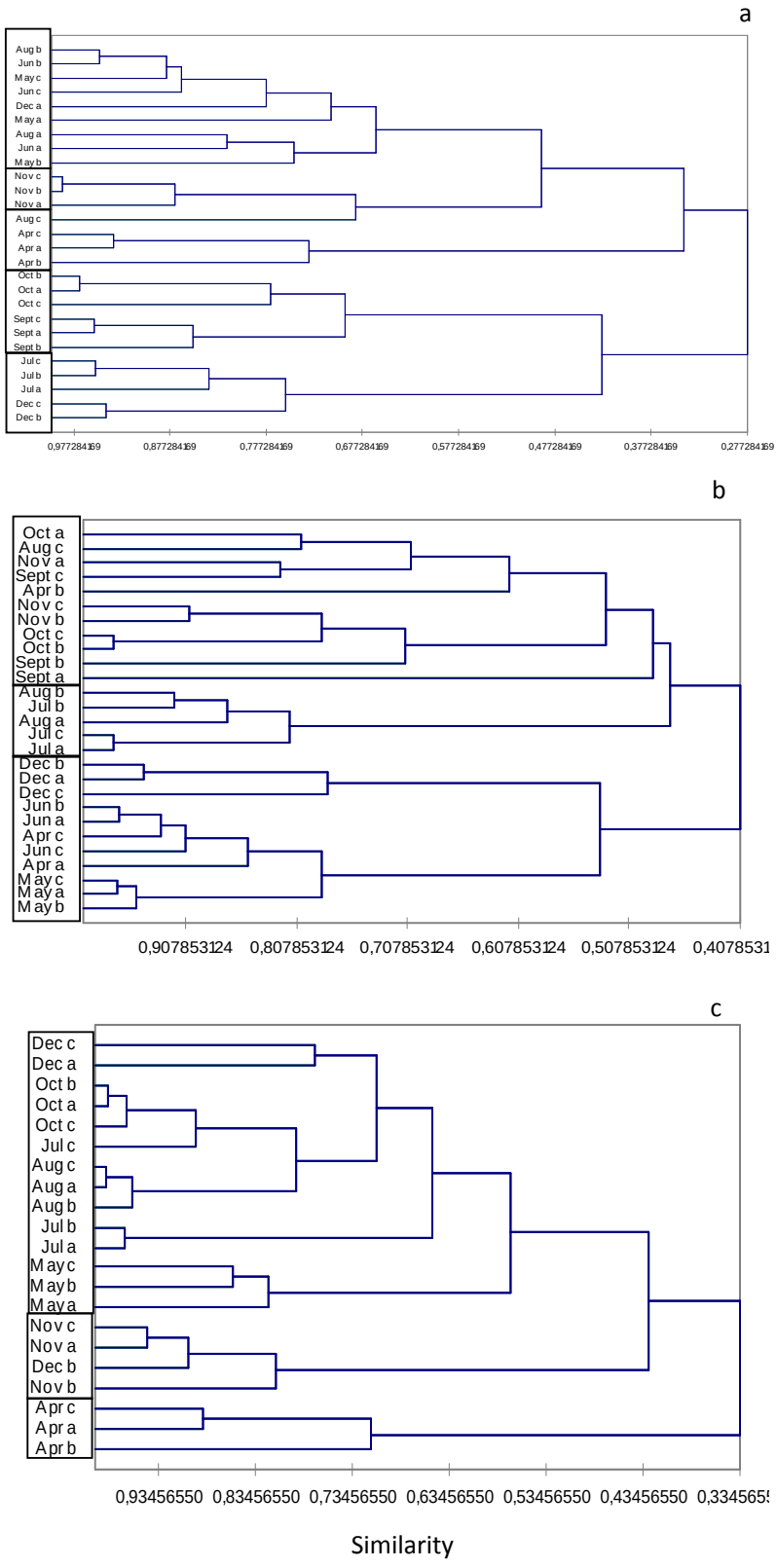

Figure 3: Cluster analysis for the discrimination of different periods for the genetic structure of fungi in Lakes Pavin (a), Aydat (b) and Vassivière (c).

Real-time PCR quantification. The use of primers targeting the whole kingdom Fungi and 3 major divisions provided estimates of the relative abundances of total fungi, Ascomycota, Basidiomycota, and Chytridiomycota in the 3 environments under study. The DNA copies from total fungi decreased from Lakes Pavin to Aydat and Vassivière, and peaked in spring in the former lake and in summer in both Lakes Aydat and Vassivière (Figure 4).

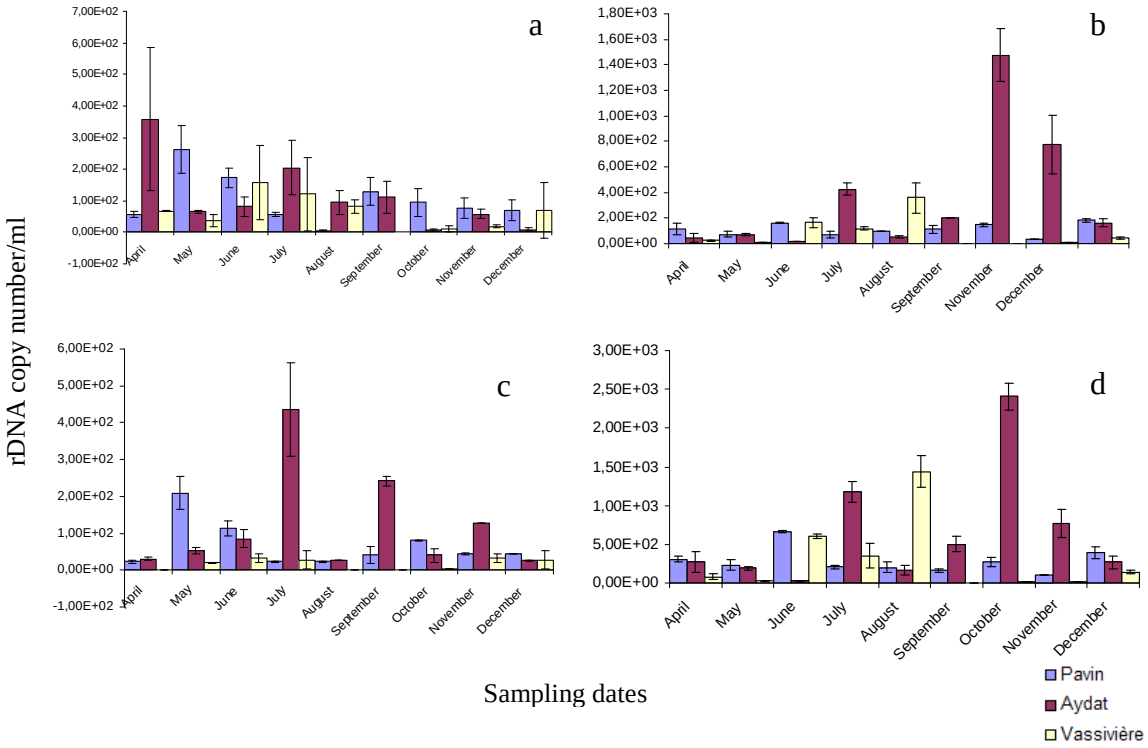

Figure 4: Dynamics of rRNA gene copy of total fungi (a), Ascomycota (b), Basidiomycota (c) and Chytridiomycota (d) in Lakes Pavin, Aydat and Vassivière during our sample campaign from April to December 2007**.**

This result was likely linked to fungal community composition that differed between the lakes. Indeed, in Lake Vassivière, although the occurrence of fungal DNA copies was scarcer compared to the other lakes, with almost no copies recorded in June, and October-November, the fungal community of this lake was dominated by Chytridiomycota which peaked in late summer, while Basidiomycota and Chytridiomycota in Lake Pavin peaked in spring. The three fungal divisions were present in Lake Aydat, with a succession from Basidiomycota in spring to Ascomycota and Chytridiomycota in autumn (Figure 4).

Application of qPCR method allowed the investigation of fungal community dynamics and these of Ascomycota, Basidiomycota and Chytridiomycota, with the same method. According to Zhu and collaborators (Zhu *et al.*, 2005), there is a good correlation between rDNA gene copies and cell size suggesting that qPCR data could be used as biomass estimation. Nevertheless, our quantification

results must be interpreted with care because we don't know the number of rDNA gene copies per targeted organisms.

Discussion

Fungal community composition. The dynamics of the fungal communities in the lakes investigated were different from one lake to another during the sampling year, but we detected approximately the same number of TRF in all lakes, considered as distinct ribotypes. T-RFLP analysis of ribosomal RNA genes is rapid, reproductible and allows assessing changes in the community structure of fungi in their environment. Our results suggest that fungus community was more particular in April for Lakes Pavin and Vassivière in terms of composition. This period of the year appear to be characterized by the spring development diatoms (Rasconi, 2010), known to produce high concentrations of transparent exopolymer polysaccharides (Arnous *et al.*, 2010, Carrias *et al.*, 2002). Spring period thus likely offers niches for both saprophytic and parasitic fungi, through the availability of organic aggregates and living diatom hosts. Indeed, it has been recently shown that intensely parasitized spring diatoms by fungal chytrids are characteristic of the euphotic layer of Lake Pavin (Rasconi, 2010; Rasconi *et al.*, 2009). Saprophytic fungi have been also detected colonizing particulate organic matter (POM), although it was difficult to quantifying the significance of this activity in the water column (Jobard *et al.*, 2010; Raghukumar, 2004).

T-RFLP method lead to the distinction of 5 periods for Lake Pavin and 3 for Lakes Aydat and Vassivière, when distinct fungal ribotype populations were observed. The enhanced fungal dynamic in Lake Pavin contrasts with the fact that this lake is weakly exposed to allochtone influences because of the absence of inflowing river and the low human impact. In this deep crater lake, fungal parasites, primarily chytrids, have been detected by both cloning sequencing and pyrosequencing of the 18s rRNA genes, and by microscopic observations (Lefèvre *et al.*, 2007, 2008; Lepère *et al.*, 2006, Rasconi *et al.*, 2009, see chapter II), and shown to have a significant impact on the seasonal development of phytoplanktonic communities (Rasconi *et al.*, 2009, 2010). Compared to Lake Pavin, Lake Aydat is a eutrophic environment with more human influence, characterized by 3 periods where the genetic structure of fungal community changed.

In contrast to Pavin, the summer periods appear to be the period of maximum fungal diversity in Aydat, with the occurrence of ribotypes 71, 305, 309, 310 (Figure 2). In Lake Aydat, this period is characterized by hard blooms of cyanobacteria, primarily of *Anabaena flosaquae*, which are intensely parasitized by the chytrid *Rhizosiphon* spp (Rasconi *et al.*, 2009), which certainly accounted for the seasonal changes in the genetic structure of fungi. It is thus likely that the differences between Pavin and Aydat in terms of the dynamics of fungal composition are probably related to the differences in the community composition of the main hosts, as a major forcing factor. This is because fungal-host relationships are generally highly species-specific (Ibelings *et al.*, 2004). Indeed, corroborating the

complexity of fungal communities, the phytoplankton community composition is more complex in Lake Pavin, with a complete series of species succession stages from small (pioneer stage) to large diatoms (intermediate stage), and then to diversified diatom and chlorophyte communities (mature climax stage), thriving in early spring, late spring, and autumn, respectively (Rasconi, 2010). In Lake Aydat, the annual phytoplankton community displayed a juvenile typology, dominated by small size species or by a hard monospecific bloom of the cyanobacterium *Anabaena flosaquae* in summer (Rasconi, 2010), thus offering less niches for the development of complex fungal populations.

The quantitative dynamics of fungal communities. Application of qPCR method allowed the investigation of fungal community quantitative dynamics. The 3 fungal divisions followed during our survey seem to differ quantitatively from one lake to another. Indeed, the maximum rDNA copies were recorded in spring and summer for total community and Basidiomycota in Pavin and Aydat, in summer for Chytridiomycota in Vassivière, and in summer and autumn for Ascomycota and Chytridiomycota in Aydat (Figure 4). These quantitative dynamics were thus also apparently related to those of the phytoplankton hosts of fungi, as discussed previously. Fungi are strict heterotrophs and need hosts (for parasites) or dead organic matters (saprophytes) to growth. However, few fungal rRNA gene copies were detected in April in the three lakes investigated, which may be related to the association of fungi with their algal hosts, which are mainly large size inedible diatoms. The fate of this host is mainly through sinking and destruction by parasites later in the season (Rasconi *et al.*, 2009; Ibelings *et al.*, 2004; Kagami *et al.*, 2007).

The abundances of Ascomycota were relatively stable during the sample period in Lakes Pavin and Vassivière. In Lake Aydat, Ascomycota peaked in October and November, likely in relation with the saprophytic competence of fungi. Indeed, intense colonisation of POM by mycelial fungi has been observed in this lake in November 2007 (Jobard *et al.*, 2010). High diversity of fungi following a cyanobacteria bloom was also recently observed in a eutrophic lake in China (Chen *et al.*, 2010). In this paper, different species of Ascomycetes (*Cladosporium cladosporioides*, *Candida atlantica*), Basidiomycetes (*Filobasidium globisporum*, *Bannoa hahajimensis*) and Chytridiomycetes (*Kappamyces laurelensis*, *Rhizophydium* sp) were detected with cloning sequencing methods, following bloom of *Microcystis sp* in mesocosm experiments (Chen et al., 2010).

Conclusions

Our survey is the first to investigate the whole fungal community composition and quantitative dynamics in 3 contrasting pelagic ecosystems during 9 months. The main findings suggest that the community composition and genetic structure, and the related functional role of fungal communities, depend on the ecosystem properties and on the availability of hosts. Fungal community composition was not stable during the sample year, and seemed to be linked to other organisms, primarily to

phytoplanktonic community which provide the most suitable living and non living resources for fungal development. Indeed, Chytridiomycota, known as fungal parasites of phytoplankton, seemed to be more important in oligotrophic conditions, whereas Ascomycota were more important in the eutrophic environments. Quantitative investigations shown that Ascomycota species were more abundant during autumn, whereas Basidiomycota and Chytridiomycota were present all over the sampling period, with enhanced development in late spring and during summer. These results thus strengthen the idea that fungi represent an important compartment in pelagic ecosystems. Future investigations have to be undertaken to explore the unknown functional diversity within these communities.

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CHAPITRE IV

Développement d'outils pour la quantification des champignons en milieux pélagiques - Applications en Hybridation Fluorescente *In Situ* et en PCR quantitative en temps réel

Préambule

Dans les chapitres I, II et III, nous avons vu qu'il était important de considérer les champignons comme des acteurs à part entière dans le fonctionnement des écosystèmes pélagiques lacustres. En effet, leur diversité phylogénétique et fonctionnelle se sont révélées plus importantes que celles décrites précédemment dans d'autres études. La stratégie d'étude, avec l'utilisation d'approches moléculaires, s'est révélée bien adaptée pour cibler l'ensemble des Eumycètes présents, sans à priori. Ceci nous a permis de mettre en évidence trois principaux phyla caractéristiques de l'écosystème pélagique des lacs étudiés. Cependant, les amorces utilisées (empruntées à la littérature) se sont révélées peu spécifiques, ciblant d'autres phyla de microorganismes comme des algues. Ceci n'est pas souhaitable dans des milieux où les blooms d'algues sont fréquents, apportant une grande quantité de microorganismes non ciblés et générant une source de biais potentiels non négligeables en écologie des champignons.

Ainsi, ce dernier chapitre a pour objectif, le développement d'outils moléculaires adaptés pour nos futures études, afin de cibler les clades d'intérêt rencontrés dans les milieux aquatiques. Il présente deux études de mise au point de méthodes moléculaires pour détecter et quantifier des champignons pélagiques. Nous avons dessiné un couple d'amorces oligonucléotidiques ciblant des champignons, connus comme parasites de nombreuses espèces d'algues. Leur importance a été négligée depuis des années, notamment en raison du manque d'outils adaptés à leur étude dans l'environnement. En effet, aucun couple d'amorces n'est disponible aujourd'hui pour étudier ces champignons, dominants dans les écosystèmes pélagiques (Rasconi *et al.*, 2009 ; Lefèvre *et al.*, 2010).

Le couple d'amorces dessiné a été développé dans le but d'une utilisation en PCR quantitative en temps réel (qPCR) et en Hybridation Fluorescent *in situ* (FISH). Ces deux méthodes permettent notamment le suivi complet et rapide de l'abondance totale des champignons ciblés (par qPCR), mais aussi la détection de ces champignons *in situ* sous forme de sporanges associé à leurs hôtes phytoplanctoniques et sous forme de zoospores à l'état libre dans le milieu (par FISH). Après les mises au point nécessaires des conditions d'utilisation du couple d'amorces dessiné, les deux méthodes ont été validées à l'aide d'échantillons environnementaux.

Première partie

Development of a Real-Time PCR assay for quantitative assessment of uncultured freshwater zoosporic fungi

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Development of a Real-Time PCR assay for quantitative assessment of uncultured freshwater zoosporic fungi

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Abstract

Recently, molecular environmental surveys of the eukaryotic microbial community in lakes have revealed a high diversity of sequences belonging to uncultured zoosporic fungi. Although they are known as saprobes and algal parasites in freshwater systems, zoosporic fungi have been neglected in microbial food web studies. Recently, it has been suggested that zoosporic fungi, via the consumption of their zoospores by zooplankters, could transfer energy from large inedible algae and particulate organic material to higher trophic levels. However, because of their small size and their lack of distinctive morphological features, traditional microscopy does not allow the detection of fungal zoospores in the field. Hence, quantitative data on fungal zoospores in natural environments is missing. We have developed a quantitative PCR (qPCR) assay for the quantification of fungal zoospores in lakes. Specific primers were designed and qPCR conditions were optimized using a range of target and non-target plasmids obtained from previous freshwater environmental 18S rDNA surveys. When optimal DNA extraction protocol and qPCR conditions were applied, the qPCR assay developed in this study demonstrated high specificity and sensitivity, with as low as 100 18S rDNA copies per reaction detected. Although the present work focuses on the design and optimization of a new qPCR assay, its application to natural samples indicated that qPCR offers a promising tool for quantitative assessment of fungal zoospores in natural environments. We conclude that this will contribute to a better understanding of the ecological significance of zoosporic fungi in microbial food webs of pelagic ecosystems.

Keywords: Aquatic chytrids**,** Fungal zoospores**,** Pelagic food webs**,** Quantitative PCR, Microbial ecology.

Introduction

Recently, molecular environmental surveys of the eukaryotic microbial community in the pelagic zone of lakes have revealed an unexpectedly high diversity of undescribed zoosporic fungi, i.e. chytrids (Lefranc *et al.*, 2005; Lefèvre *et al.*, 2007, 2008; Chen *et al.*, 2008; Lepère *et al.*, 2008). Although zoosporic fungi are commonly observed in freshwater systems as saprobes on various organic substrates (Czeczuga *et al.*, 2002, 2005; Czeczuga and Muszynska, 2000; Kiziewick and Kurzatkowska, 2004; Kiziewickz and Napela, 2008), and as parasites on various phytoplanktonic species (see review by Kagami *et al.*, 2007a), they are totally neglected in microbial food web studies. Recently, Kagami *et al.*, (2004, 2007b) showed that fungal zoospores (i.e. flagellated fungal spores) could be efficiently grazed upon by filter feeder zooplankters. Hence, zoosporic fungi, via the consumption of their zoospores, might transfer energy from large inedible algae and particulate organic material to higher trophic levels (Kagami *et al.*, 2004, 2007a, b; Lefèvre *et al.*, 2008). However, due to their small size (2-5 µm) and their lack of distinctive morphological features, traditional microscopic methods are not sensitive enough to detect fungal zoospores among a mixed assemblage of microorganisms. Recently, it has been suggested that fungal zoospores may have been misidentified as bacterivorous heterotrophic flagellates (HF) (Kagami *et al.*, 2007a; Lefèvre *et al.*, 2007, 2008). Indeed, the proportion of 'small unidentified HF' in lakes is unusually high, ranging from 10 to 90% of the total HF abundance (Carrias *et al.*, 1998; Cleven and Weisse, 2001; Domaizon *et al.*, 2003; Comte *et al.*, 2004; Sonntag *et al.*, 2006). As suggested by Lefèvre *et al.* (2008), if a portion of these unidentified HF corresponded to fungal zoospores, saprobes and parasitic fungi could be quantitatively important in pelagic systems. Unfortunately, because specific methodology for their detection is not available, quantitative data on fungal zoospores are missing.

Molecular approaches have profoundly changed our view of eukaryotic microbial diversity, providing new perspectives for future ecological studies. Among these perspectives, linking cell identity to abundance and biomass estimates is highly important for studies on carbon flows and the related biogeochemical cycles in pelagic systems. Historically, taxonomic identification and estimation of *in situ* abundances of small aquatic microorganisms have been difficult. In this context, our inability to identify and count many of these small species in the natural environment, limits our understanding of their ecological significance. Thus, new tools that combine both identification and quantification need to be developed. Fluorescent *in situ* hybridization (FISH) has been an assay of choice for simultaneous identification and quantification of specific microbial populations in natural environments (Lim, 1996; Lim *et al.*, 1993, 1996, 1999; Amann *et al.*, 1995; Massana *et al.*, 2002; Lefèvre *et al.*, 2005). However, this technique is limited because of (i) the relatively low number of samples that can be processed at a time, and (ii) its relatively low sensitivity due to the potentially low number of target rRNA molecules per cell in natural environments (Moter and Göbel, 2000). In contrast, quantitative PCR (qPCR), which has been widely used to estimate prokaryotic and eukaryotic

population abundances in marine systems (Audemard *et al.*, 2004; Skovhus *et al.*, 2004; Zhu *et al.*, 2005; Coutway and Caron, 2006; Sub *et al.*, 2006; Park *et al.*, 2007), allows the simultaneous analysis of a high number of samples with a high degree of sensitivity (Klein, 2002).

The objective of this study was to develop a qPCR assay for the quantitative assessment of uncultured fungal zoospores in natural environments. Our qPCR assay was based on the detection of the SYBR Green dye (Walker, 2002). However, because SYBR Green binds to specific and non-specific PCR products, PCR conditions and primers specificity were carefully optimized. In addition, because inhibitory factors co-extracted along with DNA during extraction are known to reduce the sensitivity of PCR (Wilson, 1997; Martin-Laurent *et al.*, 2001; Park and Crowley, 2005), extraction needed to be optimized. Finally, the method was validated on lake water samples in which the targeted zoosporic fungi were previously detected.

Materials and methods

Primer design. Primers were designed using a database containing about a hundred 18S rDNA environmental sequences recovered from surveys conducted in seven different lakes (Lefranc *et al.*, 2005; Slapeta *et al.*, 2005; Lefèvre *et al.*, 2007, 2008; Lepère *et al.*, 2008; Chen *et al.*, 2008) and sequences belonging to described fungi. Sequences were aligned using BioEdit (*http://www.mbio.ncsu.edu/BioEdit/bioedit.html*) (Hall, 1999) and the resulting alignment was corrected manually. A great proportion of the environmental chytrid sequences recovered from lakes was closely affiliated to the Rhizophydiales. Thus, Rhizophydiales-specific primers F-Chyt and R-Chyt were designed in order to fulfill three requirements: (i) a GC content between 40 and 70%, (ii) a melting temperature (T_m) similar for both primers and close to 60°C, and (iii) PCR products below 500 bp (Edwards, 2004; Table 1). Potential complementarities (hairpins and dimers) were checked using Netprimer (*http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html*).

Table 1: Properties of the primers used in this study

Name	Sequence (5'→3')	Length (bp)	Tm[a] (°C)	GC (%)	Max. number of mismatches to target sequences	Position in PFB11AU2004 sequence	Amplicon size (bp)
F-Chyt (F-Chyt'[b])	GCAGGCTTACGCTTGAATAC (GCAGGC**AT**__TT__GCT**C**GAATA-)	20 (19)	57.69 (59)	50 (47.37)	1 (0)	773	304 to 313 (308)
R-Chyt	CATAAGGTGCCGAACAAGTC	20	58.23	50	1 (2)	1076	

[a] Tm was calculated using Primer3 software.
[b] The forward primer F-Chyt', modified from F-Chyt (modified nucleotides in bold), was used with R-Chyt to amplify *Z. planktonicum*.

Optimization of DNA extraction. In order to assess the most appropriate DNA extraction method suitable to maximize genomic DNA quality, and thus enhance the downstream PCR efficiency, combinations of several cell lysis and DNA purification techniques were tested. Since the aim of this study was to quantify fungal zoospores, optimization of genomic DNA extraction was performed on the zoosporic life stage of *Zygorhizidium planktonicum* (clone FMS34600; Kagami *et al.*, 2004), a chytrid parasite on *Asterionella formosa*. Zoospores were collected on 0.6 µm pore-size polycarbonate filters (24 replicates) after removal of the algal host by prefiltration.

For cell disruption, four methods were tested: (i) an enzymatic digestion with proteinase K: 6 filters were incubated in 560 µl of a buffer containing 1% SDS (sodium dodecyl sulfate) and 1 $mg.ml^{-1}$ proteinase K and TE (Tris-EDTA, 1mM EDTA and 10mM Tris-HCl) for 1 h at 37°C. (ii) An enzymatic digestion with lyticase (Sigma) and proteinase K: 6 filters were incubated overnight at 30°C in 500 µl of a sorbitol based buffer containing 400 units of lyticase (Karakousis *et al.*, 2006), followed by the proteinase K digestion as previously described. (iii) A physical cell disruption by sonication: 6 filters were recovered with 500 µl of TE buffer and sonicated three times on ice, for 30s at 20 KHz with a power output of 30 W (model VC 50-220V, Bioblock Scientific). (iv) A physical cell disruption procedure by thermal shocks: 6 filters were recovered with 500 µl of TE buffer, frozen in liquid nitrogen and thawed three times at 37°C, and finally incubated in boiled water for 10 min.

For all the cell lysis protocols described, two purification methods were applied: (i) a phenol-chloroform extraction method previously used by Lefèvre *et al.* (2007, 2008), and (ii) a purification step using silica-membrane columns provided with the NucleoSpin Plant kit® (Macherey-Nagel). Integrity and yield of the extracted genomic DNA were visualized in a 1% agarose gel stained with 0.3 $µg.ml^{-1}$ of ethidium bromide solution (Sigma). DNA extracts were run with dilutions of calf thymus DNA (Sigma) and concentrations calculated using a standard curve of 5 to 100 ng of calf thymus DNA versus band intensity. A two way analysis of variance, ANOVA (factor 1: cell lysis procedures; factor 2: DNA purification procedures) was performed to determine significant differences in the DNA concentration obtained (at 95% confidence limit).

Material used as template for qPCR assays. As none of the chytrids targeted in this study were available in culture, the qPCR assay was optimized using (i) DNA extracted from 29 strains representative of different non-target phylogenetic groups (Table 2), (ii) 18S rDNA-containing plasmids obtained from one of our previous environmental DNA surveys on Lake Pavin, France (Lefèvre *et al.*, 2007; Table 3), and (iii) 20 environmental samples collected during our previous environmental DNA surveys in Lake Pavin (Lefèvre *et al.*, 2007; 2008). Except for one date (12/1/04), water samples were collected during spring and autumn thermal stratifications. Oxygen and temperature profiles were measured for every sampling dates.

Table 2: Negative strains used for qPCR cross-reactivity assays.

Taxonomy	Species	qPCR amplification results[a]
Fungi; Ascomycota; Eurotiomycetes	*Penicillium roqueforti*	-
	Aspergillus niger	-
Fungi; Ascomycota; Saccharomycetes	*Candida albicans*	-
Fungi; Ascomycota; Sordariomycetes	*Fusarium* sp.	-
Zygomycota; Zygomycetes	*Mortierella* sp.	+ (Ct=19.8)
Basidiomycota; Urediniomycetes	*Rhizopus nigricans*	-
	Rhodotorula muscilaginosa	-
Chytridiomycota; Chytridiales	*Zygorhizidium planktonicum*	-
Chytridiomycota; Spizellomycetales	*Rhizophlyctis rosea*	-
Heterokonta; Bacillariophyta	*Asterionella formosa*	-
	Synedra sp.	-
	Fragilaria crotonensis	-
	Melosira sp.	-
Heterokonta; Chrysophyceae	*Synura* sp.	-
	Chilomonas sp.	-
Cryptophyta; Cryptophyceae	*Cryptomonas* sp.	-
	Gonium sp.	-
Viridiplantae; Chlorophyta	*Choricystis minor*	-
	Ankistrodesmus sp.	-
	Kirchneriella sp.	-
	Staurastrum sp.	-
	Scenedesmus sp.	-
	Volvox sp.	-
	Closterium sp.	-
Alveolata; Dinophyceae	*Peridinium* sp.	-
Alveolata; Ciliophora	*Paramecium caudatum*	-
Prokaryotes	*Flavobacterium* sp.	-
	Synechococcus elongatus strain SAG89.7	-
	Synechococcus elongatus strain B8809	-

[a] Binary results from the qPCR using the optimized conditions defined in this study: '-' means that no amplification occurred at the end of the 40 cycles. '+' means that amplification occurred and the Ct value is given between brackets.

Table 3: Primer regions aligned against the 4 target and 4 non-target sequences from different phylogenetic groups displaying variable numbers of mismatches with our primers. Sequences presented in this table were used as control for qPCR optimizations in this study. Bold and underlined nucleotides indicate mismatches with the primers.

Genbank Acc. No.	Sequence id.	Alignment with F-Chyt (5'→3')	Alignment with R-Chyt (5'←3')	Mismatches with F-Chyt	Mismatches with R-Chyt	Phylogenetic affiliationa	Type of control
		GCAGGCTTACGCT--TGAATAC	**GACTTGTTCGGCACCTTATG**				
DQ244014	PFB11AU2004	GCAGGCTTACGCT--TGAATAC	GACTTGTTCGGCACCTTATG	0	0	Targeted Chytrid	+
DQ244004	PFF6AU2004	GCAGGCTTACGCT--TGAATAC	GACTTG**A**TCGGCACCTTATG	0	1	Targeted Chytrid	+
DQ244009	PFH1AU2004	GCAGGCTTACGCT--TGAATAC	GACT**C**GTTCGGCACCTTATG	0	1	Targeted Chytrid	+
DQ244008	PFE7AU2004	GCAGGC**C**TACGCT--TGAATAC	GACT**C**GTTCGGCACCTTATG	1	1	Targeted Chytrid	+
DQ244002	PFF2AU2004	GCAGGCTTA**T**GCT--**C**GAATAC	GACT**C**GT**C**CGGCACCTTATG	2	2	Ascomycota	-
FJ99981	PG4AU2004	GCAGGC**C**TACGCT--TGAATAC	GACT**C**CA**T**TGGCACCTT**G**TG	1	5	Cryptomonad	-
FJ99982	PG8AU2004	GCAGGC**C**TACGCT-**C**TGAATAC	GAC**C**T**C**T**G**CAGCACCTTATG	2	4	Chlorophyceae	-
FJ99983	PF7AU2004	GC**G**GTTAT**G**G**TGAT**TGAATA**G**	GA**GCGCA**T**G**GG**G**ACC**G**T**GA**G	11	10	Choanozoa	-

[a] Phylogenetic affiliations as defined in Lefèvre *et al.* 2007

QPCR assays: optimization, specificity and sensitivity. The PCR mastermix contained SYBR Green (Sigma), 200 µM of each dNTPs, 10 pM of each primer, 2.5 units of HotStarTaq DNA polymerase (Qiagen), the PCR buffer supplied with the enzyme (1.5 mM final $MgCl_2$, provided in the PCR Buffer), and variable quantity of DNA (5 ng for environmental samples, 10 ng for cultures, and 50 pg for plasmids) was used as template in a final volume of 25 µl. Quantitative PCR reactions were carried out using a Mastercycler ep realplex detection system (Eppendorf). To confirm the absence of primer dimers and unspecific PCR products, immediately following each qPCR assay, a melting curve analysis was performed by increasing the incubation temperature from 50 to 95°C for 20 min.

In order to optimize the qPCR specificity, annealing temperature (T_a) was determined using four positive (PFB11AU2004, PFF6AU2004, PFH1AU2004 and PFE7AU2004) and three negative (PFF2AU2004, PG4AU2004, PG8AU2004) plasmids (Table 3). The cycling conditions consisted of an initial HotStarTaq activation at 95°C for 15 min, followed by 40 cycles with denaturation at 95°C for 1 min, annealing with a gradient temperature from 54 to 65°C for 1 min, and elongation at 72°C for 1 min. An additional elongation step at 72°C for 10 min was finally performed. Fluorescence data were collected at each PCR cycle during the elongation step. In order to maximize the specificity, an additional assay with an annealing time of 30 s was also performed using the optimal T_a previously determined. The maximum number of cycles for which the qPCR should be run was determined in order to exclude potential amplifications of negative plasmids. Then, using the number of cycles determined, the specificity of our primers was tested with a range of 29 non-target organisms (Table 2). Genomic DNA was extracted using the DNA extraction protocol previously selected (in 'Optimization of DNA extraction' section) and 10 ng of genomic DNA was used as template in the qPCR assays.

The detection limit of the qPCR assay was determined using the plasmid PFB11AU2004. Serial 10-fold dilutions of the plasmid were made, ranging from 1 to 10^9 dilution factors corresponding to 1 x 10^9 to 1 copy μl^{-1}, respectively. The number of copies in the standard was calculated using the equation: Molecules μl^{-1} = a / [3931 + 1815] x 660 x $6.022.10^{23}$, where a is the plasmid DNA concentration ($g.\mu l^{-1}$), 3931 the plasmid length without the cloned 18S rDNA insert, 1815 the 18S rDNA insert length in bp (PFB11AU2004), 660 the average molecular weight of one base pair, and 6.022 x 10^{23} the Avogadro constant (Zhu *et al.*, 2005).

Because background DNA has been observed to dramatically decrease the sensitivity of qPCR (Skovhus *et al.*, 2004; Sub *et al.*, 2006), we tested the effect of the addition of different amounts of non-target DNA to target DNA. Mixtures of one positive (PFB11AU2004) and 4 negative plasmids (PFF2AU2004, PG8AU2004, PG4AU2004 and PF7AU2004) were made at different proportions (50, 25, 10 and 5% of the positive plasmid). QPCR assays were performed using 50 pg of each mixture as template and using the qPCR conditions previously defined. Resulting amplified products were then purified using QIAquick PCR purification kit (Qiagen) and sequenced by MWG Biotech service (Paris, France). Resulting sequences were aligned and compared to our 18S rDNA sequence database.

To test the effect of the DNA extraction protocol on the qPCR efficiency, genomic DNA obtained from *Z. planktonicum* zoospores (see section 'Optimization of DNA extraction') was used. The 18S rRNA gene from the non-target chytrid *Z. planktonicum* was cloned in order to obtain a plasmid for this chytrid, and sequenced. Blastn analysis revealed that *Z. planktonicum* is not a member of the Rhizophydiales, but a member of the Chytridiales, which explained why our Rhizophydiales-specific primers F-Chyt and R-Chyt displayed 4 and 2 mismatches with *Z. Planktonicum* 18S rRNA sequence, respectively. Thus, we designed a specific *Z. planktonicum* primer, F-Chyt' (slightly modified from F-Chyt), displaying approximately the same T_m as the F-Chyt primer (Table 1). This strategy limited the mismatches of the reverse primer to only 2, which is acceptable for amplification. Five ng of each triplicate of *Z. planktonicum* genomic DNA obtained from the successful DNA extraction protocols tested were used as template. In order to compare more easily the effect of DNA extractions on qPCR efficiency, 50 pg of the *Z. planktonicum* plasmid was also used as reference in one well of the same qPCR plate and δCt values were calculated for each extraction treatment using the following equation : $\delta Ct = [Ct_{ref}] - [Ct_{treatment}]$, where $[Ct_{ref}]$ is the Ct value (the first PCR cycle for which fluorescence crosses a pre-determined level) calculated for the *Z. planktonicum* 18S rDNA-containing plasmid, and $[Ct_{treatment}]$ is the Ct value calculated for each extraction test (the higher the δCt, the more efficient the PCR reaction). QPCR assays were performed using the previously optimized PCR conditions (annealing temperature and time, number of cycles). A two way analysis of variance (factor 1: cell lysis procedures; factor 2: DNA purification procedures) was performed to determine significant differences in the δCt values obtained (at 95% confidence limit).

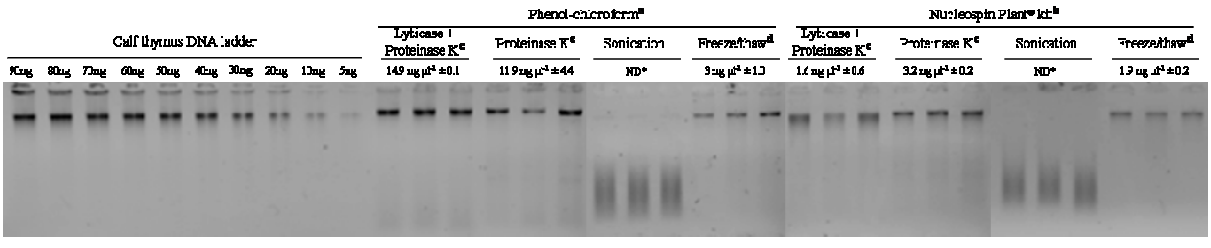
Application to natural samples. Five ng of two-fold diluted environmental DNA was added in qPCR reactions. Target copy numbers for each quantification reaction were calculated from a standard curve generated using triplicates of the 10-fold dilutions of the plasmid PFB11AU2004 (from 100 to 1 x 10^8 copies μl^{-1} which corresponded to 0.63 fg and 0.63 ng of target DNA, respectively). The initial concentration of target 18S rDNA (copies ml^{-1}) in the environmental genomic DNA was calculated using the formula: [(a / b) x 10 x 50] /c, where a is the 18S rDNA copy number estimated by qPCR, b is the volume of the environmental genomic DNA added in the qPCR reaction (µl), 10 corresponds to the dilution factor of the environmental genomic DNA once added in the qPCR reaction, 50 is the volume into which the environmental genomic DNA was resuspended at the end of the DNA extraction (µl), and c the volume of lake water filtered from which DNA was extracted (ml).

Nucleotide sequences used or obtained in this study are available in Genbank under accession numbers DQ244002 (PFF2AU2004), DQ244004 (PFF6AU2004), DQ244005 (PFB1AU2005), DQ244008 (PFE7AU2004), DQ244009 (PFH1AU2004), DQ244013 (PFD11AU2004), DQ244014 (PFB11AU2004), EU162636 (PFB4SP2005), EU162640 (PFD5SP2005), FJ799981 (PG8AU2004), FJ799982 (PG4AU2004), FJ799983 (PF7AU2004) and FJ799984 (*Z. planktonicum*).

Results

QPCR primer design. The two primers designed in this study fulfill all the requirements needed for PCR application (Table 1). For both primers, T_m is close to 60°C and the GC content is 50%. PCR products are between 304 bp and 313 bp in size, depending on the sequence considered. Netprimer software analysis did not reveal primer hairpins and dimers which were confirmed by inspection of the melting curves (data not shown). Although Blastn analysis revealed that F-Chyt also matched to non-targeted sequences belonging to the fungal phylum Glomeromycota, R-Chyt matched to 100% of environmental and known chytrid sequences within the Rhizophydiales. The only exception was *Chytridium polysiphoniae* (within the '*C. angularis clade*'; James *et al*., 2006) which presented a perfect match to R-Chyt primer and only two mismatches to F-Chyt primer. However, because this chytrid is one of the rare species exclusively found in marine systems, its amplification from freshwater samples is considered unlikely. As shown by sequence alignments in Table 3, our primers display a maximum of 2 mismatches (one for each primer) with the target sequence PFE7AU2004 and a minimum of 4 mismatches (two for each primers) with the non-target Ascomycota PFF2AU2004 sequence.

Optimization of DNA extraction. Because the cell disruption treatment using sonication resulted in the degradation of DNA (Figure 1), this method was not selected. Similarly, because the freezing/ thawing cell disruption treatment yielded significantly lower DNA concentrations than the enzymatic methods **(**ANOVA $P < 0.05$; Figure 1), this method was also not selected. For each of the enzymatic cell disruption methods tested, the phenol-chloroform purification procedure yielded significantly higher amounts of DNA than the commercial kit (ANOVA $P<0.05$; Figure 1).



*ND : DNA concentrations for the treatment using sonication were not determined because the DNA was degraded.

Figure 1: Agarose electrophoresis of DNA extracted from *Z. planktonicum* zoospores using 8 extraction methods. The ten first lanes show the DNA quantification ladder with the quantity of calf thymus DNA. Following lanes show triplicate of DNA extracted using the different methods used. Concentration ($ng.\mu l^{-1}$) of genomic DNA from *Z. planktonicum* is given above each line. Data are presented as means ± SD for triplicate extractions. Treatments followed by the same letter (a and b for the comparison of DNA extraction methods, and c and d for the comparison of cell disruption treatments) were not significantl different (ANOVA, $P> 0.05$).

However, when the genomic DNA extracts were used as template in PCR reactions, the DNA purification method using the commercial kit gave significantly better results (higher δCt) than the phenol-chloroform method (ANOVA $P<0.05$; Table 4). Consequently, although it was not the one yielding the highest quantity of DNA, the method using the commercial kit was selected. Although no significant difference was shown between the two enzymatic cell disruption methods tested, the one step proteinase K was selected because it yielded higher amount of genomic DNA than the lyticase method and had a better reproducibility (as shown by the SD calculated in Figure 1). In addition, compared to the other cell lysis treatments, single-step proteinase K did not degrade DNA (as shown by the smear pattern of the DNA obtained with the three other treatments tested; Figure 1). Consequently, the DNA extraction method using Proteinase K and the commercial kit was selected and considered the best overall.

Table 4: Effect of DNA extraction methods on qPCR amplification of *Z. planktonicum* zoospores. δCt values presented were calculated using the following formula: $\delta Ct = [Ct_{ref}] - [Ct_{treatment}]$, where Ct_{ref} is the Ct value displayed by 50 pg of the plasmid containing the 18S-rDNA *Z. planktonicum* insert, and $Ct_{treatment}$ is the Ct value displayed by 5 ng of *Z. planktonicum* genomic DNA extracted using the different treatments. Treatments followed by the same letter (a, b or c) do not differ significantly ($P > 0.05$).

Cell disruption treatments	DNA purification methods	
	Phenol - Chloroform [a]	NucleoSpin® Plant kit [b]
Enzymatic cell disruption :		
Lyticase + Proteinase K [c]	-0.2 ±0.8	4.0 ±1.1
Proteinase K [c]	0.9 ±0.3	2.5 ±0.4
Physical cell disruption:		
Freezing / thawing [c]	2.1 ±1.4	0.9 ±0.7

Optimization of qPCR assay. In order to optimize the specificity of our qPCR assay, 12 annealing temperatures (T_a; from 54 to 65°C) were tested on 4 target and 3 non-target plasmids (Table 3). For the 12 T_a tested, all the target plasmids displayed a Ct value below 24 and were detected after the same number of PCR cycles (Ct~21; Figure 2). The negative plasmid PG8AU2004 was never amplified and the negative plasmid PG4AU2004 was amplified very late (Ct = 38) only for one T_a (59°C). The negative clone PFF2AU2004 which displayed the fewest mismatches to our two primers was amplified for all T_a tested but always after (i.e. at least of 5 cycles above) the positive plasmids. Thus, a T_a of 63.3°C was selected because it is the lowest T_a which allows us to perform the longest qPCR (35 cycles) without amplifying PFF2AU2004.

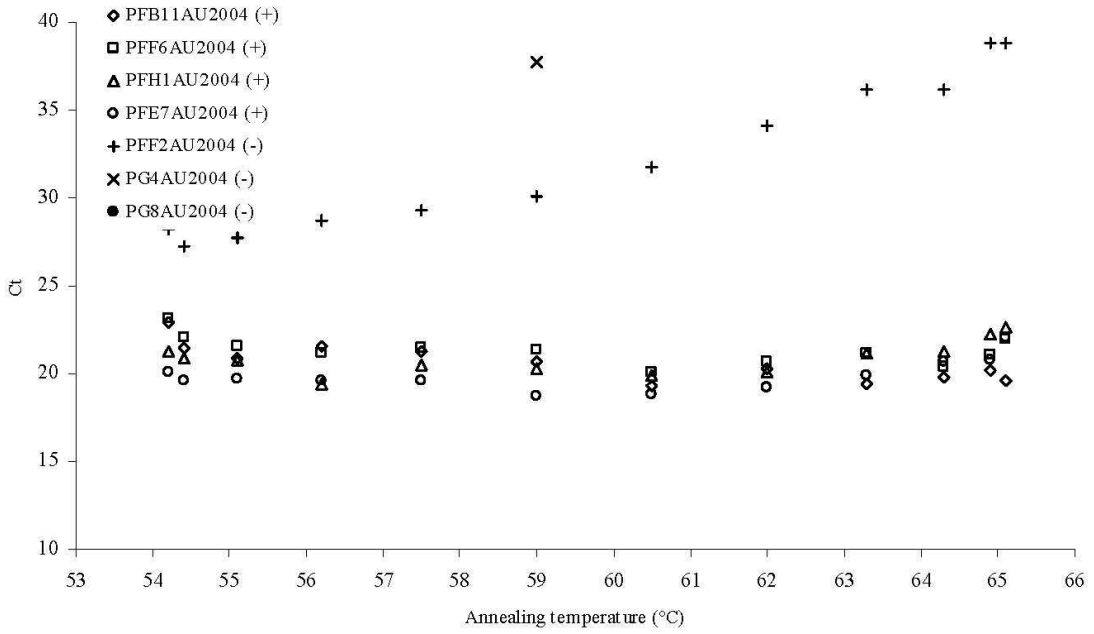


Figure 2: Effect of annealing temperature on the threshold cycle (Ct) for 4 positive and 3 negative plasmids. In the legend, positive and negative plasmids are followed by '+' and '–' signs, respectively.

In order to increase the specificity of our qPCR, the annealing time was also tested. Two further qPCR assays were performed using the T_a previously defined (63.3°C) at annealing times of 30 s and 1 min. For the two annealing times tested, the positive plasmids displayed Ct values around 20 and the negative plasmids displayed Ct values around 36 cycles (Supplemental material Figure S1). However, because Ct of the target plasmids were advanced by an average of 0.7 cycle and Ct of the non-target plasmid PFF2AU2004 was delayed by 1 cycle, an annealing time of 30 s was selected. Overall, an annealing temperature of 63.3°C and an annealing time of 30 s for 35 cycles were selected as the best PCR conditions. Melting curves and agarose electrophoresis performed after each PCR did not reveal any non-specific amplifications and primer dimers (data not shown). The analysis of the amplified fragments by agarose electrophoresis indicated that PCR products had the expected size, i.e. between 304 and 312 bp (data not shown).

QPCR sensitivity and specificity. Standard curves generated using 10-fold serial dilutions of plasmids were used to calculate the range of detection and the sensitivity of our qPCR assay (Supplemental material Figure S2). DNA was detected across a range of dilutions to 6 orders of magnitude (from 100 to 1 x 10^8 copies μl^{-1}), with a coefficient of determination of 0.991 and a PCR efficiency of 90.23%. To evaluate the sensitivity of our qPCR assay, mixtures of one positive plasmid and 4 non-target plasmids were tested with the previously defined qPCR conditions. For all mixtures the amplification started before 30 PCR cycles (data not shown). The sequencing of the PCR products

for each plasmid mixture tested revealed that only the positive plasmid was amplified, indicating that no amplification of the negative plasmid occurred. In addition, using the same qPCR conditions, the genomic DNA of 29 non-target organisms (Table 2) was tested. Although one non-target fungal strain isolated from soil (*Mortierella* sp.) was amplified, the other 28 organisms did not amplify at all.

Application of qPCR to natural samples. Figure 3 presents the results obtained when our optimized qPCR was applied to natural samples. Fungal zoospores were detected in 11 of the 20 samples analyzed. Target chytrids were detected more often and at higher densities in the thermocline. Each time target organisms were detected in the thermocline, they were also detected at 30 m depth, but at lower densities. Among the 7 dates sampled, 4 corresponded to samples for which picoeukaryotic 18S rDNA clone libraries were previously constructed (underlined dates in Figure 3; Lefèvre *et al.*, 2007, 2008). For two of these dates (i.e. 10/06/04 and 06/16/05), target chytrids were detected using both the qPCR method developed in this study, and the 18S rDNA cloning-sequencing approach previously conducted. However, because our cloning-sequencing surveys in Lake Pavin were conducted using universal eukaryote primers, it is difficult to infer any further comparison between the present study and our previous ones.

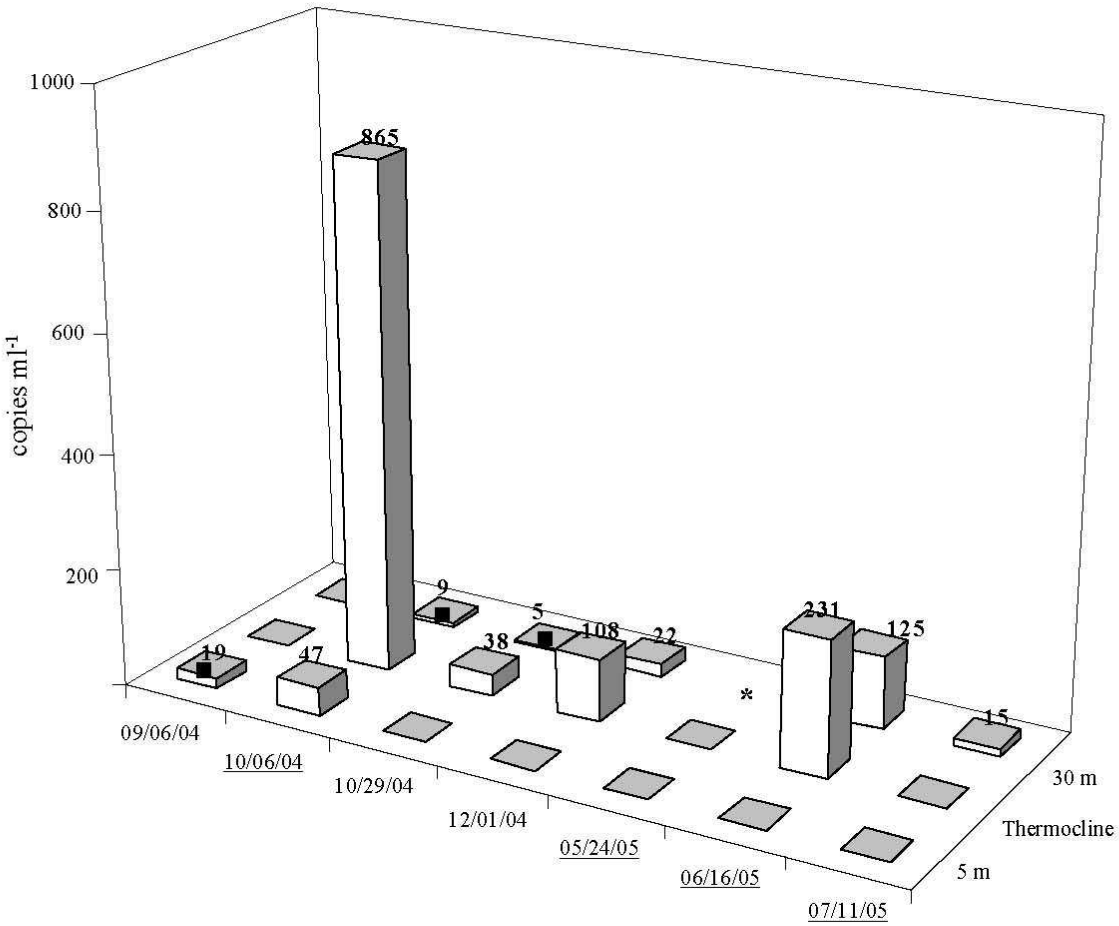


Figure 3: Semi-quantification of fungal zoospores (copies ml^{-1}) at three depths (5m; Thermocline, 30m) on 7 different occasions. Underlined dates correspond to date from which clone libraries were constructed in previous studies (Lefèvre *et al.*, 2007, 2008).
'*' indicates a missing sample; plain squares indicate that the number of copies calculated was below the detection limit of our method.

Discussion

It is now largely recognized that the sensitivity and success of PCR-based methods strongly depend on the quality of amplifiable DNA (Wilson, 1997). Extraction methods are therefore a key step in the success of such PCR-based methods and must be optimized before downstream PCR optimization. Indeed, phenolic compounds used in the phenol-chloroform procedure can inhibit PCR reactions by binding to or denaturing the polymerase (Young *et al.*, 1993). In this study, we compared two DNA purification methods. As expected, the phenol-chloroform procedure significantly decreased the efficiency of the qPCR assay. Thus, a combination of the single-step proteinase K extraction method followed by the purification method using the commercial kit was selected because it enhanced the PCR reaction and was reproducible. Our results are in accordance with Karakousis *et al.* (2006) who found that optimal DNA extraction of several fungal species for downstream PCR application was achieved using enzymatic cell disruption along with a commercial kit.

Blastn analysis showed that a significant portion of the environmental zoosporic fungal sequences recovered from molecular surveys conducted in lakes were closely affiliated to the Rhizophydiales. Although Rhizophydiales members are known as saprobes in soil, numerous species within this order have been exclusively observed in aquatic systems as both saprobes and algal parasites (Letcher *et al.*, 2006, 2008), hence our effort to design specific primers for this order. Although less specific, F-Chyt displayed affinities only to sequences belonging to the kingdom Fungi. When combined, the lack of specificity of the F-Chyt primer is largely compensated for the high specificity of the R-Chyt primer and thus the use of both primers together would exclusively amplify species within the Rhizophydiales.

Although some factors such as primer and magnesium chloride concentrations can be tested to increase the sensitivity of the PCR, annealing temperature and time are usually the easiest factors to modify (Sipos *et al.*, 2007). The sensitivity of the method is another important aspect we considered and can be enhanced by increasing the number of PCR cycles. However, a high number of cycles and long annealing times can also lead to the amplification of non-target DNA and, as a result, decrease the qPCR specificity (Edwards, 2004). In this study, a good compromise was reached using a shorter annealing time and a lower number of cycles than in previous studies (1 min and 40-50 cycles; Skovhus *et al.*, 2004; Sub *et al.*, 2006; Park *et al.*, 2007). Using these optimized conditions, only one of the 29 negative cultures (i.e. 3.5 % false positive rate), a species within the genus *Mortierella* (phylum Zygomycota), was amplified. Because of aerial spore dispersal, the recovery of DNA of this species in aquatic system is not impossible. However, because *Mortierella* sequences were never detected in previous cloning-sequencing surveys conducted in lakes (Lefranc *et al.*, 2005; Slapeta *et al.*, 2005; Lefèvre *et al.*, 2007, 2008; Lepère *et al.*, 2008; Chen *et al.*, 2008), it is unlikely to recover *Mortierella* DNA in freshwater samples.

In order to validate our method, we analyzed water samples from Lake Pavin, from which Rhizophydiales sequences were previously detected. Because the target group was detected in 11 of the 20 samples analyzed, we consider that the method is validated and can be applied to natural samples. The highest copy concentrations were found in the metalimnion (thermocline sample). Because it has been suggested that fungal zoospores could have been misidentified as HF, we compared our results to a study previously conducted by Carrias *et al.* (1996) on the vertical distribution of HF during thermal stratification in Lake Pavin. These authors reported that HF abundances were always maximal in the metalimnion of the lake. In addition, they mentioned that 24% of the HF observed in their study corresponded to small uniflagellate heterotrophic cells (2-4 µm) which were not found to graze on bacteria. However, although this suggests that fungal zoospores may have been misidentified as HF, our data is too preliminary to validate this hypothesis.

Zoosporic fungi targeted in this study are known as saprobes and algal parasites in aquatic systems (Letcher *et al.*, 2008). Thus, finding the highest abundances of zoospores in the thermocline is not surprising. Indeed, this layer of the water column acts as a physical barrier to particulate sedimentation. As a result, sinking organic material accumulates at this depth, attracting saprobe organisms, including zoosporic fungi. In addition, the depth at which our thermocline samples were collected also corresponded to the metalimnic oxygen maximum (see Supplemental material Figure S3), which is due to the intense activity of algae. Thus, because some members of Rhizophydiales are known as algal parasites, finding the highest density of fungal zoospores at this depth is not unexpected. It is however difficult to draw firm ecological conclusions from our limited data set.

Our zoospore abundances are semi quantitative because they only provide the number of rRNA genetic units per ml of water as opposed to the number of zoospores per ml of water. We intended to use *Z. planktonicum* to estimate the number of rRNA operons per zoospore. Unfortunately, parasitic chytrids in culture with their algal hosts are hard to maintain and the culture was lost during the course of our study. The number of rRNA operons per cell has been estimated for some fungal taxa. However, it is largely variable within the kingdom Fungi (ranging from 50 to 220 depending on the species; Lozupone and Klein, 2002) and has mostly been estimated for 'higher Fungi' (Subkingdom Dikarya). More recently, it has been estimated at 169 copies per zoospore for the chytrid *Batrachochytrium dendrobatidis* (Kirshtein *et al.*, 2007). Thus, because this chytrid is a member of the Rhizophydiales (Letcher *et al.*, 2008), if we assume that the number of rRNA operons within the same phylogenetic order is similar for all species, the density of zoospores in our samples would vary from 40 to 5000 L^{-1}. These densities fall outside the range of a previous study that only estimates the zoospore densities of *B. dendrobatidis* in natural environments as 50 to 454 zoospores L^{-1} (Kirshtein *et al.*, 2007). However, in contrast to Kirshtein *et al.* (2007) who targeted only one species, the present study targets all species of an entire order, which may explain our higher density estimates. Moreover, although *B. dendrobatidis* belongs to the Rhizophydiales, Letcher *et al.* (2008) showed that this species is highly divergent from the other members of this order. Thus, giving our assumption that all

the species within the Rhizophydiales have the same number of rRNA operons per cell, our density estimates must be interpreted with caution.

QPCR was recently used to estimate fungal biomass in a stream during leaf decomposition (Mayura *et al.*, 2008) and in biological soil crusts (Bates and Garcia-Pichel, 2009). The interpretation of the semi quantitative data obtained in these studies was relatively difficult because the whole fungal community was targeted (including, unicellular, multicellular and multinuclear fungal species). Thus, an estimation of fungal density or even fungal biomass was not possible. In our case, because zoospores are unicellular, qPCR data can be directly converted into cell density estimates. Thus, the transformation of our semi quantitative data into absolute cell densities (by multiplying them by a number of rRNA copies per cells) will not change the relative proportion of the densities obtained. Overall, we (i) believe that qPCR offers a good tool for the quantification of fungal zoospores in natural environments, and (ii) consider that its application will contribute to a better understanding of the ecological significance of environmental chytrids, and primarily of their role in microbial food webs of pelagic ecosystems.

Acknowledgements

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Supplemental materials

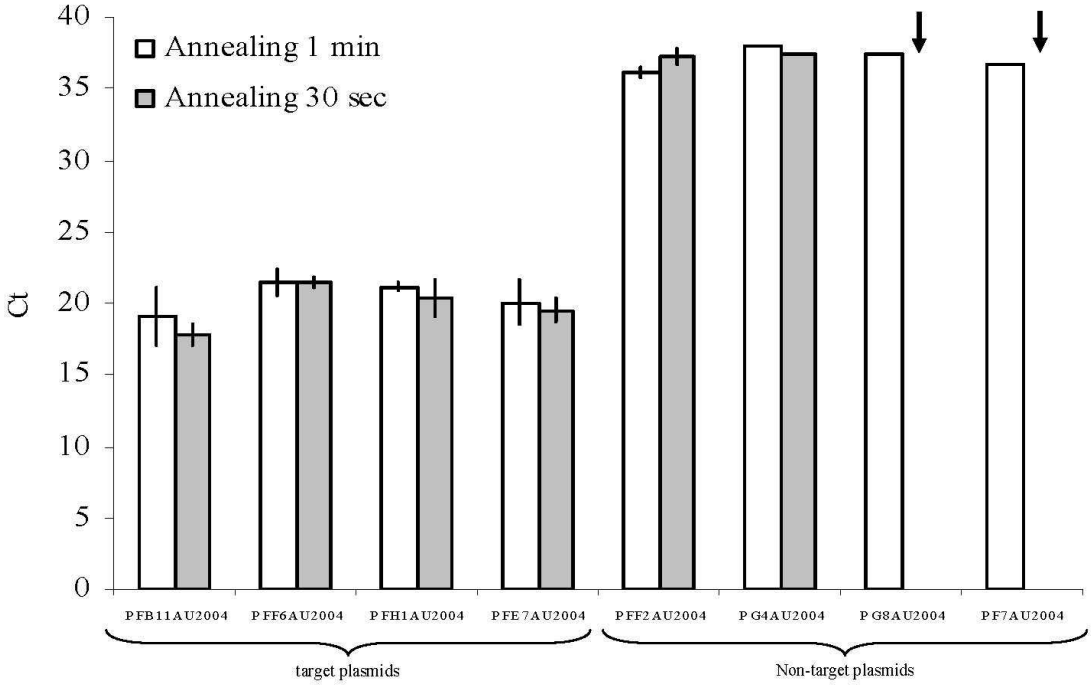

Figure S1: Effect of annealing time on the threshold cycle (Ct) of a set of 8 target and non-target plasmids. Arrows indicate that none of the triplicate was amplified after 40 cycles. No standard deviation was calculated for the three last non-target plasmids because less than three replicates were amplified during the PCR.

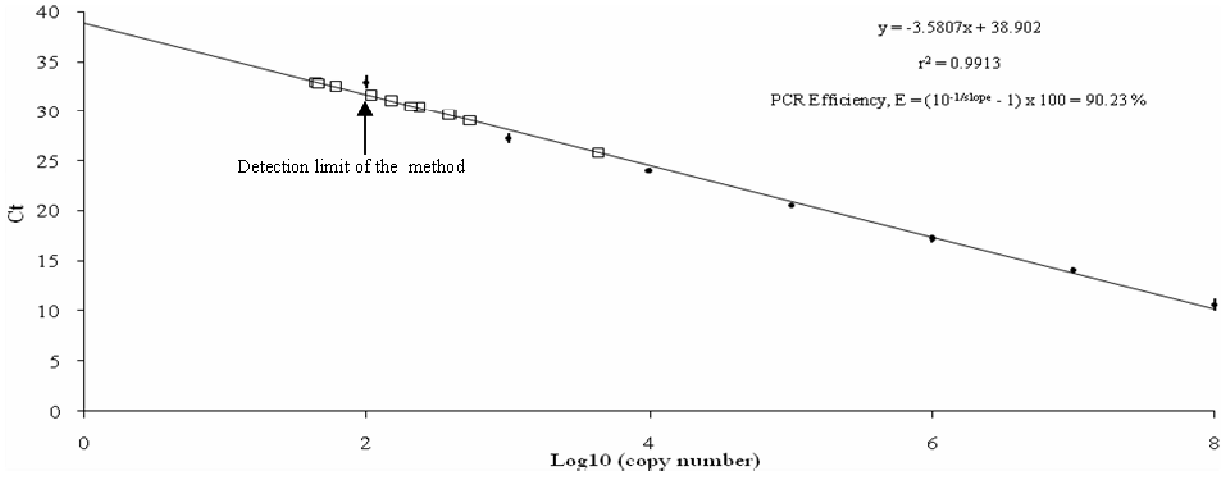

Figure S2: Linear relationship between threshold cycles (Ct) and the input copy number of 18S-rDNA from the PFB11AU2004 plasmid. Error bars represent the standard deviation from triplicate of input 10-fold dilution of PFB11AU2004 plasmid. Linear regression (r^2 =0.9913) results in an equation of y = -3.5807 x + 38.902. PCR efficiency (E) was calculated from the slope of the standard curve (-3.5807). Open squares indicate the unknown natural samples.

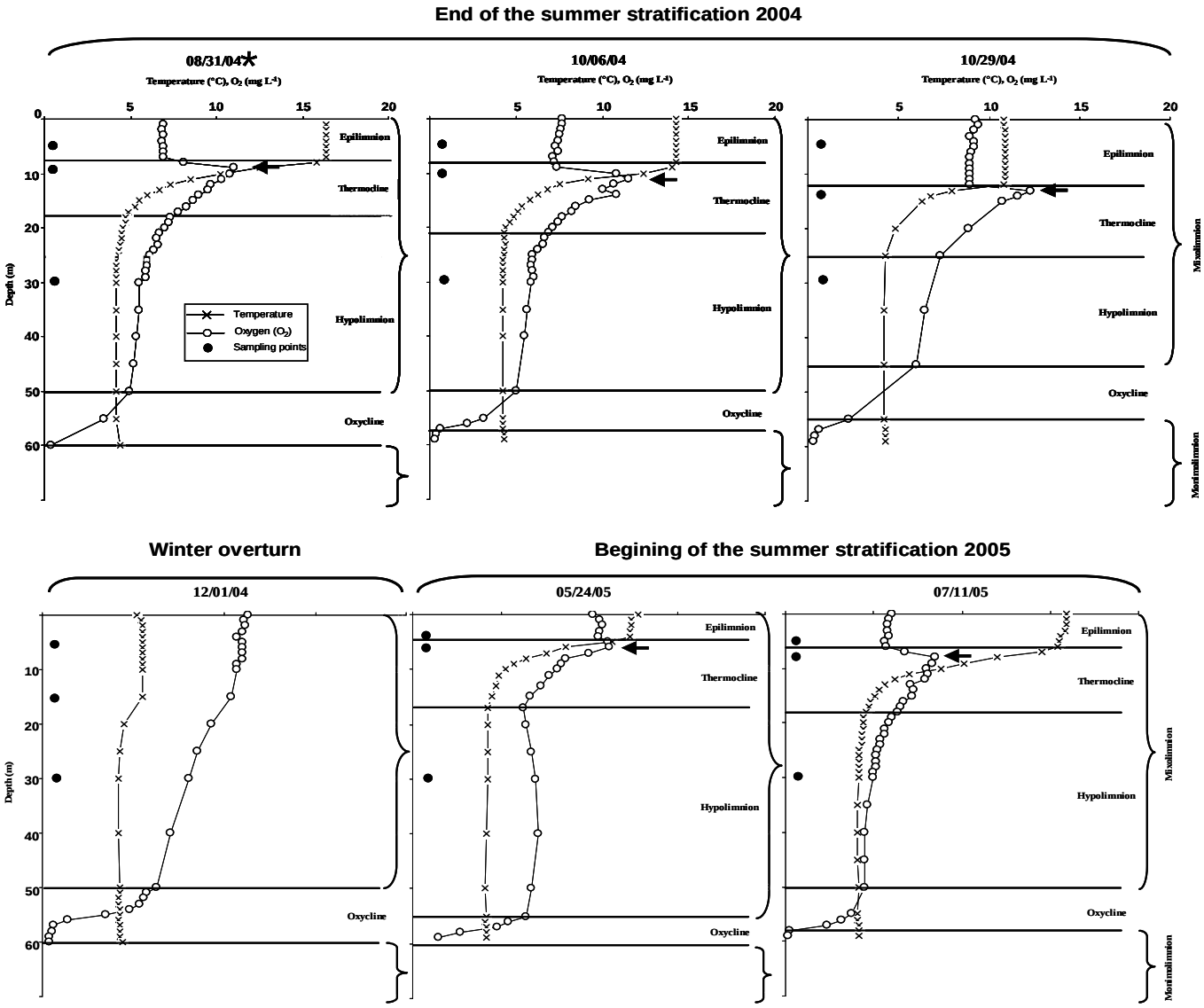

Figure S3: Vertical profiles of temperature and oxygen concentration measured at the six sampling dates. Arrows indicate the oxygen concentration peaks in the upper part of the thermocline.
* The first graph (08/31/04) corresponds to a profile measured six days before our first sampling date (10/06/04).

Deuxième partie

Fluorescence *in situ* hybridization of uncultured zoosporic fungi: testing with clone-FISH and application to freshwater samples using CARD-FISH

Fluorescence *in situ* hybridization of uncultured zoosporic fungi: testing with clone-FISH and application to freshwater samples using CARD-FISH

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Short Title: Fluorescence *in situ* hybridization of chytrids

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Abstract

Recently, molecular environmental surveys of the eukaryotic microbial community in lakes have revealed a high diversity of sequences belonging to uncultured zoosporic fungi commonly known as chytrids. These microorganisms have two different stages in their life cycle and are known as algal parasites (i.e. host-attached infective sporangia) and as food sources for zooplankton (i.e. free-living zooflagellate propagules) in aquatic systems. However, because of their small size and their lack of distinctive morphological features, traditional microscopy does not allow the detection of chytrids, particularly of zoospores which have probably been misidentified as phagotrophic flagellates in previous studies. Hence, quantitative data on chytrids in natural environments is missing. We have adapted a clone–fish approach known from prokaryotes to optimize the hybridization conditions of a designed oligonucleotidic probe specific to Chytridiales (i.e. the largest group of the true-fungal division of Chytridiomycota), before application to natural samples using the CARD-FISH approach. When these conditions were applied, the CARD-FISH assay demonstrated high specificity and sensitivity, and offers a promising tool for quantitative assessment of natural zoosporic fungi, primarily of zoospores which contributed up to 60% of the total abundance of heterotrophic flagellates. Although the field results from the CARD-FISH approach were considered preliminary and mainly as 'proof of concept', findings were consistent with ecological considerations known from pelagic habitats and host versus parasite populations, with recurrent ecological patterns in two contrasting lake ecosystems. We conclude that this approach will contribute to a better understanding of the ecological significance of zoosporic organisms in microbial food webs of pelagic ecosystems.

Keywords: Chytrids, Sporangia, Zoospores, Clone-FISH, CARD-FISH, Aquatic microbial ecology.

Introduction

In pelagic systems, small heterotrophic flagellates (HF) are considered the main grazers of bacteria and other picoplankton (Fukami *et al.*, 1996). In addition, they are prey sources for microbial grazers and for metazoa (Arndt, 1993; Premke and Arndt, 2000), thereby representing key intermediates in aquatic food webs. HF cells have few conspicuous structural features for accurate identification based on morphological characters (*cf.* Sime-Ngando *et al.*, 2010). This is why 18S rDNA surveys have been conducted but relatively recently in freshwater systems, to assess the taxonomic composition of natural communities (Lefranc *et al.*, 2005; Richards *et al.*, 2005; Slapeta *et al.*, 2005; Lepère *et al.*, 2008), often dominated by small unidentified picoplanktonic items (Lefèvre *et al.*, 2007, 2008). These studies have demonstrated that HF assemblages form a heterogeneous polyphyletic group of microorganisms, including a large number of genetically unrelated organisms from many different phyla and with unexpected, neglected putative functions. For example, we have recently unveiled lineages belonging to Fungi, primarily to the division of Chytridiomycota (i.e. chytrids, *sensu* Barr, 2001) as important members of freshwater HF communities (Lefèvre *et al.*, 2007), which included typical protistan bacterivores but also saprobes and facultative or obligate parasites, primarily of phytoplankton, such as chytrids (Ibelings *et al.*, 2004). Chytrids are omnipresent in pelagic environments (Kagami *et al.*, 2007; Gleason *et al.*, 2008) where they can have a significant impact on the dynamics of host populations (Canter and Lund, 1951; Van Donk and Ringelberg, 1983), and provide valuable food source to zooplankton (Kagami *et al.*, 2007; Gleason *et al.*, 2008; Gleason and Lilje, 2009). However, they have received little attention in terms of quantitative ecology, mainly because of methodological difficulties and the complexity of life cycles (Rasconi *et al.*, 2009). Major methodological difficulties are related to the facts that chytrid zoospores are small in size (2-5 µm, Canter and Jaworski, 1980) and lack phenotypic characteristics for easy identification and counting (Kudoh and Takahashi, 1990). For these reasons, they probably have been misidentified and included in total counts of phagotrophic HF (i.e. protists) in previous studies (see Figure 2 in Sime-Ngando *et al.*, 2010). Methods for distinguishing chytrids from protistan HF are thus strikingly lacking in aquatic microbial ecology.

Sporangia and the associated rhizoidal system are characterized by a chytinaceous wall (a common fungal structure element for many species) which can be targeted by specific fluorochromes such as calcofluor white (Rasconi *et al.*, 2009). In contrast, because the chitinaceous wall springs out after zoospore encystment (Selitrennikoff *et al.*, 1976; Powell, 1993), chytrid zoospores completely lack cell wall and chitin, precluding any simple use of fluorochromes for their quantitative assessment in aquatic environments. Molecular approaches, primarily fluorescence *in situ* hybridization (FISH), offer an alternative for quantitatively probing both chytrid sporangia and zoospores in nature. FISH method is based on the detection of targeted nucleic acid sequences by the use of oligonucleotide probes labelled by a fluorochrome, usually Cy3 (Amann *et al.*, 1995; Baschien *et al.*, 2008). However,

its application to the recently unveiled zoosporic chytrids in lake pelagial is still problematic, because of the lack of laboratory cultures which are necessary to test and validate the specificity and hybridization conditions of FISH probing. That is why we have adapted an alternative approach called clone–FISH, known from prokaryotes (Schramm *et al.*, 2002), for the testing of an oligonucleotidic probe that we have recently designed and optimized but for qPCR (Lefèvre *et al.*, 2010). This approach is based on the genetic modification of a clone of *Escherichia coli* by inserting plasmid vector containing the target 18S rDNA sequence. One of the major limitations of FISH-based methods for natural samples is the autofluorescence interference from autotrophic organisms. During the past few years, numerous efforts have been made to improve the sensitivity of monolabeled probes for FISH assay, including the use of brightener fluorochromes (Glöckner *et al.*, 1996), or of signal amplification with reporter enzymes (Schönhuber *et al.*, 1999). Of particular interest is the hybridization method using horseradish peroxidase (HRP) labelled probes activated by fluorescent tyramide (also known as catalyzed reporter deposition, CARD-FISH), which is very efficient in overcoming the interference from natural fluorescence (Schmidt *et al.*, 1997). The method is based on the fact that each HRP-labelled probe catalyzes the deposition of many labelled tyramides, so that numerous fluorescent molecules are introduced at the hybridization site, resulting in net fluorescence signal amplification, compared to the simple Cy3-monolabelled FISH probes (Not *et al.*, 2002).

In this study, (i) an oligonucleotidic probe for aquatic chytrids was tested in laboratory conditions and with enriched cultures of natural infected diatoms, using the clone-FISH approach. Following validation, (ii) the probe was then applied to natural communities collected in two contrasting lake ecosystems, using the CARD-FISH approach. In addition, (iii) the total counts of HF communities were determined and the interference from fungal zoospores assessed, for evidence of the relative abundances of fungal chytrids versus phagotrophic protists within these communities. The present study has resulted in the development of a simple method to detect and quantify an important group of actively growing zoosporic organisms in pelagic environments, which is being investigated at an increasingly rapid rate, almost exclusively in freshwater lakes (Jobard *et al.*, 2010; Rasconi *et al.*, *in press*; Sime-Ngando *et al.*, 2010).

Materials and methods

***In silico* probe design.** The designed probe (Table 1) targets fungal species in the order Chytridiales (Lefèvre *et al.*, 2010), the largest order of the division Chytridiomycota (chytrids) (Gleason *et al.*, 2008), mainly represented by phytoplanktonic parasites in aquatic environments (Huber-Pestalozzi, 1944; Canter, 1950; Sparrow, 1960). It was designed using the alignment of rDNA sequences of Chytridiales obtained from GenBank (*http://www.ncbi.nlm.nih.gov/*), together with 106 sequences derived from 18S rDNA PCR surveys of freshwater picoeukaryotes conducted in French Lakes Pavin (Lefranc *et al.*, 2005; Lefèvre *et al.*, 2007, 2008), Godivelle and Aydat (Lefranc *et al.*,

2005). Distinct rDNA sequence unique to target organisms was localized and imported in Primer3 software (*http://fokker.wi.mit.edu/primer3/input.htm*) in order to design a probe with size between 18 and 27 bases, probe melting temperature (Tm) between 57°C and 63°C, and GC percentage at about 50%. The probe was analyzed for potential complementarities (hairpins and dimers) using Netprimer software (*http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html*). The probe was commercially synthesized by MWG-biotech Company (Germany) and labelled with the fluorochrome Cy3 for the testing of hybridization conditions using clone-FISH and enriched natural isolates, before application to environmental samples using the CARD-FISH approach.

Table 1: Probe Chyt1061 sequence, percentage of G+C in the sequence, and dissociation temperature (Td) value estimated experimentally with variable formamide concentrations in hybridization buffer.

Probe	**Sequence**	**Size**	**GC %**	***S. cerevisiae* position**		**Td value estimation**
	5'-3'	(bp)		5'	3'	(°C)
Chyt1061	CATAAGGTGCCGAACAAGTG	20	50	1061	1080	61
Chyt1061 rv	GACTTGTTCGGCACCTTATG	20				

Probe evaluation

The clone-FISH approach. In the absence of cultures of chytrids to evaluate our probe before application to environmental samples, laboratory tests were performed using Fluorescent *in situ* Hybridization (FISH) targeting rRNA that has been transcribed *in vivo* from a vector containing the 18S rRNA gene sequence of interest. The vector was introduced into prokaryotic cells (i.e. *E. coli*). This approach is known as Clone-FISH and was first developed with 16S rRNA gene inserts by Schramm *et al.* (2002). In our adaptation of the approach, cells of *E. coli* clone BL21 star were genetically transformed by inserting plasmid vector containing 18S rDNA sequence from several different target fungal cells. Specific plasmid inserts came from freshwater lake surveys of picoeukaryote 18S rDNA (Lefèvre *et al.*, 2007, 2008) and fungal 18S-ITS rDNA as well (Jobard *et al.*, in prep) (Table 2).

The vector pCR®2.1-TOPO® (Invitrogen) with the insert of 18S rDNA was reinserted into a chemically competent *E. coli* strain (OneShot® BL21 Star™ (DE3) Invitrogen, Carlsbad, CA) containing a genomic copy of IPTG (Isopropyl-beta-thio-galactoside) inducible T7 RNA polymerase (DE3 Strains), to generate sufficient transcript targets for hybridization signal. Top10 F strain (no-DE3) was used as host negative control. Genetically modified bacteria (i.e. *E. coli* clone BL21 star with target insert) were grown in LB (Luria Broth) medium containing 100 $\mu g.ml^{-1}$ ampicillin to an OD_{600} of 0.3-0.4 from 1:50 dilution of an overnight culture, at 37°C on a shaker (180 rpm). IPTG

(1mM) was added into the cultures in order to induce the *in vivo* transcription of the rRNA gene inserts. Various times (1 h, 2 h, 3 h, 4 h and 5 h) of IPTG induction were tested with the *E. coli* clone DE3 for determining optimal induction time. Chloramphenicol (170 $mg.l^{-1}$) was then added for 4 h before the cultures were fixed with paraformaldehyde (3% final concentration) and stored at 4°C overnight. After this delay, clones were pelleted by centrifugation (4000 x g) for 5 min at 4°C, and resuspended in 8 ml of PBS 1x (phosphate buffer saline) to wash cells. A second washing was performed before resuspending cells in 500 µl of PBS 1x. Approximately 10^6 cells were then filtered on white 0.6 µm pore size polycarbonate membranes and stored at -20 °C until the following tests.

Table 2: Phylogenetic affiliation and accession number of clones BL21 containing eukaryotic 18S rDNA insert from Lake Pavin used in this study, and signal intensity of FISH (estimated from microscopic observation) after hybridization with probe Chyt1061 and its reverse complement

Name	Phylogenetic affiliation	Accession no	Trophic state	Mismatch number	Hybridization with probes		References
					Chyt1061	Chyt1061rv	
Positives[a]							
PFD11AU2004	*Rhizophidium*	DQ244013	Oligomesotrophic	1	ND	+++	Lefèvre et al. 2007
PFB11AU2004		DQ244014		0	+++	-	
PFF6AU2004		DQ244004		1	-	+++	
PF6SP2007			Oligomesotrophic	2	-	+++	
VBA7SP2007			Humic	0	ND	ND	
AB7SP2007			Eutrophic	2	ND	ND	
AG1SP2007				2	ND	ND	
Negatives [b]							
PFH8AU2004	*Microallomyces*	DQ244002	Oligomesotrophic	5	-	-	
PFA4AU2004		DQ244003	Oligomesotrophic	6	-		
PFA12AU2004	Fungi novel clade	DQ2244011		4	-	-	
PFB7SP2005	Fungi novel clade	EU162635.1		5	ND	ND	
PCF12AU2004	Cercomonadida	DQ3998		4	-	-	
PG4P06	Chlorophyceae			5	-	-	

+++ very bright signal; ++ good signals; - no signal. ND, not determined

[a] Clones containing vector with insert from Chytridiales

[b] Clones containing vector with insert from eukaryotes excepted Chytridiales

Hybridization stringency. The *in vivo* hybridization tests for detecting *E. coli* targeted clones were conducted under different levels of stringency, the conditions being adjusted by varying the formamide concentration from 0 to 70% (v/v) with an increment of 10%. The signal intensity of at least 20 single bacterial cells containing a positive insert (namely PFB11AU2004, affiliated to *Rhizophidium* spp.) was measured at fixed hybridization temperature (46°C) with increasing concentrations of formamide in the standard hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH7.2), 0.01% SDS, and 2.5 $ng.\mu l^{-1}$ of Cy3-labelled probe), assuming an increase of the effective

hybridization temperature of 0.5°C per 1% of added formamide (Stahl and Amann, 1991). Tm could thus be experimentally estimated with the formula: Tm = 46 + 0.5 x [% formamide], for testing of the hybridization stringency. In parallel, *E. coli* clone PG4P06 containing non-target inserts (i.e. affiliated to *Cryptophyceae*), was used as a negative control. Hybridization time was set at 120 min at 46°C, followed by 30 min washing at 48°C. The washing buffer consisted of 20 mM Tris-HCl (Ph 7.2, 5 mM EDTA, 0.01% SDS, and variable concentration of NaCl depending on the initial percentage of formamide in hybridization buffer) (Pernthaler *et al.*, 2001). Hybridized clones were counterstained with 4.6-diamidino-2-phenylindole (DAPI; 0.5 $\mu g.ml^{-1}$) and hybridization signal evaluated in a dark room under an inverted epifluorescence microscope (Leica DMIRB model) equipped with 1250 x (i.e. 100x1.25) objective lens, a mercury lamp HBO-100W, and a set of different optic filters, including filters (340-380 nm) for UV light excitation. For picture capture and processing, the microscope was equipped with a Leica color video camera (model DC 300F) and a Leica Q500 personal computer. Filters were visualized under green light for Cy3 fluorescence (excitation filter: 535-550 nm; collecting filter 610-675 nm) and under UV light for DAPI. Differences in fluorescence signal intensity from binding probe, with increasing formamide concentrations, were evaluated by epifluorescence microscopy by plotting the fluorescence relative intensity of *E. coli* clones versus the formamide percentage. Optimal hybridization stringency was defined as the highest concentration of formamide in the hybridization buffer that does not result in loss of fluorescence intensity from target clones.

Probe specificity. The specificity of the designed probe was checked in *silico* with basic local alignment search tool (BLAST, Altschul *et al.*, 1997) and by screening of clone libraries with the clone-FISH approach. Clones containing 18S rRNA or 18S-ITS rRNA gene inserts from different eukaryotes closely related to microorganisms of interest (Table 2) were FISH-targeted following the protocol described previously (with 30% formamide in the hybridization buffer). In addition, the *in vivo* transcription of the 18S rRNA gene insert was induced with IPTG (1mM) for 1 h. The designed probe or its reverse complement probe (Table 1) was used, depending on the orientation of insertion into the vector (i.e. 3'→5' or 5'→3' way downstream the T7 promoter).

Application to enriched culture of natural infected diatoms. To investigate whether hybridization conditions developed using the clone-FISH approach could be used with the targeted eukaryotic cells, FISH was performed on two diatom species isolated from samples collected during spring in Lakes Pavin and Aydat, and grown together with their parasites in enriched filtered and autoclaved lake water as suggested by Lund *et al.* (1975). Spring corresponds to the period of maximum development of chytrid parasites in the two lakes, and the diatoms species were chosen for their high sensitivity to chytrid parasites (Rasconi *et al.*, 2009). The corresponding parasite - host systems isolated in laboratory cultures were as follows: *Rhizophidium* sp and *Asterionella formosa* in Lake Pavin, and *Chytridium* sp and *Fragilaria crotonensis* in Lake Aydat.

Both systems were harvested on polycarbonate white filters (pore size 0.6 µm, catalog no. DTTP02500, Millipore). FISH targeting of fungal zoospores and sporangia with Cy3-labelled oligonucleotide probe was performed in the standard hybridization buffer (containing 30% formamide). The reverse complement probe was used as a negative control and to check the autofluorescence interference from fungi and other natural plankton present in the culture medium. Hybridization time lasted 3 h at 46°C, followed by rinsing of filters in the washing buffer for 30 min at 48°C, and by staining with DAPI before mounting between slides and glass cover slips. Observations were carried out in a dark room under the Leica epifluorescence microscope.

Application to environmental samples and total heterotrophic flagellate counts. Samples were collected in two freshwater lakes which differed in trophic status and were located in the French Massif Central. Lake Pavin (45° 29' 41" N, 002° 53' 12" E) is an oligo-mesotrophic deep volcanic mountain lake (Zmax = 92 m), characterized by small surface (44 ha) and drainage basin (50 ha) areas. Lake Aydat (45° 39' 48" N, 002° 59' 04" E) is a small eutrophic lake (Zmax = 15 m, surface area = 60 ha), with a larger catchment area (3 x 10^4 ha). Samples were collected in triplicate near the centre of both lakes, at the point of maximum depth. Both lakes were sampled twice in 2007 during spring and autumn (22 March and 2 October 2007) for Lake Pavin, and during spring and late-summer (22 May and 28 August 2007) for Lake Aydat. During all sampling occasions, ca 21 L of the whole euphotic layers of the two lakes (estimated from Secchi depth) was sampled manually and concentrated using the size-fractionated community approach (< 25 µm fraction for parasitized small algae and zoospores, and > 25 µm for large parasitized algae), as described previously (Rasconi *et al.*, 2009). Aliquots of both fractions were collected on polycarbonate white filters (pore size 0.6 µm, catalog no. DTTP02500, Millipore) for CARD-FISH.

For CARD-FISH analyses, we applied the protocol proposed by Not *et al.* (2002). Briefly, filters were covered with the hybridization buffer (30% deionized formamide, 0.9 M NaCl, 20 mM Tris-HCl [pH 7.5], 0.01% sodium dodecyl sulfate, 10% blocking reagent [Roche Diagnostics/Boehringer]) and the oligonucleotide probe labelled with horseradish peroxidase (100 ng.µl^{-1}) commercially synthesized by Biomers (Germany). The mixture was left to hybridize at 35°C for 3 h. After two successive 20 min rinses in the dark at 37°C in a washing buffer (112 mM NaCl, 5 mM EDTA, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl [pH 7.5]; the volume of NaCl depends on the percentage of formamide in the hybridization buffer), samples were later equilibrated to increase enzyme activity in TNT buffer at room temperature for 15 min. Signal amplification was performed by incubation in TSA mixture (1:1) of 40% dextran sulphate (Sigma-Aldrich) and 1 x amplification diluent (Perkin-Elmer LAS), to which fluorescein isothiocyanate coupled with tyramide (1 x; Perkin-Elmer LAS) was added (1:50 vol/vol). Filters were transferred in two successive 5-ml TNT buffer baths at 55°C for 20 min, in order to stop the enzymatic reaction and remove the dextran sulphate. Finally, filters were counterstained with DAPI (Porter and Feig, 1980), mounted between slides and glass cover slips, and observed under ultraviolet and blue light in a dark room using the Leica epifluorescence microscope.

Finally, the total abundance of HF was determined and the percentage of zoospores estimated, in order to evaluate the quantitative importance of chytrids amongst natural HF assemblages. Natural raw samples were concentrated on nucleopore polycarbonate black filters (pore size 0.8 μm, catalog no. 110659, Whatman) by using gentle vacuum (< 0.2 bar or 20 kPa) and total HF counted under the epifluorescence microscope after coloration with primuline (Caron, 1983). For all samples, at least 200 individuals were counted, a number that gives a counting error of less than 15% at 95% confidence limits, according to Lund *et al*. (1958). Counts of fungal zoospores via CARD-FISH were subtracted from those of the total HF, in order to estimate the interference of chytrids in natural HF assemblages.

Results

Characteristics and specificity of the probe. The probe used matches a unique region of the 18S rRNA of the fungal order of Chytridiales and was named Chyt1061, because of the sequence position (1061 base pairs) on *Saccharomyces cerevisiae* small-subunit rDNA molecule (GenBank accession no. J01353) (Table 1). According to Behrens and collaborators (2003), this position provides a good accessibility for FISH probing. There were 2 mismatches in the middle of the probe with sequences of chytridiale species (Table 2), which did not result in loss of positive signal. Negative clones displayed more mismatches (> 4) in the middle and at the 3' boundary. Using BLAST (NCBI) search against the GenBank, a non-redundant nucleotide database, we found a strong specificity of our probe for Chytridiales 18S rDNA sequences from 6 different environments and seven different world regions (Table 3). No dimers or hairpins were found using Netprimer software. In addition, among clones containing eukaryote 18S rDNA insert tested with probe Chyt1061 and its reverse complement, five were FISH-positive and gave good hybridization signals while other clones showed no signal. Depending on the orientation of insertion into the plasmid, signal was obtained with Chyt1061 probe for positive clone PFB11AU2004 or with its reverse complement for positive clones PFD11AU2004, PFF6AU2004, A20407 and PF60407. FISH with probe targeting the antisense strand of the plasmid but not the transcribed rRNA gave no signal (Table 2), confirming that observed signals came from specific probe hybridization.

The clone-FISH approach with fungal rRNA gene. The clone-FISH approach showed that the hybridization fluorescent signal depended on the duration of IPTG induction, the duration of incubation of culture with chloramphenicol, and the concentration of formamide in the hybridization buffer. Indeed, the signal intensity was the best with BL21 star strain for 1 h IPTG induction, followed by 4 h incubation with chloramphenicol. Hybridization tests with non-DE3 strain (top 10F) gave no signal, implying that signal came from transcripts. Fluorescent signal intensity decreased drastically at formamide concentrations between 20 and 30%, and then continued to decrease more slowly at percentages. Accordingly, all subsequent tests were performed at 30% formamide concentration in the hybridization buffer. Assuming an increase of the effective hybridization temperature of 0.5°C per 1% of added formamide, the melting temperature (Tm) of the probe Chyt1061 was experimentally calculated at 61°C (Table 1).

Table 3: Results of checking Chyt1061 probe specificity from the first hits in the Genbank Database. These data are the percentages (number of results) of identical matches between the Chyt1061 probe and sequences in the GenBank (NCBI), a non-redundant nucleotide database (September 2008), for Chytridiale, Fungi and Eukaryotes (bold), together with the details for the different genera and (or) species.

Taxon Closest relative			Identical matches with Chyt1061 expressed as percentage (and number of results)	Origin		References
				Ecosystem	Region	
Chytridiale			**89.83 (97)**			
		Rhizophidium sp	4.63 (5)		USA	
		Chytridium polysiphoniae	0.93 (1)		UK	
		Boothiomyces macroporosum	0.93 (1)		USA	
	Uncultured chytridiale		0.93 (1)	River sediment	Switzerland	Berney et al., 2004
	Uncultured chytridiomycota	*Rhizophidium sp*	82.41 (89)	Freshwater	France	Lefèvre et al., 2007, 2008
				Hydrothermal	North Atlantic	LeCalvez et al., 2009
				Anoxic sediments	USA	Dawson et al., 2002
				soil	USA	O'Brien et al., 2005
Fungi			**2.78 (3)**			
	Uncultured fungus	*Geopyxis carbonaria*	1.85 (2)	Spring hyporheic zone		Zhang et al., 2008 Bärlocher et al., 2008
	Uncultured Basidiomycete		0.93 (1)			
Eucaryotes			**7.41 (8)**			
	Uncultures eukaryotes	*Chytridium polysiphiniae*		Oxygen-depleted marine sediment	Artic	Stoeck et al., 2007
		Spizelliomycete		Freshwater	France	Lefranc et al., 2005
		Rhizophyctis harderi		Anoxic Fjord	Denmark	Zuendorf et al., 2006
		Spizelliomycete		Marin		Massana et al., 2004

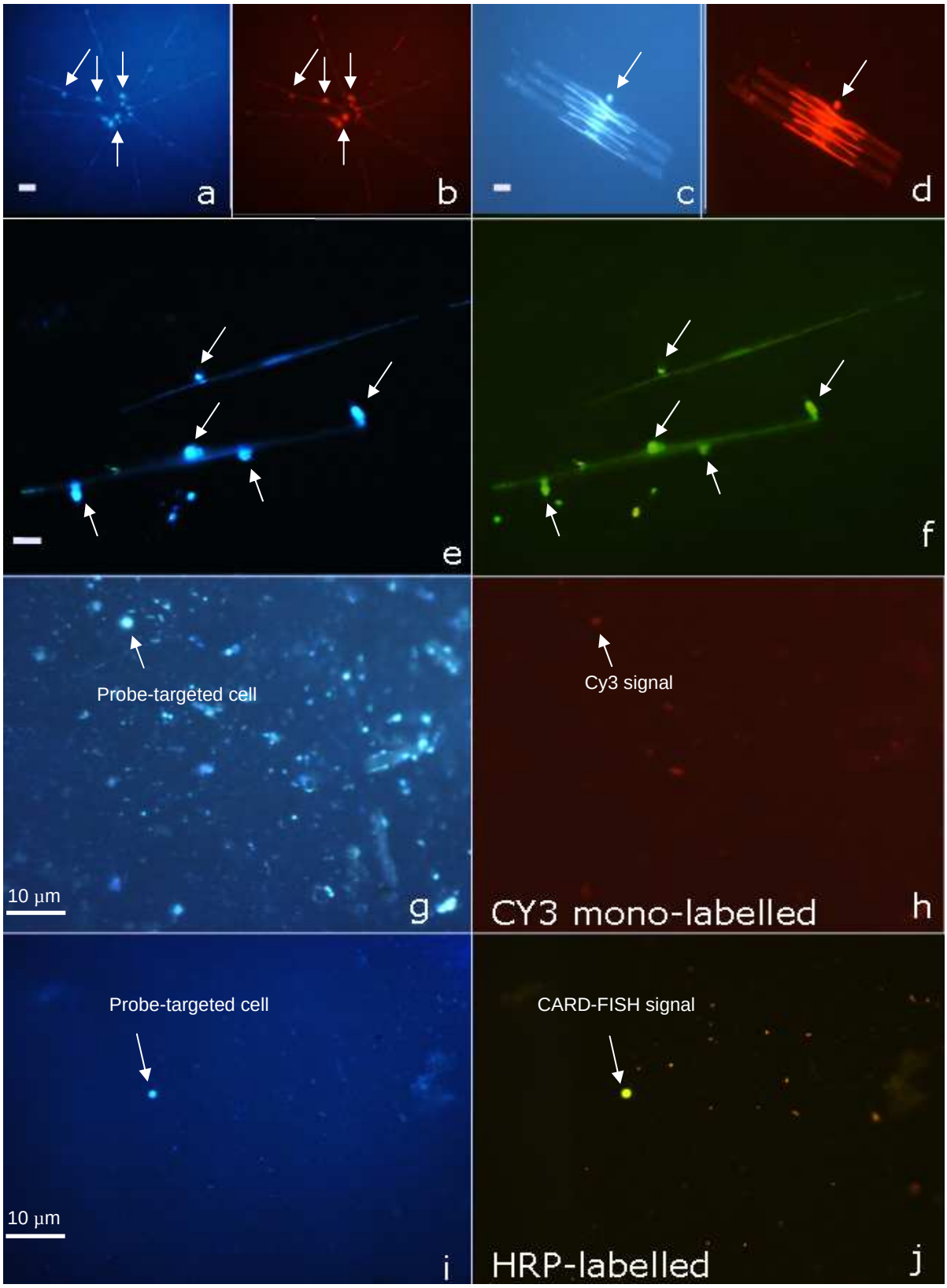

Figure 1: Examples of microscopic micrographs of DAPI stained (a,c,e,g,i) and Chyt1061 probing organisms (b,d,f, h, j) from enriched laboratory cultures (a-d), Lake Pavin (e,f), and Lake Aydat (g - j). Under green light, hybridization with the probe Chyt1061 revealed chytrid parasites (arrows: sporangia) on different species of host algae: the diatoms *Asterionella* sp (a,b), *Fragilaria* sp (c,d), and *Synedra* sp (e,f). The weak signal of the monolabelled Cy3 probe (g,h) was improved (arrows: picoplanktonic cell targeted with the probe) when applying the Card-FISH approach (i,j).

Probe Chyt1061 and the quantitative ecology of environmental chytrids.

Enriched cultures of diatoms. Enriched cultures of two species of diatoms from Lake Pavin and Lake Aydat were used for hybridization of eukaryotic parasites of interest with the FISH probe Chyt1061. Observation of parasitized phytoplanktonic cells, i.e. *A. formosa* parasitized by *Rhizophidium* sp. in Lake Pavin and *F. crotonensis* parasitized by *Chytridium* sp. in Lake Aydat, revealed that the sporangia of both species were FISH-positive after hybridization with Chyt1061, and bright signal was recorded (Figure 1a-d). It was also possible to assess the numerical abundances of zoospores in the same enriched samples, which were at 1059 ± 107 spores ml^{-1} in Lake Pavin, and at 935 ± 323 spores ml^{-1} in Lake Aydat.

Environmental samples. CARD-FISH analyses of > 25 µm fraction of environmental samples revealed that sporangia from several species of fungal parasites were attached on different algal hosts, given a highly bright signal. In samples collected in March and May, diatoms were dominant (> 90% of total phytoplankton abundance) in both lakes. In Lake Pavin, positive signals were found on diatoms *A. formosa* and *Fragilaria* sp, and multiple infections were found on *Synedra acuus* (Figure 1 e, f). In Lake Aydat, hybridized sporangia were found on *Melosira italica* (data not shown). In summer/autumn, phytoplankton community composition changed in both lakes. In Lake Pavin, Chlorophyta became dominant and hybridized parasites were found on the desmid *Staurastrum* sp. and on the chlorococcale *Oocystis* sp. In Lake Aydat, a cyanobacterial bloom occurred and hybridized parasites were found on *Anabaena flosaquae*. Overall, the numerical abundances of sporangia in natural samples were similar (t test, $p > 0.05$) to those previously recorded in the same samples using the calcofluor approach (Rasconi *et al.*, 2009). However the CARD-FISH resolution for fungal images and species identification was poor compared to the calcofluor approach. For this reason, and because both calcofluor and CARD-FISH approaches allow the distinction of fixed chytrid sporangia from attached protists (e.g. the group of *Bicosoeca*), we will exclude sporangia from comparisons thereafter and focus on the zoospore stages and their interference in total counts of heterotrophic flagellates in the < 25 µm sample fractions.

The Cy3-monolabelled FISH probing of our natural samples clearly showed that the fluorescence of targeted chytrid zoospores was quite similar to the autofluorescence from natural picoautotrophs (Figure 1 g, h). The application of the CARD-FISH approach clearly has improved the detection and the recognition of chytrid zoospores, because of the enhanced signal conferred by HRP-labelled probes (Figure 1 i, j), compared to monolabelled oligonucleotides (Figure 1 g, h). In addition, the choice of fluorescein as stain (emission in the green spectrum at 520 nm) has reduced significantly the interference from natural fluorescence of autotrophic organisms, thereby preventing the use of the deductive approach based on a double counting of the same sample, i.e. with and without hybridization (Lefèvre *et al.*, 2005). Quantitatively, zoospore abundance was higher in October (573 ±

68 spores ml^{-1}) than in March (52 ± 11 spores ml^{-1}) in Lake Pavin. In Lake Aydat, abundances were lower compared to Pavin, and were at 156 ± 51 spores ml^{-1} in spring and at 89 ± 11 spores ml^{-1} in summer (Figure 2). Compared with the total counts of heterotrophic nanoflagellates (HF), the interference from fungal zoospores in total HF ranged from 5 to a value as high as 60%, the values being higher in Lake Pavin than in Lake Aydat. Interestingly, the highest level of zoospore interference was noted in the sample with the lowest number of HF, this being valid for each of the sampled lake (Figure 2).

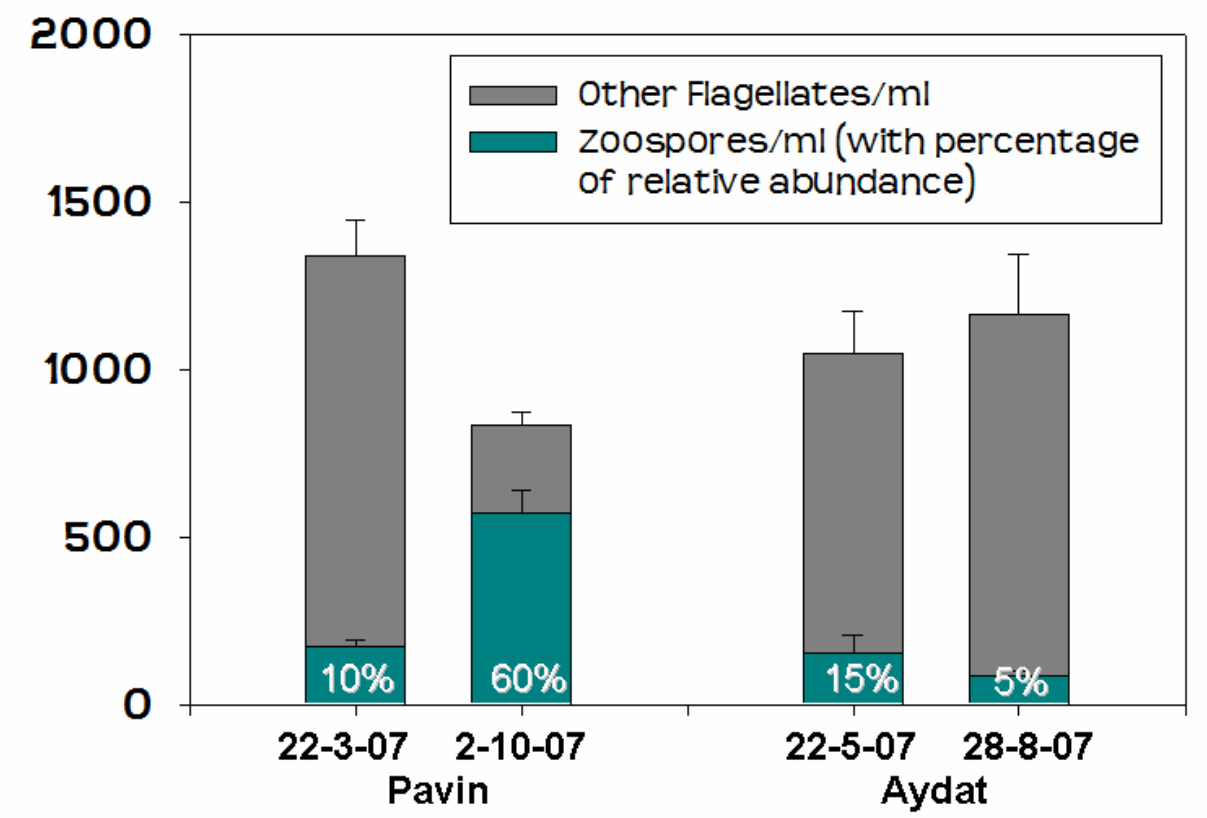

Figure 2: Abundances of total heterotrophic flagellates (HF) and of fungal zoospores in two freshwater lakes. The percent contributions of zoospores to total HF are included in the graph.

Discussion

The aim of this study was to develop a method to identify and count chytrid parasites of phytoplankton, including both host-attached sporangium and free-living zoospore stages in aquatic environments. To our knowledge, no molecular quantitative probe for visualizing and direct-counting specific chytrid parasites of algae in aquatic environments is available. In this study**,** we propose a FISH approach for the quantitative ecology of an important group of aquatic fungi, the Chytridiomycetes order of Chytridiales. This group is commonly found growing in aquatic ecosystems (Barr, 1987), especially in freshwaters (Sparrow, 1960), and includes many parasites of a wide range

of phytoplankton species (Sigee, 2005). Calcofluor white staining has been reported as being the best method for the qualitative ecology of sporangia (Rasconi *et al.*, 2009), yielding the highest quality images for fungal identification, compared to the molecular probing. Zoospores however lack chitin, thus rendering them incompatible with the calcofluor staining method. Fluorescent *in situ* hybridization (FISH) technique, a now widely used approach for the direct, quantitative study of specific uncultured microbial populations in environmental samples, appeared as a valid alternative.

In the absence of laboratory cultures of chytrids, we adapted the clone-FISH approach (Schramm *et al.*, 2002) to test and validate our probe. Our experimentation revealed that clone-FISH is a valid alternative method to test molecular probes before application to environmental eukaryotic samples. The approach is cheap and rapid, and allows an easy testing of the characteristics and specificity of eukaryotic oligonucleotidic probes, from FISH-targeting of prokaryotic cells containing the rRNA target sequence of interest (i.e. *Rhizophidium* insert in our case study). This is a sizeable advantage because uncultured organisms largely dominate the microbial communities in environmental samples (Rappé and Giovannoni, 2003). In our case study, this approach has allowed us to set the best hybridization conditions, i.e. 30% formamide concentration and a melting temperature of 61°C, for our probe Chyt1061. These conditions agree well with those described by Pernthaler *et al.* (2001) who calculated that a 18-base oligonucleotide sequence with a G+C content between 50 and 60% will start to dissociate at a formamide concentration of approximately 30-40% in the hybridization buffer. Clone-FISH with the probe Chyt1061 resulted in good fluorescence signal by conventional epifluorescence microscopy (Figure 1), while the reverse complement of Chyt1061 showed no detectable signal. Similar results were also obtained with enriched diatom cultures from natural lakes. BLAST search revealed strong specificity of Chyt1061 for chytrid sequences from different worldwide origins, including six different ecosystems (Freshwater, marine, lotic, anoxic, soil, and hydrothermal systems) and four geographical regions in the Northern hemisphere (North America, Europe, North Atlantic, and Arctic) (Table 3). These findings corroborate the idea that chytrids and other zoosporic organisms represent an important group of microbes in the environment (Powell, 1993), particularly in pelagic ecosystems where they still are largely neglected (Sime-Ngando *et al.*, 2010; Jobard *et al.*, 2010).

For the application of the tested FISH-probe to environmental samples, CARD-FISH clearly appeared as the most appropriate method that directly combine identification and counting of chytrids at the complex natural community level, because of the minimization of the interference from natural autofluorescence (Figure 1 h, j). Hybridization with HRP-labelled probe can enhance fluorescence intensity by 10 to 20 times (Not *et al.*, 2002), greatly facilitating the detection of target cells. This approach is thus particularly adapted to small size living particles such as fungal zoospores. Chytrid sporangia have larger dimensions than zoospores, are attached on their algal hosts, and are easier to detect under the microscope. The main advantage of FISH method is that FISH allows the detection of both sporangium and zoospore fungal life stages (Figure 1). However, an alternative staining method,

i.e. calcofluor white, exists for the detection and the quantification of sporangia (Rasconi *et al.*, 2009), and this method was preferred over the CARD-FISH-targeting which is much less efficient in terms of species identification. We believe that the probe Chyt1061 is specific to the two life stages of chytrids, although more indicated for the quantitative ecology of zoospores.

The CARD-FISH approach was applied to four natural samples from two contrasting lake ecosystems. Because few samples were collected (i.e. two dates per site), the findings are considered preliminary and mainly a "proof of concept" rather than a valid comparison of sites versus seasons. The findings were consistent with ecological considerations known from pelagic habitats and host versus parasite populations, with recurrent ecological patterns in both lakes. In Lake Pavin, the abundances of CARD-FISH targeted zoospores were lower in spring than in autumn, contrasting with the abundances of sporangia in the same samples as given elsewhere (Rasconi *et al.*, 2009). This is consistent with the biological cycle of development in zoosporic fungi, that includes a delay between the sporangium growing phase and the release of zoospores (Beakes *et al.*, 1993). Interestingly, the total abundances of heterotrophic flagellates (HF) in Lake Pavin increased from spring to autumn while the contribution of zoospores to these abundances decreased. Spring period is characteristic of the phytoplankton growing period in temperate lakes (Sommer *et al.*, 1986), when the availability of resources for bacteria and their grazers is enhanced compared to the autumn limiting period. A similar finding was recorded in the productive Lake Aydat where the resource limitation for HF growth was less marked as in other eutrophic lakes (Simek *et al.*, 1997), with comparable HF abundances in spring and in late summer. However, the relative contribution of zoospores to HF abundances increased with decreasing HF abundance, also suggesting that fungal zoospores may represent an important fraction of HF assemblages particularly when these assemblages are resource-limited. These limiting periods likely also coincided with a resource-limited phytoplankton community that comprise an important fraction of physiologically weak cells, considered the most exposed to parasitic attacks (Canter and Lund, 1948; Canter and Lund, 1969; Kagami and Urabe, 2002). The fact that fungal zoospores are an important component of pelagic communities at a time when the classical microbial loop is weakened by low resource availability supports our "*parasite/saprotroph-dominated HF hypothesis*" (Lefèvre *et al.*, 2008). This also supports the idea that bacterivory is not the sole trophic strategy for the transfer of matter and energy from the microbial food web to the higher trophic levels.

In conclusion, this study demonstrates a successful application of the FISH approach to the direct detection and counting of chytrid sporangia and zoospores in aquatic samples. The probe tested and validated for FISH could be useful to confirm our previous conclusion that zoosporic organisms, primarily chytrids, are sizeable members of the heterotrophic flagellate community in pelagic ecosystems (Lefèvre *et al.*, 2007, 2008), via their quantitative assessment at the complex natural community level. Such information is crucial to improve our knowledge about fungal parasitism of phytoplankton communities and to evaluate the quantitative importance of fungal zoospores as nutritive resources for zooplankton (Kagami *et al.*, 2007; Jobard *et al.*, 2010; Sime-Ngando *et al.*,

2010). Overall, we (i) believe that FISH offers a good tool for the quantification of zoosporic organisms in natural environments, and (ii) consider that its application will contribute to a better understanding of the ecological significance of environmental chytrids, primarily of their roles in microbial food webs of pelagic ecosystems.

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CONCLUSIONS GENERALES ET PERSPECTIVES

Récemment, plusieurs auteurs ont pris en considération l'importance des champignons dans les écosystèmes pélagiques. Depuis le début de ce travail de thèse, plusieurs travaux sur la diversité ou le rôle potentiel des champignons dans les écosystèmes pélagiques ont été publiés (Gao *et al.*, 2009 ; Tang *et al.*, 2006 ; Wurzbacher *et al.*, 2009 ; Jobard *et al.*, 2010 ; Chen *et al.*, 2010), ce qui témoigne de l'intérêt scientifique de la prise en compte de ces organismes dans le fonctionnement des écosystèmes pélagiques. Le travail réalisé au cours de cette thèse a mis en évidence la présence et une diversité fongique non négligeable dans les écosystèmes lacustres étudiés, ce qui suggère l'omniprésence de ces organismes dans l'environnement pélagique.

La structuration génétique de la communauté fongique étudiée par t-RFLP, ainsi que l'importance quantitative des différentes divisions estimée par qPCR (**Chapitre III**), nous ont indiqué que la composition de la communauté fongique varie fortement au cours d'une année, sans doute en relation avec les facteurs du milieu et les autres communautés microbiennes. Ces changements de structure correspondent à des périodes clés du fonctionnement des écosystèmes étudiés. Par exemple, le mois d'Avril est une période particulière dans les lacs oligotrophes tempérés, comme le lac Pavin et le lac de Vassivière, notamment par l'installation des populations printanières de diatomées. Ces populations, généralement de grande taille, constituent alors des hôtes préférentiels pour les champignons parasites, notamment de la division des Chytridiomycota (Rasconi *et al.*, 2009). L'exploration de la diversité par clonage séquençage de l'ADN ribosomique 18S au cours du printemps 2007 dans les écosystèmes pélagiques lacustres étudiés (**Chapitre II-1**) a, par ailleurs, confirmé la présence de chytridiomycètes mais aussi d'autres taxons de champignons, les ascomycètes et les basidiomycètes. Ces deux dernières divisions étant totalement ignorées dans les études pélagiques, beaucoup d'hypothèses accompagnent leur détection. Notre étude et d'autres études en milieux lacustres (Lefèvre *et al.*, 2007, 2008 ; Lefranc *et al.*, 2005 ; Lepère *et al.*, 2006 ; 2008) montrent que ces divisions de champignons sont omniprésentes au cours de l'année dans les milieux pélagiques. Cependant, l'hypothèse d'une origine allochtone de ces champignons n'est pas à exclure complètement. L'étude de la diversité spatiale des Eumycètes dans les lacs Pavin et Aydat a, en effet, révélé que la détection d'ascomycètes et de basidiomycètes étaient plus importantes dans les eaux proches de la zone littorale des lacs (**Chapitre II-2**). En effet, certaines espèces de champignons colonisant d'autres milieux comme le sol ont déjà été retrouvées en milieux aquatiques (Shearer *et al.*, 2007 ; Seena *et al.*, 2008 ; Damare *et al.*, 2008). Ces espèces allochtones sont capables de s'adapter à la vie aquatique et pourraient donc jouer un rôle fonctionnel dans le milieu pélagique, notamment en tant que saprophytes (Jobard *et al.*, 2010). En effet, la morphologie et la taille des espèces de champignons ascomycètes et basidiomycètes retrouvées dans les écosystèmes lacustres peuvent rendre ces microorganismes réfractaires à la prédation. Une question se pose alors sur l'importance de ces « acteurs allochtones » dans le fonctionnement des écosystèmes aquatiques pélagiques. Des études complémentaires sont nécessaires pour y répondre.

A la diversité phylogénétique des champignons correspond sans doute une diversité fonctionnelle, puisque les séquences dominantes retrouvées ont été affiliées à des espèces connues comme étant potentiellement saprophytes (e.g. *Catenomyces* sp., *Tetracladium* sp.) ou parasites, notamment du phytoplancton (*Rhizophydium* sp., *Zygorhizidium planktonicum*) ou d'autres champignons *(Rozella allomycis*). Par ailleurs, l'étude préliminaire de la dynamique quantitative des différentes divisions de champignons a montré l'importance des ascomycètes dans le lac eutrophe d'Aydat en fin de période estivale et au début de l'automne. Cette période correspond à l'efflorescence de la cyanobactérie *Anabaena flosaquae*, caractéristique de la phase saisonnière tardive de ce lac (Rasconi, 2010). Les champignons présents pourraient donc jouer un rôle important dans la minéralisation de cette matière organique connue pour être réfractaire à la prédation, notamment dans la phase de sénescence de l'efflorescence. Cette observation renforce l'idée du rôle des champignons dans le recyclage de la matière organique, particulièrement suite à l'apport de biomasse résultant des efflorescences algales (Jobard *et al.*, 2010). De plus, au cours de notre campagne d'échantillonnage, le développement de champignons mycéliens a été observé en microscopie sur des agrégats de matière organique prélevés suite au bloom de la cyanobactérie *Anabaena flosaquae* au lac d'Aydat (Jobard *et al.*, 2010). Le rôle des champignons mycéliens dans l'épuration des eaux suite aux efflorescences phytoplanctoniques a récemment été confirmé par une étude expérimentale conduite lors d'une efflorescence de la cyanobactérie *Microcystis* sp. (Chen *et al.*, 2010). Ces recherches posent la question de l'importance des champignons saprophytes dans la minéralisation de la matière organique pélagique. Ces champignons saprophytes sont donc potentiellement des partenaires synergistiques ou antagonistes des bactéries hétérotrophes, acteurs essentiels de la boucle microbienne en milieux pélagiques.

A la diversité phylogénétique des champignons correspond également une complexité morphologique de ces microorganismes. En effet, les champignons regroupent des espèces présentant différentes caractéristiques morphologiques, variables selon le stade de développement. Les séquences retrouvées lors de nos études ont pu être affiliées à des espèces de champignons possédant trois morphologies connues. En effet, des séquences affiliées à des espèces de champignons développant des formes flagellées (Chytridiomycota), mycéliennes (Ascomycota) et sphériques (i.e. levures : Ascomycota et Basidiomycota) ont été retrouvées dans les lacs étudiés (**Chapitre II-1**). L'intégration de ces champignons dans le fonctionnement des écosystèmes pélagiques dépendrait sans doute des différents phénotypes en présence, connus pour avoir différents modes de colonisation de la matière organique (Figure 1).

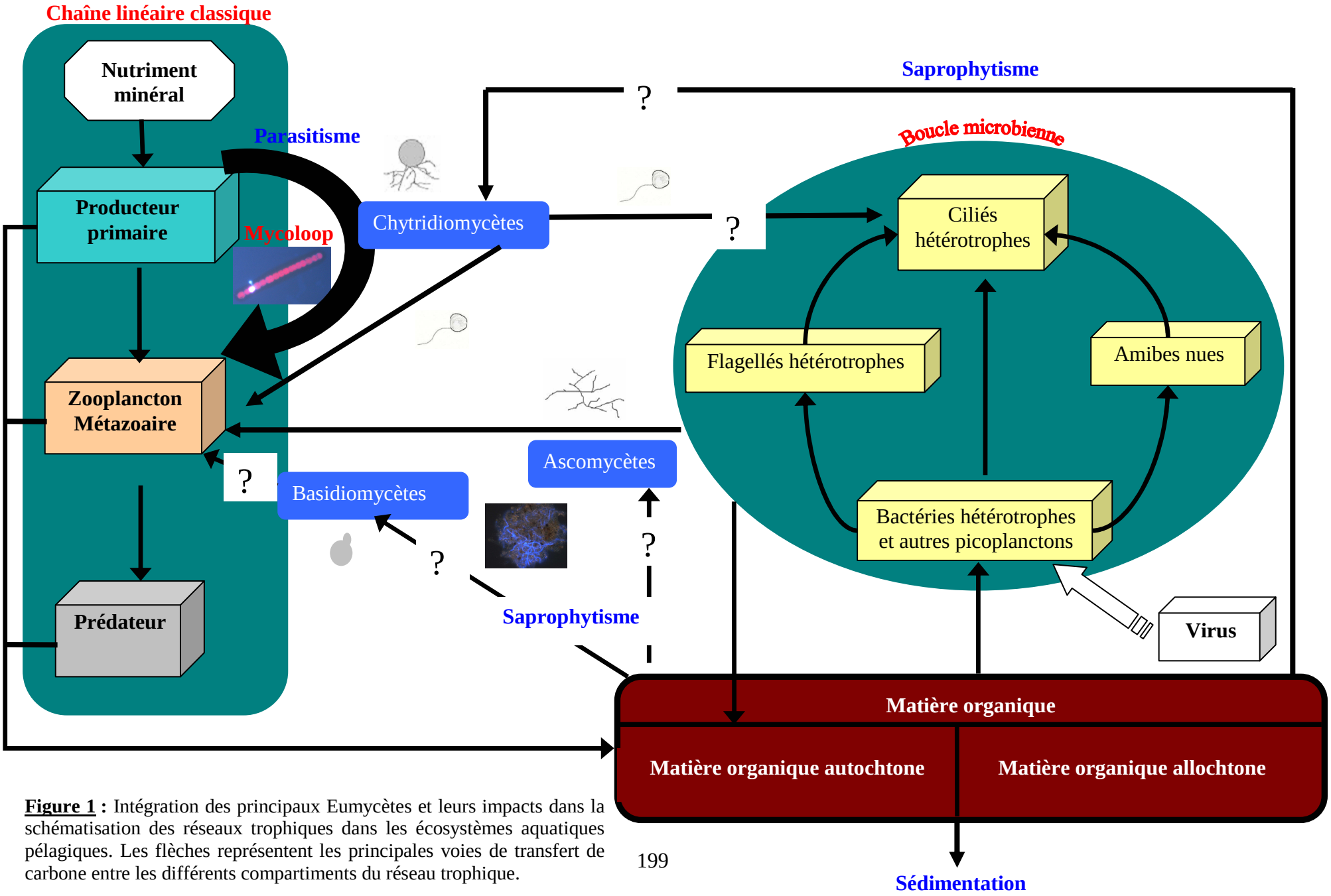

Figure 1 : Intégration des principaux Eumycètes et leurs impacts dans la schématisation des réseaux trophiques dans les écosystèmes aquatiques pélagiques. Les flèches représentent les principales voies de transfert de carbone entre les différents compartiments du réseau trophique.

Les chytridiomycètes sont des champignons dont la phase de dispersion dans le milieu correspond à une cellule uniflagellée (la zoospore), qu'il est difficile de distinguer des protistes flagellés bactérivores (Lefèvre *et al.*, 2007). La présence de ces champignons flagellés dans le milieu pélagique remet en question les flux de matière dans la conception actuelle du fonctionnement des écosystèmes pélagiques, notamment ceux transitant par la boucle microbienne (Figure 1). En effet, ces champignons sont connus pour être parasites des plantes et des animaux, ou saprophytes. Ils peuvent donc intervenir, comme précédemment mentionné, dans la régulation du phytoplancton filamenteux ou de grande taille, dont les efflorescences sont actuellement considérées comme de véritables impasses trophiques, en raison de la résistance au zooplancton herbivore.

Quant à la forme mycélienne, elle est partagée par des espèces de champignons ascomycètes et basidiomycètes. Cette forme leur procure un avantage dans la colonisation et l'expansion dans les agrégats de matière organique, comme le montre la photographie du **Chapitre I**, prise dans un échantillon prélevé au lac d'Aydat en Novembre 2007 (Jobard *et al.*, 2010). L'estimation quantitative des champignons dans cet échantillon, par PCR en temps réel, laisse apparaître un pic du nombre de copies du gène de l'ARNr appartenant aux ascomycètes, qui est en effet un groupe de champignons présentant un développement essentiellement mycélien.

En ce qui concerne les levures, ce sont des champignons particuliers qui possèdent une forme unicellulaire avec un mode de reproduction par bourgeonnement. Dans les milieux aquatiques, cette forme, que l'on retrouve uniquement chez les ascomycètes et les basidiomycètes, peut occuper les mêmes niches écologiques que les bactéries, puisqu'elles sont capables d'incorporer la matière organique par osmotrophie, directement ou après dissolution par des exoenzymes (Raghukumar, 2006). Ceci peut amener à des interactions complexes (antagonisme, synergie) entre les bactéries et les champignons dégradant la matière organique, comme cela a déjà été prouvé en rivière (Mille-Lindblomm and Tranvik, 2003 ; Mille-Lindblomm *et al.*, 2006).

La nouveauté du sujet de thèse a fait que les amorces préalablement définies comme spécifiques des champignons (pour la plupart du sol) et souvent utilisées dans les études de diversité dans les écosystèmes aquatiques se sont révélées peu adaptées à nos échantillons. En effet, de nombreuses amplifications croisées avec les amorces NS1 et ITS4 (White *et al.*, 1990) ont été observées, notamment avec des espèces phytoplanctoniques chlorophycées. Les travaux futurs devront donc avoir pour priorité le développement d'outils, notamment de nouvelles amorces, afin d'étudier plus précisément les groupes d'intérêt retrouvés dans les milieux pélagiques. La stratégie de clonage séquençage de l'ADNr 18S et de l'ITS mise en place durant ce travail de thèse nous permet, aujourd'hui, de bénéficier d'une base de données de séquences importante, pour le développement de telles amorces. Ce travail a été initié à la fin de la thèse (**Chapitre IV**) par le développement de sondes oligonucléotidiques ciblant un clade de chytridiomycètes, les Rhizophydiales (Lefèvre *et al.*, 2010 ; Jobard *et al.*, *in press*). Ce clade est prépondérant dans les écosystèmes lacustres (Lefèvre *et al.*, 2007,

2008 ; **Chapitre IV-1**) où il est principalement associé au parasitisme du phytoplancton. Ces amorces ont été dessinées avec pour objectif le suivi quantitatif des formes végétatives (sporanges) et dispersives (zoospores) de ce taxon. La détection et la quantification des sporanges de Rhizophydiales permettent également la détection *in situ* des algues hôtes de ces parasites, et le calcul de variables fonctionnelles (prévalence et intensité de l'infection) nécessaires à l'étude de l'impact des chytridiomycoses sur les communautés phytoplanctoniques et la production primaire dans les écosystèmes naturels. En parallèle, la détection et la quantification de zoospores de chytrides permettent d'évaluer leur importance dans les lacs, notamment comme sources de nourriture pour le zooplancton. Il est à noter que les amorces ont été dessinées à partir du gène codant pour l'ARNr 18S, ce qui a rendu leur utilisation possible en PCR quantitative en temps réel, et par les approches FISH.

En résumé, au vue des différentes approches moléculaires mises en application et des différentes échelles spatiales et temporelles examinées, les résultats acquis dans le cadre de ce travail de thèse nous permettent d'affirmer que les Eumycètes constituent une part importante de la diversité biologique des écosystèmes pélagiques lacustres, où leur importance quantitative laisse supposer un rôle essentiel dans le fonctionnement de ces écosystèmes. Ce travail ouvre donc des perspectives intéressantes sur le rôle potentiel d'une ressource génétique méconnue (les Eumycètes) dans les écosystèmes pélagiques, notamment en tant que parasites et saprophytes, et sur les interactions trophiques (compétition, prédation, lyse cellulaire, etc.) avec les autres composantes biotiques et avec les facteurs du milieu. Les résultats préliminaires laissent supposer que ce rôle des Eumycètes est loin d'être négligeable.

Enfin, de manière générale et du point de vue du fonctionnement des communautés, la prise en compte des stratégies trophiques négligées, notamment celles liées au développement des champignons microscopiques, devrait changer, profondément, la vision conceptuelle que nous avons des flux de carbone dans les réseaux trophiques aquatiques. Pour cela, il est impératif de favoriser l'acquisition de connaissances fine et approfondie de tous les acteurs impliqués dans les processus de transferts de matière et d'énergie dans les écosystèmes aquatiques, notamment pélagiques. C'est dans ce cadre scientifique général que se situent l'intérêt et la pertinence des résultats présentés.

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ANNEXES

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Use of Calcofluor White for Detection, Identification, and Quantification of Phytoplanktonic Fungal Parasites[▽]

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We propose a routine protocol based on size fractionation of pelagic samples and the use of the fluorochrome calcofluor white (which binds to β-1,3 and β-1,4 polysaccharides) for diagnosing, identifying, and counting chitinaceous fungal parasites (i.e., the sporangia of chytrids) of phytoplankton. The protocol was applied to freshwater samples collected during different seasons (spring and summer/autumn) in two lakes whose trophic statuses varied. Because few samples were collected (i.e., two dates per site), the findings are considered preliminary and mainly a "proof of concept" rather than a valid comparison of sites versus seasons. The results from the proposed protocol indicate higher diversity of infected host and parasite communities than in previous studies. Chytrid epidemics were omnipresent, infecting diverse phytoplankton host communities, primarily diatoms, chlorophytes, and colonial and filamentous cyanobacteria. The diversity and numerical abundance of sporangia and of hosts, and the prevalence of infection (range, <1 to 24% of total host cells) as well, increased from the oligotrophic Lake Pavin to the eutrophic Lake Aydat, while the temporal changes in parasites were apparently more influenced by the host community composition. We conclude that the proposed protocol offers a valid method for the quantitative ecology of chytrid epidemics in aquatic ecosystems and food web dynamics.

Fungal infections are recurrent in aquatic ecosystems (15, 42, 46). Organisms belonging to the order of *Chytridiales* (i.e., chytrids) are known mainly as phytoplankton parasites (10, 22, 44). Recently, we have unveiled a large reservoir of unexpected fungal diversity in freshwater lakes, primarily of chytrids (31–33). Parasitic chytrids and other zoosporic true fungi sensu Barr (3) produce zoospores and are often host specific, highly infective, and extremely virulent (13, 16). They are considered relevant not only for the evolution of their hosts but also for the population dynamics and successions of phytoplankton communities (14, 48). Studies of chytrid fungal parasitism carried out in the English Lake District indicate that infection of diatoms, desmids, and other green algae is fairly common (10). Significant chytrid parasitism has also been recorded in other lakes, affecting primarily the diatom *Asterionella formosa* (12, 29, 37, 48). However, fungal parasitism on plankton has rarely been studied and has mostly been restricted to descriptive taxonomy. Full descriptions of parasitic chytrids have been given since the middle of the last century (8–9) but even today, their impact on the dynamics of host populations and the related biogeochemical cycling and energetics remain largely unknown (16), mainly because of methodological difficulties (17).

Various approaches have been used to study fungal parasites, but routine techniques for reliably identifying and counting these organisms are lacking in the context of aquatic microbial ecology (31–33). Some of them have been misidentified as protistan bacterivorous nanoflagellates, e.g., flagellates in the group of *Bicosoeca*, which are attached to phytoplankton but do not harm the algae (31–33). A few studies have demonstrated epidemic outbreaks of chytrids on phytoplankton, but mainly with particular emphasis on host-parasite interactions and coevolution (21, 30, 48). Thus far, observations of parasitic fungi were obtained by using phase-contrast light microscopy with live or Lugol's iodine preserved samples (30, 39, 48). Such conventional microscopy allows observation of fungal sporangia or similar forms (especially in laboratory cultures) but is a poor approach for characterizing chytrid parasites in natural samples, at the complex community level. For example, a simple light microscopy observation of fungal rhizoidal systems, i.e., a pertinent criterion for identifying chytrids (10, 13, 44), is very difficult, a situation which usually leads to confusion of chytrids with protistan flagellates such as choanoflagellates (31–33). Most chytrids occupy the most basal branch of the kingdom *Fungi*, a finding consistent with choanoflagellate-like ancestors (25, 26).

Methodological limitations for the study of the ecological dynamics of chytrid populations can be overcome with epifluorescence microscopy coupled to a specific fluorochrome targeting molecular tracers (i.e., some types of polysaccharides) of chitinaceous fungal structures, primarily sporangia, the presence of an opercule, and the extent of the rhizoidal system. We propose here a simple procedure based on the fluorochrome calcofluor white (CFW) and epifluorescence microscopy for diagnosing, identifying, and counting parasitic chytrids within phytoplanktonic communities.

MATERIALS AND METHODS

Sites and sampling. Samples were collected in two freshwater lakes, which differed in trophic status and were located in the French Massif Central. Lake

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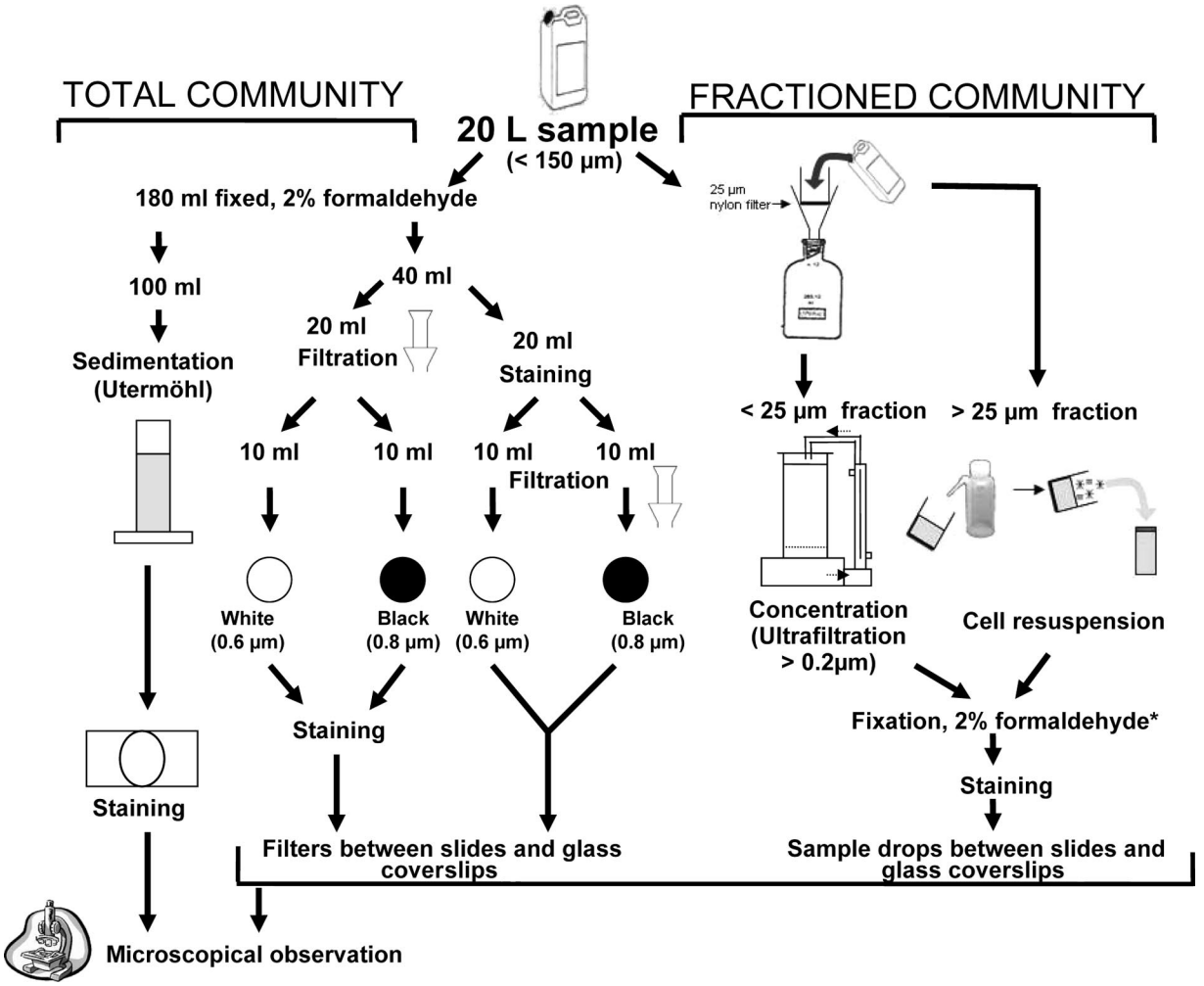

FIG. 1. Sample partitioning and different concentration procedures tested for CFW staining and epifluorescence microscopy observation of phytoplankton parasitic chytrids. *, the fixation step for the fractionated community approach is facultative, i.e., can be avoided when observations are made without delay.

Pavin (45°29′41″N, 002°53′12″E) is an oligotrophic deep volcanic mountain lake (Z_{max} = 92 m), characterized by small surface (44 ha) and small drainage basin (50 ha) areas. Lake Aydat (45°39′48″N, 002°59′04″E) is a small eutrophic lake (Z_{max} = 15 m, surface area = 60 ha), with a larger catchment area (3×10^4 ha). For both lakes, samples were taken near the center of the lake, at the point of maximum depth. Lake Pavin was first sampled on 26 October 2006 for testing different methodological protocols, and both lakes were subsequently sampled twice in 2007 for the application of the optimal protocol retained (i.e., the size-fractionated community approach using 1% CFW final concentration; see below) to the natural community dynamics. The latter samples were collected during the spring and autumn (22 March and 2 October 2007) for the oligotrophic Lake Pavin, and during spring and late summer (22 May and 28 August 2007) for the eutrophic Lake Aydat. All samples were collected in triplicates, i.e., from three independent sampling operations per sampling date.

During all sampling occasions, ca. 21 liters of the whole euphotic layers of the two lakes (estimated from Secchi depths) was sampled manually using a flexible plastic tube (diameter, 4 cm) provided by a rope connecting the ballasted bottom of the tube with a surface manipulator. With this system, the euphotic water column samples are collected by simple capillarity. This technique of sampling is rapid, easy, and inexpensive (43). Analytic samples were thus considered as integrated samples representative of the euphotic layers of the lakes, i.e., 0 to 20 m for Lake Pavin and 0 to 5 m for Lake Aydat. Collected samples were immediately prefiltered through a 150-μm-pore-size nylon filter (to eliminate the predatory metazoan zooplankton) when poured into clean transparent recipients previously washed with the lake water and then transferred immediately to the laboratory for processing.

Protocols tested for concentrating and staining fungal parasites. Back in the laboratory, different procedures were tested on the experimental samples collected on 26 October 2006 in Lake Pavin for concentrating phytoplankton cells before the staining tests of chytrids. Concentrating phytoplankton is essential for an accurate assessment of morphological characteristics (general shape of sporangia, the rhizoidal system, the attachment of peduncle, and the presence of operculum), which include typical identification keys of fungal parasites. The sample processing is summarized in Fig. 1.

Concentration of host cells. Two different approaches were tested on samples collected on 26 October 2006 in Lake Pavin: the total community approach and the size-fractionated community approach (Fig. 1). For the former approach, 180-ml portions of experimental samples were fixed with formaldehyde (2% final concentration), and aliquots were concentrated in three different ways: (i) by simple gravity according to the Utermöhl method (47) before the chyrids were stained, (ii) by vacuum pressure on two different filters before staining directly onto filters, and (iii) by vacuum pressure on the same two types of filters but after being stained in solution. For the Utermöhl method, 100 ml of fixed samples was settled for at least 24 h. For each of the two filter-vacuum pressure methods, 10 ml of fixed samples was filtered onto polycarbonate white filters (pore size, 0.6 μm; catalog no. DTTP02500 [Millipore]) and Nuclepore polycarbonate black filters (pore size, 0.8 μm; catalog no. 110659 [Whatman]) by using gentle vacuum (20 kPa).

For the size-fractionated community approach, 20 liters of the experimental samples was passed through a 25-μm-pore-size nylon filter (Fig. 1). Large phytoplankton cells in the >25-μm size fraction were collected by washing the filter with 40 ml of 0.2-μm-pore-size-filtered lake water and fixed with formaldehyde (2% final concentration) before staining and analysis. Nanoplanktonic cells in the <25-μm size fraction (i.e., the 20-liter filtrate) were concentrated ~20-fold by ultrafiltration to a volume of ~1 liter using a high-performance concentration/diafiltration system (model DC 10LA; Amicon, Epernon, France) equipped with a reusable hollow fiber cartridge (0.2-μm cutoff, surface area of 0.45 m^2; Amicon) and an entry pressure of 0.9 bar. About 180 ml of the ultrafiltrate retentate was then fixed with formaldehyde (2% final concentration) before staining and analysis. The size-fractionated community approach gave optimal results and was used for the samples collected in 2007 in both Lake Pavin and Lake Aydat.

Staining of parasites. Sporangia and rhizoids of parasitic chytrids in concentrated subsamples were stained with the fluorochrome CFW ($C_{40}H_{44}N_{12}O_{10}S_2$ Fluorescent Brightener 28; Sigma catalog no. F3543). CFW is used as whitening agent by the paper industry and selectively binds to cellulose and chitin. The dye fluoresces when exposed to UV light and offers a very sensitive method for direct microscopic examination of skin scrapings, hairs, nails, and other clinical specimen for fungal elements (19, 20). Here, we have adapted the technique to environmental aquatic samples. Before staining, a stock solution of CFW was prepared as modified from an original protocol (20; http://www.mycology.adelaide.edu.au/) by adding 35 mg of CFW to 7 ml of sterile distilled water and a few drops of 10 N NaOH to increase the pH to between 10 and 11, because CFW does not dissolve well in neutral solutions. The final volume of stock solution was then adjusted to 10 ml with sterile distilled water, divided into small aliquots (150 μl), and stored in the dark at −20°C until use.

For the experimental samples collected on 26 October 2006 in Lake Pavin, four final concentrations of CFW (20, 10, 3, and 1% [vol/vol]) relative to the stock solution were tested. For the Utermöhl method, settled phytoplankton cells were stained by replacing an appropriate volume of the supernatant water with the stock solution of CFW directly into the Utermöhl chamber, so as to yield the target dye final concentrations tested. For the vacuum pressure method, phytoplankton cells concentrated onto DTTP and black filters were stained by flooding the filters with the dye (CFW stock solution was diluted at the four final concentrations tested) for 10 min, followed by thorough washing with <0.2-μm-pore-size-filtered lake water. In the variant of this method, CFW stock solution was added to subsamples of suspended phytoplanktonic cells (to reach the appropriate concentrations) for 10 min, before concentrating stained cells onto DTTP and black filters, following by washing with <0.2-μm-pore-size-filtered lake water. All filters were then mounted between microscope slides and glass coverslips using a nonfluorescent immersion oil (Cargille type A). For the fractionated samples (i.e., >25 μm and <25 μm), aliquots (150 μl) of concentrated and fixed materials were stained in solution for 10 min (as previously described for suspended cells), and drops (10 μl) of the stained samples were mounted between glass slides and coverslips for observations and counting (Fig. 1).

The optimal CFW final concentration (i.e., 1%) staining and the size-fractionated community approach were then applied to the samples collected in 2007 in both Lake Pavin and Lake Aydat, for diagnosing, identifying, and counting phytoplankton fungal parasites using direct epifluorescence microscope observations.

Direct observation and counting. For all samples, stained chytrids were observed in a dark room under an inverted epifluorescence microscope (Leica DMIRB model) equipped with a ×1,250 (i.e., 100/1.25) objective lens, an HBO-100W mercury lamp, and a set of different optic filters, including filters (340 to 380 nm) for UV light excitation. For picture capture and processing, the microscope was equipped with a Leica color video camera (model DC 300F) and a Leica Q500 personal computer.

Experimental samples were mounted between slides and glass coverslips either as concentrates onto filters (i.e., the total community approach) or directly as concentrated liquid drops (5 to 15 μl, i.e., the size-fractionated approach). Slides were then inverted for light excitation and observation under the inverted Leica epifluorescence microscope. For each replicate analyzed, at least 500 phytoplanktonic cells (calculated standard error of <10%) were inspected for fungal infection (i.e., the presence of fixed sporangia), and the morphological characteristics of parasites were noted for identification using the software for image analysis Leica Qwin. The natural abundance and composition of phytoplankton hosts were determined from raw fixed parallel samples by using the Utermöhl method (47).

Species identification and fungal infectivity parameters. During microscopic observations, phytoplanktonic cells were identified, often to the species level, using morphological taxonomic keys known from references, e.g., Bourelly (6), Huber-Pestalozzi (23), and Prescott (38). For fungal parasites, identification was similarly based on phenotypic keys known from classical manuals, primarily those in Sparrow (44), Canter (10), and Canter and Lund (13). To estimate the infectivity parameters of ecological interest, several algorithms were used according to formulas proposed by Bush et al. (7). These parameters include the prevalence of infection (Pr), i.e., the proportion of individuals in a given phytoplankton population with one or more sporangia or rhizoids, expressed as $\Pr(\%) = [(N_i/N) \times 100]$, where N_i is the number of infected host cells, and N is the total number of host cells. The second parameter is the mean intensity of infection (I), calculated as $I = N_p/N_i$ where N_p is the number of parasites, and N_i is the number of the infected individuals within a host population.

RESULTS AND DISCUSSION

Handling, staining, and observation of chytrids. In the present study CFW clearly appears to be a good candidate for diagnosing, identifying, and counting phytoplankton parasitic chytrids in pelagic samples (see, for example, Fig. 2 and 3). This complements the idea that this dye offers a very sensitive method for direct microscopic examination of skin scrapings, hairs, nails, and other clinical specimens for fungal elements known from clinical mycology (19, 20), cytopathology (34, 35), ophthalmology (49), or parasitology (36). CFW binds to β1-3 and β1-4 polysaccharides such as those found in cellulose or in chitin which commonly occur in the fungal cell wall (42, 46). CFW also stains tissue elements such as keratin, collagen, and elastin, providing useful markers for their examination (35). The absorption spectrum for aqueous CFW solution peaked at 347 nm (19, 20) and, when excited with UV radiation, fluoresces with an intense blue color (e.g., Fig. 2 and 3). In our effort to search for an accurate routine procedure for simultaneous study of population dynamics of parasitic chytrids and their hosts in the plankton, it was clear that the quality of observations and counting depended on the concentration of the stain and on the approach used for concentrating phytoplankton hosts.

For the total community approach using the classical Utermöhl method (47), visualization of fungal parasites was very difficult, and most of the time it was practically impossible for all of the stain concentrations tested. The main reason was that staining directly in the Utermöhl chamber resulted in very poor-quality specimens of parasites observed in any given sample. Other disadvantages of the procedure include the relatively long sedimentation time and the difficulty of increasing the volume analyzed. For these reasons, we decided to exclude the procedure based on the Utermöhl method from the comparisons. The alternative total-community approaches based on vacuum pressure concentrations on polycarbonate filters, i.e., white (0.6-μm-pore-size) and black (0.8-μm-pore-size) filters, yielded similar-quality images of fungal parasites, either when CFW staining was done before (i.e., in solution) or after (i.e., on filters) concentrating phytoplankton host cells onto filters. However, substantial differences were noted depending both on the type of the filter and on the concentration of the stain. In general, for the two types of filters, high levels of background fluorescence were obtained when CFW was used at final concentrations of 3, 10, or 20%, precluding any accurate assessment of numerical and phenotypic characteristics of both host cells and their fungal parasites (data not shown). For this reason, experimental samples stained at these CFW concentrations were not analyzed further. Staining with 1% CFW final concentration substantially improved the viewing of chytrids on filters, with an increasing contrast from the white

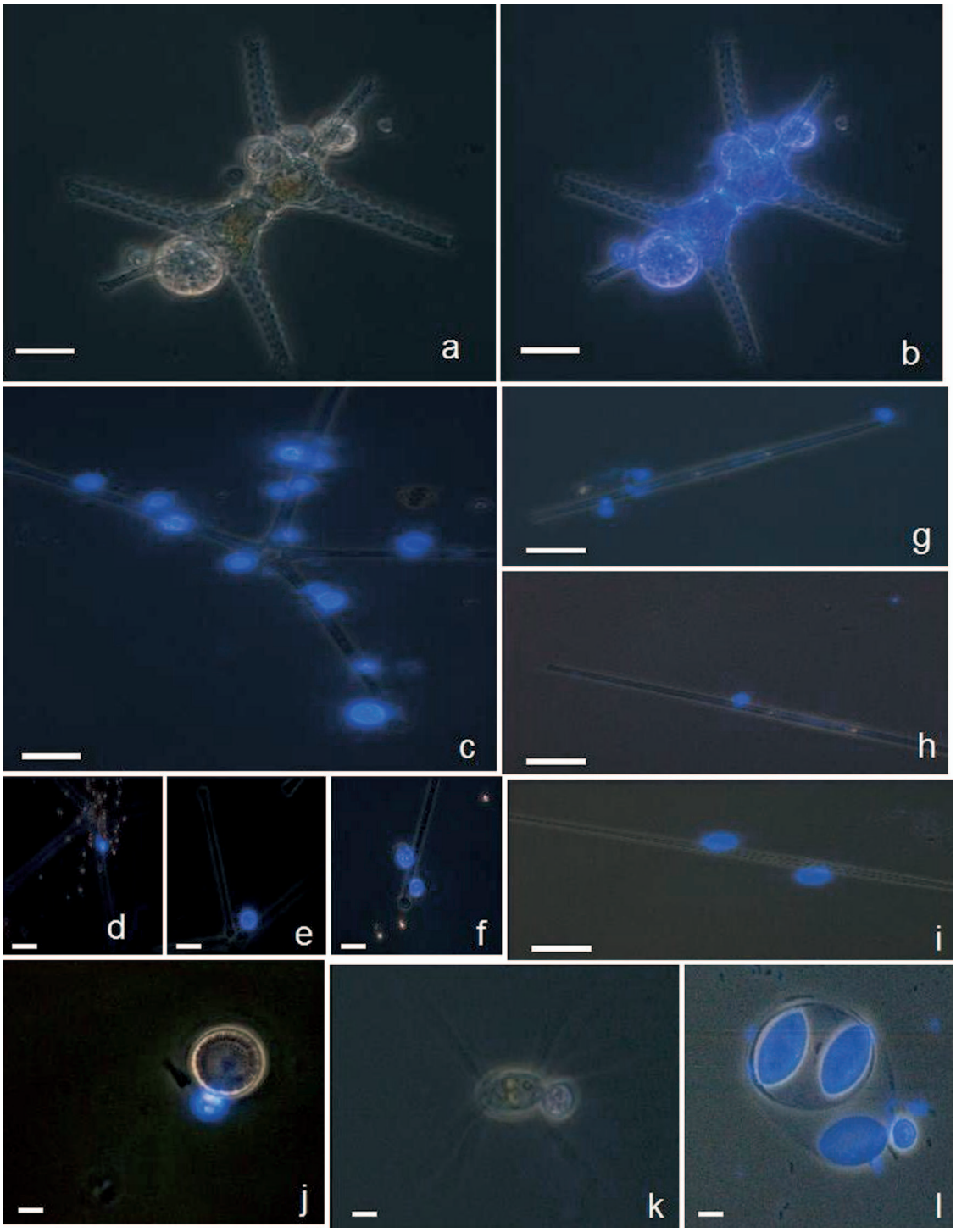

FIG. 2. Examples of microscopic micrographs of phytoplankton eukaryotes with CFW-stained chytrid parasites, obtained via the fractionated community approach. Typical morphological taxonomic keys are visible under white light for host cell (i.e., the chlorophyte *Staurastrum* sp.) (a) and under UV light for the specific parasitic chytrids (b). Under UV light, chytrid epidemics were diagnosed for a diversified host populations, including both large size (e.g., the chlorophyte *Staurastrum* sp. [a and b] and the diatoms *A. formosa* [c to f] and *Synedra* sp. [g to i]) and small size (e.g., the diatom *Cyclotella* sp. [j] and the chlorophytes *C. ciliata* [k] and *O. lacustris* [l]) hosts. Multiple infectious chytrids are visible in most micrographs and different development stages as well, e.g., young sporangia with visible rhizoidal system (d and g), mature sporangia containing zoospores (e and h), a mature sporangium discharging its zoospore contents (f), and empty sporangia with chitinaceous wall visible (i). Scale bar, 10 μm.

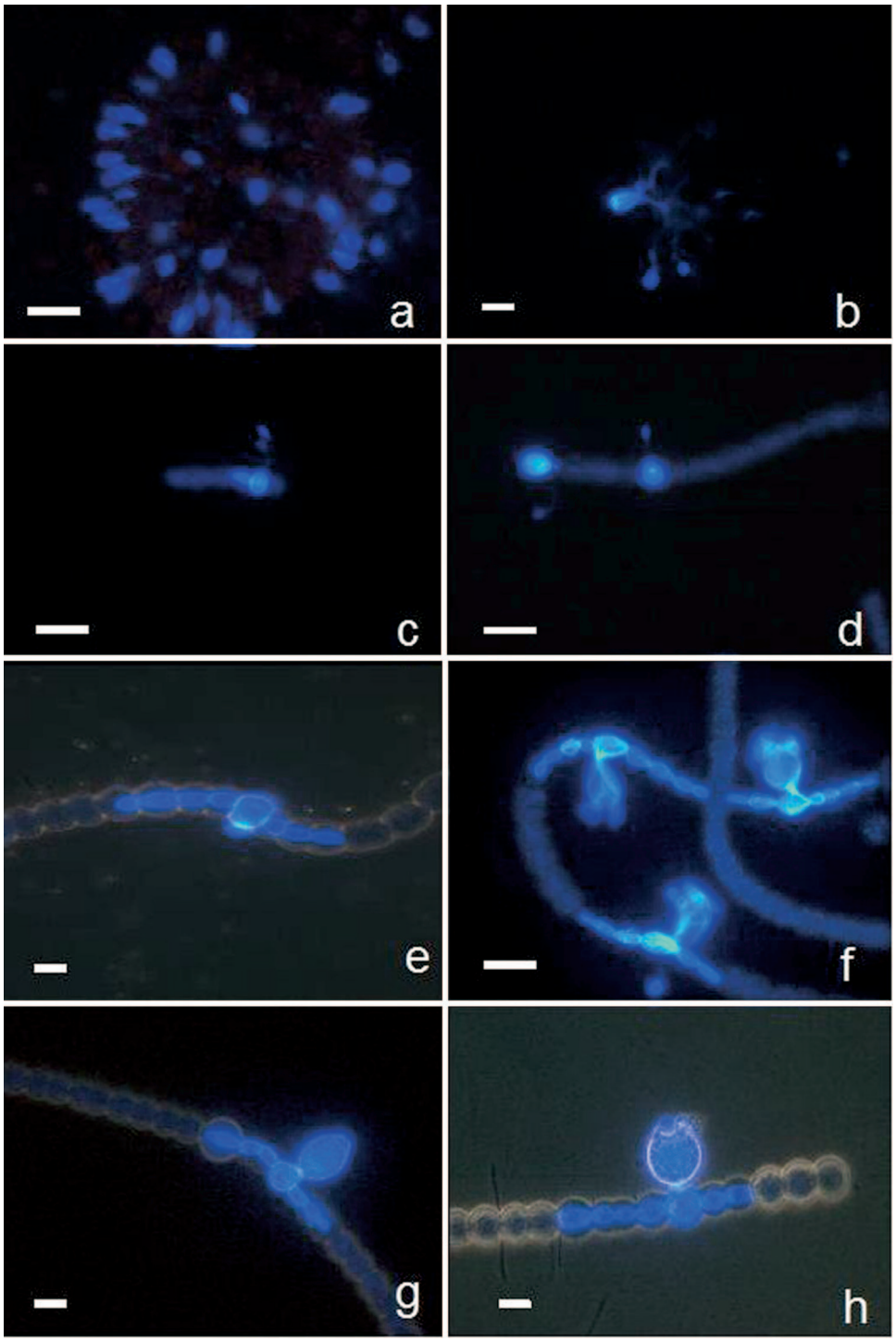

FIG. 3. Examples of microscopic micrographs of phytoplankton prokaryotes (cyanobacteria) with CFW-stained chytrid parasites, obtained using the fractionated community approach. Typical morphological taxonomic characteristics are visible under white light for host cells (micrographs not shown) and under UV light for parasitic chytrids on their hosts identified as colonial *Gomphosphaeria* sp. (a) and *Microcystis* sp. (b) and as the filamentous *Anabaena flosaquae* (c to h). The branched rhizoidal system of the parasite is visible on *Microcystis* sp. (b). On *A. flosaquae*, young endobiotic thalli and encysted zoospores attached by long slender penetration tubes to the host are visible (c and d). In addition, tubular vegetative structure (e), mature sporangia of irregular pyriformic shape (f), and mature sporangia with protruding papilla for discharging zoospores (g and h) are also visible. Scale bar, 10 μm.

DTTP Millipore filters to the black Whatman filters (data not shown). However, none of the membrane-retaining approaches yielded satisfactory images of morphological and cellular features of the host cells, e.g., the presence of chloroplast or viability of the host cell. In addition, almost all of the infected phytoplankton individuals observed with the total community approach appeared to be large diatoms. Accordingly, procedures from the total community approach were

excluded from comparisons. We will thereafter focus on the size-fractionation approach using a 1% (vol/vol) CFW final concentration (from the stock solution) which substantially enhanced the observational results. We consider this protocol to be optimal for the diagnosis and quantitative assessment of phytoplanktonic chytrid infections in natural samples.

Indeed, the latter procedure yielded the best images for identification and quantitative assessment of both phytoplankton host cells present in the two size classes (>25 μm and < 25 μm) under white light illumination, and their fungal parasites after switching light to UV excitation. Illustrations from samples collected in 2006 in Lake Pavin and in 2007 in Lakes Pavin and Aydat are provided on Fig. 2 and 3. Under white light, diverse phytoplankton host cells were identified based on phenotypic features and on their viability through the integrity of cell wall and the presence of chloroplasts. Under UV excitation, the wall of chytrid sporangia is well visible because of the presence of chitin (19, 20), allowing the assessment of phenotypic keys for identification. These keys include the shape of thallus (e.g., Fig. 3c,d), the rhizoidal system (e.g., Fig. 3e to h) and, at times, different development stages of parasites (e.g., Fig. 2d to f and 3e to f) (10, 13, 44). Furthermore, the size-fractionation approach allowed the diagnosis of fungal phytoplankton infections not only for large hosts such as diatoms and colonial and filamentous cyanobacteria in the >25-μm size fractions but also for small nanophytoplankton cells such as *Cyclotella* sp., *Chodatella ciliata*, or *Oocystis lacustris* (e.g., Fig. 2j to l) in the <25-μm size fractions.

Comparative data from the optimal protocol proposed are presented in the following sections only for the field survey, i.e., samples collected in 2007 in both lakes, mainly as a "proof of concept" rather than a valid comparison of sites versus seasons.

Preliminary data on the natural dynamics of hosts versus parasites using the proposed protocol. (i) Host community composition. Our size-fractionation approach resulted in an apparent increase in the diversity of infected phytoplankton populations, compared to previous studies. With this approach, a total of nine phytoplankton species belonging to *Cyanobacteria*, Chrysophyceae, and Chlorophyceae were found with fungal parasites (Fig. 2 and 3). The major hosts were the diatoms *A. formosa*, *Synedra* spp., *Fragilaria crotonensis*, and *Cyclotella* sp., and the Chlorophyceae *Staurastrum paradoxum*, *Staurodesmus incus*, and *C. ciliata* (see Table 2). For a long time, diatoms, primarily *Asterionella* spp., have been described from light microscopy as the preferred hosts for chytrid infections in several lakes: English Lake District (12) and Shearwater Lake (39), United Kingdom; Lake Leman, Switzerland (37); Lake Maarsseveen, The Netherlands (48); and two lakes in Colorado (29). Reports on the chytrid epidemic on green algae are more episodic, including host species *O. lacustris* in Lake Walensee, Switzerland (22), and the desmids *Staurastrum* spp. in Lake Windermere (11) and Shearwater Lake (40), United Kingdom. Our finding of chytrid infections among prokaryotes is new. This occurred only in summer in the eutrophic Lake Aydat and affected three species of cyanobacteria: *Anabaena flosaquae*, *Gomphosphaeria* sp., and *Microcystis* sp. (Fig. 3). The latter two species were detected once in one of the replicate samples from Lake Aydat. This corroborates our recent suggestion that chytrid infections and the related biogeochemical processes in aquatic systems, primarily through parasitisms and saprophytism, may represent ecologically important driving forces in the food web dynamics (17, 31–33).

(ii) Parasite community composition. Based on morphological features, the majority of fungal parasites were tentatively identified as monocentric (i.e., with one center of growth and development) and eucarpic (using part of the thallus for the fruit-body and with a specialized rhizoidal system). This is characteristic of the order Chytridiales, with two families, four genera, and about ten different species (cf. Table 1 and the identification keys) recorded in our natural samples. The family Phlyctidiaceae contains two genera: *Rhizosiphon*, which comprised typical parasites of cyanobacteria and normally harbors tubular rhizoids that radiate from the bodies of sporangia (Fig. 3c to h), and *Rhizophidium*, which is the largest and most complex genus of chytrids parasitizing diatoms and chlorophytes, especially desmids (10, 44). The second family (i.e., Chytridiaceae) was represented by two genera (*Chytridium* and *Zygorhizidium*) (Table 1) with species known mainly as parasites of diatoms and green algae, which have the ability to maintain the wall of their sporangia after zoospore discharge, as does *Rhizophidium* (e.g., Fig. 2i) (10, 44).

Based on the limited number of samples analyzed, few differences in the occurrence of parasites were noted between the two lakes sampled, where the more common species were in the genera *Rhizophidium* and *Chytridium* (Table 1). The *Rhizosiphon* species (i.e., *R. crassum*) was observed only in the eutrophic Lake Aydat as a parasite of the cyanobacteria *A. flosaquae* (Fig. 3c to h), while *Rhizophidium fulgens* and *Chytridium oocystidis* were observed only in Lake Pavin (Table 1) as a parasite of the small chlorophyta *O. lacustris* and *C. ciliata* (Fig. 2k and l). Only one species in Lake Pavin (*R. planktonicum*) and two in Lake Aydat (*Rhizophidium cyclotella* and *C. versatile*) were present during the two sampling times (Table 1). In our samples, one parasite species often was found on one host species. An exception was *R. planktonicum*, which infected different species of diatoms (*A. formosa* and *Synedra* spp). In contrast, the diatom *F. crotonensis* was found infected by two different parasite species in Lake Aydat, i.e., *Rhizophidium fragilaria* and *C. versatile.* These findings corroborate the complexity of parasitic ecology (2, 5, 15, 17) and lifestyles in chytrids, which can be facultative parasitic (1), hyperparasitic (28, 41), promiscuous (18), symbiotic (45), and/or multispecific within a genus (4) but species specific in the majority of cases (24, 27).

(iii) Quantitative data. In Lake Aydat, the total abundance of phytoplankton increased from 4.5 to 18.5 $\times$ 10^6 cells liter^{-1} between May and August and were higher than those recorded in Lake Pavin in March (2.5 $\times$ 10^6 cells liter^{-1}) and October (3 $\times$ 10^6 cells liter^{-1}) (Fig. 4a). In samples collected during spring, phytoplankton communities were dominated by diatoms in both lakes, accounting for ca. 75 and 65% of the total abundance in Lakes Pavin and Aydat, respectively (Fig. 4b). The main diatom species were *A. formosa* (32% of total phytoplankton abundance) and *Synedra* spp. (24%) in Lake Pavin and *Melosira italica* (57%) in Lake Aydat. In the summer and autumn, diatoms were replaced by cyanobacteria, the species *A. flosaquae* in Lake Aydat and *Cylindrospermum maius* (i.e., with no infection noted) in Lake Pavin, with an increasing relative abundance from ca. 65% (of the total abundance) in

TABLE 1. Morphological features and occurrence of phytoplankton chytrid parasites in the euphotic layer of Lakes Pavin and Aydat sampled each on two occasions during different seasons[a]

Classification	Sporangium	Thallus	Rhizoid	Lake Pavin		Lake Aydat	
				Mar.	Oct.	May	Aug.
Order Chytridiales	Operculate or inoperculate Spherical or pyriform Sessile or epibiotic	Holocarpic or eucarpic Monocentric	Branched or unbranched				
Family Phlyctidiaceae	Inoperculate Sessile or epibiotic	Eucarpic	Branched or unbranched				
Genus *Rhizophydium*	Spherical						
R. planktonicum	Sessile or epibiotic		Unbranched	X	X	X	
R. couchii	Sessile		Branched		X		X
R. cyclotellae	Sessile-subspherical		Branched		X	X	X
R. melosirae	Epibiotic		Branched	X		X	
R. fulgens	Sessile				X		
R. fragilariae	Spherical to subspherical		Unbranched			X	X
Genus *Rhizosiphon*	Sessile		Unbranched				
R. crassum	Pyriform		Unbranched and tubular				X
Family Chytridiaceae	Operculate Sessile or epibiotic	Eucarpic					
Genus *Chytridium*	Spherical or pyriform Epibiotic		Extremely variable				
C. versatile	Obpyriform		Branched			X	X
C. oocystidis			Unbranched		X		
Genus *Zygorhizidium*	Sessile						
Z. melosirae	Ovate		Unbranched	X		X	

[a] Tentative identifications are based on phenotypic keys (see the text).

Lake Pavin to 95% in Lake Aydat. In addition, chlorophytes were also quantitatively important in Lake Pavin in autumn (27% of the total abundance), with *O. lacustris* (23% of total abundance) as the major species.

The total abundance of fungal sporangia increased from the oligotrophic Lake Pavin to the eutrophic Lake Aydat, similar to the total abundance of phytoplankton. This may represent a universal pattern, although few quantitative seasonal studies on fungal parasites are sufficiently complete to permit generalization (21). Indeed, the abundance of sporangia was higher in spring than in summer-autumn. The counts were 32.9 (March) and 3.9 (October) $\times 10^3$ sporangia liter^{-1} for Lake Pavin and 10.7 (May) and 1.1 (August) $\times 10^3$ sporangia liter^{-1} for Lake Aydat. The fluctuation in the number of sporangia thus seems to increase with the trophic status of the lake but may instead be related to the seasonal changes in the host

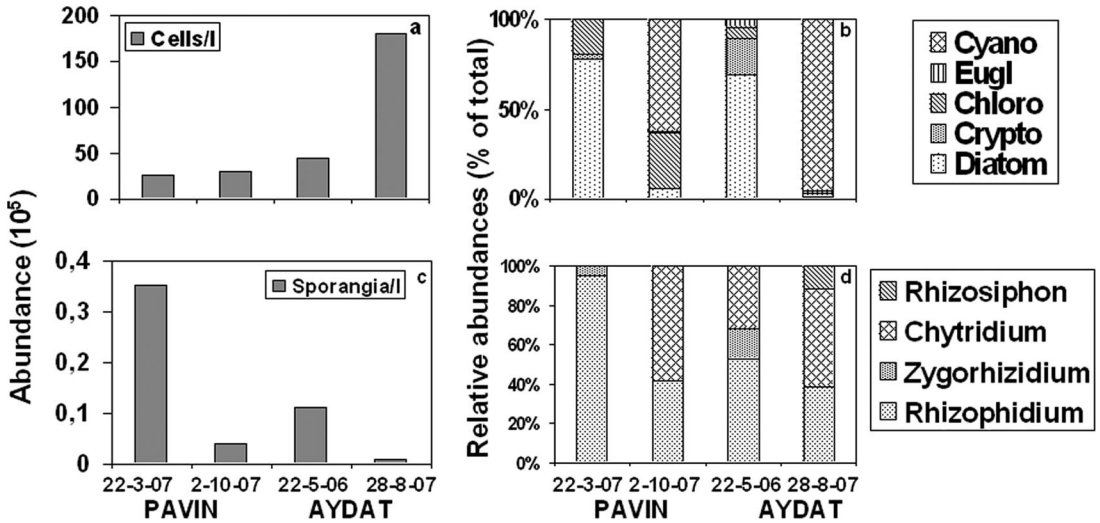

FIG. 4. Variations in the numerical (a and c) and relative (b and d) abundances of phytoplankton and their chytrid parasite (i.e., sporangia) communities. Samples were collected in triplicate (i.e., from three independent sampling operations for each sampling date) on two occasions during two different seasons in the eutrophic Lake Aydat and in the oligotrophic Lake Pavin. The variability between replicates was low, with a coefficient of variation that was always <10%.

TABLE 2. Prevalence and intensity of chytrid infection for different phytoplanktonic populations[a]

Species	Lake Pavin			Lake Aydat		
	Date	Pr (%)	I (%)	Date	Pr (%)	I (%)
Asterionella formosa	22/03/2007	6.32	1.09	22/05/2007	24.19	1.21
Synedra sp.	22/03/2007	3.23	1	22/05/2007	10	1
Melosira italica	22/03/2007	1	1	22/05/2007	1.43	1
Fragilaria crotononensis				22/05/2007	3.03	1.07
				28/08/2007	4.95	1
Cyclotella sp.	02/10/2007	<1	1			
Staurastrum sp.	02/10/2007	<1	1	28/08/2007	10.82	1.25
Oocystis lacustris	02/10/2007	4.63	1.28			
Chodatella ciliata	02/10/2007	15.8	1			
Anabaena flosaquae				28/08/2007	<1	1

[a] Samples were collected on two occasions during different seasons in the euphotic layer of Lake Pavin and Lake Aydat, and the percent prevalence (Pr) and intensity (I) values were determined. Dates are expressed in the format day/month/year.

community composition. Indeed, the numerical abundance of sporangia appeared to increase with the increasing relative importance of diatoms within the phytoplankton communities due to the infection from *Rhizophidium* spp., *Zygorhizidium melosirae*, and *C. versatile* (Fig. 4b to d). Diatoms are well known as preferred hosts for chytrid epidemics in the plankton (24, 27), likely because of the large cell size and capacity of diatoms to form blooms, thereby increasing the probability for fungal propagule attachment and development (39). This may help explain why, in contrast to their abundances, the host community was less diverse in Lake Pavin, where fewer species dominated host communities than in Lake Aydat (Fig. 4b and d).

The data on the prevalence and intensity of chytrid infection show that the prevalence increased with increasing trophic status and ranged from <1 to 16% in Lake Pavin and from 1 to 24% in Lake Aydat. The more vulnerable populations were the chlorophyte *C. ciliata* recorded in October in Pavin and the diatom *A. formosa* recorded in May in Aydat. Other highly exposed host populations (i.e., prevalence > 5%) included the *A. formosa* diatom in Lake Pavin and the *Synedra* spp. diatoms and *Staurastrum* spp. chlorophytes in Lake Aydat (Table 2), confirming the importance of diatoms and other bloom-forming phytoplankton as preferred hosts for chytrid epidemics (10, 24, 27, 39, 44). The calculation of the intensity of infection revealed the occurrence of multiple infections (i.e., >1 parasites per host cell) for *A. formosa* in both lakes, *O. lacustris* in Lake Pavin, and *F. crotonensis* and *Staurastrum* spp. in Lake Aydat (Table 2 and Fig. 2).

Conclusions. The present study describes a routine size-fractionation, CFW staining approach for diagnosing, identifying, and counting phytoplankton chytrid parasites in pelagic samples. The approach is based on the concentration of large initial volumes and size partitioning of samples, a step that we judged necessary in order to yield good analytic images of infectious sporangia for an accurate diagnosing and identification of parasites. In addition, our approach yields freeze-conserved particulate DNA samples for quantifying the propagule stages (i.e., zoospores) of chytrids via FISH targeting of a specific rRNA oligonucleotide probe that we have recently designed (M. Jobard et al., unpublished data). Our protocol can therefore be combined with modern molecular biology protocols such as fluorescence in situ hybridization-targeting or cloning/sequencing. Applied to field samples, the approach provides quantitative preliminary data on infectious sporangia within phytoplankton communities in two contrasted lake environments, which were consistent with ecological considerations known from pelagic habitats and host versus parasite populations. When the samples are analyzed immediately, the approach does not require toxic fixatives and the related disadvantages such as losses during sample storage.

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Real-time PCR assay for identification and quantification of *Rhizoctonia solani* AG-2-2 in soil

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Abstract: *Rhizoctonia solani* AG-2-2 causes severe damages on sugar beet, which appear in the field as dispersed and unpredictable patches of diseased plants. Knowledge of the ecology of this pathogen is limited by the absence of diagnostic tool. We have developed a specific real-time PCR assay enabling the identification of *R. solani* AG-2-2 and its direct quantification in soil.

Key words: anastomosis group AG-2-2, molecular detection, quantification, real-time PCR, *Rhizoctonia solani*, soil

Introduction

Rhizoctonia solani Kühn is a soilborne fungus pathogenic on a wide range of plants. It includes thirteen anastomosis groups (AG) and additional subgroups (Ogoshi, 1987; Gonzalez Garcia *et al.*, 2006). *R. solani* AG-2-2 is responsible for severe damages on sugar beets (*Beta vulgaris* L.) causing damping-off of seedlings and crown and root rot of mature plants (Herr, 1996). Both subgroups AG-2-2 IIIB and AG-2-2 IV are pathogenic on sugar beets (Engelkes and Windels, 1996; Panella, 2005). In fields, the damages appear as dispersed and unpredictable patches of diseased plants (Herr, 1996). Ecological studies are limited by the absence of a diagnostic tool for *R. solani* AG-2-2. The detection of the fungus in soil using cultivation methods is difficult because there is no selective medium allowing its isolation from the whole microflora (Van Bruggen *et al.*, 1996). Baiting or soil pelleting methods have been used to recover *R. solani* from soil but their utilization in complex environments is limited by their lack of specificity toward the subgroup of interest (Neate and Schneider, 1996). Molecular methods can differentiate AG and subgroups in cultures but were not designed for diagnostic purpose (Carling *et al.*, 2002; Guillemaut *et al.*, 2003). Investigations of the ecology of *R. solani* AG-2-2 and prediction of disease incidence relies on the availability of a diagnostic tool for the pathogen. The objective of the present study was to set up a specific and quantitative assay enabling the identification of *R. solani* AG-2-2 and quantification in soil. The assay combines specific primers matching the ribosomal internal transcribed spacer (ITS) region and real-time polymerase chain reaction (PCR).

Material and methods

Fungal strains and DNA extractions

The fungal strains used in this study are listed in Table 1. DNA was extracted using the rapid procedure described by Edel *et al.,* (2001) with the following modifications. DNA was

Table 1. Fungal strains used in this study

Species	Anastomosis group/ subgroup	Strain	Collector or supplier (original strain designation)[1]	Origin
Rhizoctonia solani	AG-1-1C	D2	E. Butler (43)	Pine, Canada
	AG-2-1	F1	CBS 208.84	Flax, Japan
	AG-2-2	G6	FPFS	Sugar beet, France
	AG-2-2 IIIB	G3	A. Ogoshi (C-96)	Mat rush, Japan
	AG-2-2 IV	G5	A. Ogoshi (B.62)	Sugar beet, Japan
	AG-2-2	CG30	FPFS	Sugar beet, France
	AG-2-2	CG31	FPFS	Sugar beet, France
	AG-2-2	CG32	FPFS	Sugar beet, France
	AG-2-2	CG34	FPFS	Sugar beet, France
	AG-2-2	CG36	FPFS	Sugar beet, France
	AG-2-2	CG37	FPFS	Sugar beet, France
	AG-2-2	CG66	FPFS	Sugar beet, France
	AG-2-3	H1	M. Hyakumachi (23R01)	Soybean, Japan
	AG-3	I1	CBS 211.84	Potato, Japan
	AG-4	K1	M. Hyakumachi (Chr-3)	Chrysanthemum, Japan
	AG-5	M1	CBS 212.84	Soybean, Japan
	AG-6	N1	A. Ogoshi (OHT1-1)	Soil, Japan
	AG-7	O3	M. Hyakumachi (1529)	Soil, Japan
	AG-8	P1	(A68)	Wheat, Australia
	AG-10	R2	CBS 971.96	Soil, USA
	AG-11	S1	CBS 973.96	Rice, USA
Rhizoctonia oryzae-sativae	AG-Bb	C3	IMI 375130	Rice, Japan
	AG-Bb	C6	IMI 375133	Rice, Japan
Fusarium oxysporum		Fo47	FPFS	Soil, France
Fusarium solani		FS1	CBS 318.73	Cucurbitaceae, India
Gliocladium roseum		GLIO1	MNHN 87.3499	Soil, France
Gliocladium virens		GLIO3	MNHN 82.3356	Soil, France
Acremonium kiliense		ACR1	MNHN 52.1067	Unknown
Cladosporium cucumerinum		CLA1	ATCC 38584	Unknown
Cylindrocarpon cylindroides		CYL1	CBS 239.29	Picea, UK
Helminthosporium carbonum		HEL1	ATCC 38585	Unknown
Verticillium alboatrum		VERT1	IACR	Unknown
Verticillium chlamydosporum		VERT3	IACR	Unknown
Trichoderma harzianum		TRI1	FPFS	Unknown
Trichoderma viride		TRI2	FPFS	Unknown

[1] CBS: Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; FPFS: Collection of Flore Pathogène et Faune du Sol, INRA, UMR Microbiologie du Sol et de l'Environnement, Dijon, France; IACR: Institute of Arable Crop Research-Rothamsted, Herpenden, UK; IMI: International Mycological Institute, Surrey, UK; MNHN: Museum National d'Histoire Naturelle, Paris, France.

extracted from cultures on malt agar and purified with chloroform/isoamyl alcohol (24:1, v/v) before being precipitated. The good quality of the DNA for PCR reactions was confirmed using the general fungal primers ITS1 and ITS4 and classical PCR conditions (White *et al.*, 1990).

Design of specific primers for Rhizoctonia solani AG-2-2 and conventional PCR

The ITS fragment of *R. solani* AG-2-2 strain G6 was excised from the gel, purified using the QIAEX II gel extraction kit (Qiagen), cloned with the pGEM-T Easy Vector System (Promega) and sequenced using the primers SP6 and T7. The resulting ITS1-ITS4 sequence was aligned with other *R. solani* ITS sequences found in Genbank. Criteria for primer optimization were both the specificity toward *R. solani* AG-2-2 and the efficiency for real-time PCR. The primers were selected with a maximum of 2°C between their melting temperatures (Tm) and a resulting amplicon of less than 200 bp. Primers compatibility was evaluated using Primer 3 (Center for Genome Research, Cambridge, MA). The specificity of the designed primers toward *R. solani* AG-2-2 was checked by conventional PCR. The 25-µl PCR mix contained 1 µl of fungal DNA, 0.2 µM of each primer, 100 µM each of dATP, dCTP, dGTP and dTTP, 3 U of *Taq* DNA polymerase (Q-BIOgene, France) and PCR reaction buffer containing 1.5 mM $MgCl_2$. DNA amplifications were performed in a Mastercycler (Eppendorf) with a denaturation of 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C and a final extension of 10 min at 72°C.

Development of a real-time PCR assay

Primers G6-F2 and G6-R2 were used to set up a real-time quantitative PCR assay. Real-time PCR was performed on an ABI Prism 7900 (Applied Biosystems) using the fluorescent dye SYBR Green. The threshold cycle Ct at which the fluorescence intensity can be distinguished from the background and enters a logarithmic linear phase was automatically calculated by the SDS 2.2. software (Applied Biosystems). In order to construct a standard curve based on Ct values versus known quantities of target DNA, the plasmid containing the cloned ITS region of the strain G6 was extracted using the QIAfilter plasmid purification kit (Qiagen), linearized using the restriction enzyme *Sal*I and quantified using a biophotometer (Eppendorf). Ten-fold dilution series of plasmid DNA were prepared to obtain 10^8 to 10^2 copies of target DNA per PCR reaction. Real-time PCR was performed in microplates in 25-µl mix containing 12.5 µl of QuantiTect™ SYBR® Green PCR Master Mix (Qiagen) with 2.5 mM $MgCl_2$, 0.5 µM of each primer and 5 µl of DNA (or water in the negative control). The program included 15 min at 95°C and 35 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. Each plasmid dilution was analyzed three times in each microplate. Fungal DNA sets were analyzed in triplicate in independent real-time PCR experiments.

The real time PCR assay was used for direct detection of *R. solani* AG-2-2 in soils collected in an experimental farm in Epoisses (France): i) in a plot cultivated with sugar beets showing symptoms of Rhizoctonia crown and root rot from July onwards and ii) in a plot where no disease was previously observed. The soils were sampled in June and September. Each sample was a composite of 10 sub-samples collected from the top layer (0-10 cm) in the same plot. Soils were passed through a 2-mm sieve and stored at -20°C. DNA was extracted from 1 g of soil in triplicate according to the method described by Edel-Hermann et al. (2004) and purified twice using a polyvinylpolypyrrolidone spin column and once using a Geneclean® Turbo kit (Q-BIOgene). Samples of soil DNA (20 ng) were analyzed in triplicate in independent real-time PCR experiments.

Results and discussion

Primer design and conventional PCR

Sequencing of the ITS region of the strain G6 of *R. solani* AG-2-2 revealed a 737-bp sequence between primers ITS1 and ITS4. Alignment of the sequence with ITS sequences of other AG of *R. solani* revealed a higher polymorphism in the ITS1 region than in the ITS2 region. The optimized primers designed to amplify an ITS1 fragment of 143 bp and to develop a specific real-time PCR assay for *R. solani* AG-2-2 were G6-F2 (5'-AGGTTGTAGCTGGCTCCATTAG-3') (Tm = 59.8°C; 50% G+C content) and G6-R2 (5'-GTAGGGGTCCCAATCATTCA-3') (Tm = 59.6°C; 50% G+C content) (Figure 1). The primer pair was tested in conventional PCR against DNA extracted from strains of *R. solani* belonging to different AG. The PCR reaction resulted in a single DNA fragment of about 145 bp for the strains belonging to AG-2-2 but no product for the other strains.

Real-time PCR assay

The standard curve showed a linear correlation between the Ct value and the number of copies of target DNA with a good efficacy of PCR (Figure 2). The Ct values were 17.63 ± 0.04 for the strain G6 of *R. solani* and between 17.96 ± 0.46 and 21.03 ± 0.12 for the other strains of *R. solani* AG-2-2. Dissociation curves confirmed the presence of a unique peak corresponding to the specific PCR product. In contrast, no amplification was detected for the 25 strains belonging to AG other than AG-2-2 or to other fungal species than *R. solani*.

Anastomosis group and subgroup	Strain	Geographic origin	Genbank accession ITS sequence	Sequence at the position of primer G6-F2	Sequence at the position of primer G6-R2
AG-2-2	G6	France	This study	AGGTTGTAGCTGGCTCC------ATTA--------G	TGAATG-ATTGGGACCCCTAC
AG-2-2 IIIB	65-L-2 (ATCC66489)	USA	AB054858	.....-------....--------.	.....-.....
	87-36-1	USA	AB054855	.....-------....--------.	.....T.....
	C-96	Japan	AB054854	T.....-------....--------.	.A....T.....T.C..
	88-40-1	USA	AB054856	.....-------....--------.	.....-.....
	89-21-4	USA	AB054857	.....-------....--------.	.....-.....
AG-2-2 IV	RI-64 (ATCC 76125)	Japan	AB054865	.....-------....--------.	.A....-.....
	W-22 (ATCC 18619)	USA	AB054864	.....-------....--------.	.....-.....
	87-24-4A	USA	AB054859	.....-------....--------.	.....-.....
	H-3-77	USA	AB054862	.....-------....--------.	.....-.....
AG-2-1	PS-2	Japan	AB054846	T.....C.----------....TTCA---TA	-A..G.C.A.A..TTATTGGA
AG-2-3	H5-307	Japan	AB054870	T.....-------.----TCG---TT	-A.GAAC.A.T..-TG.TGGA
AG-2-4	221	USA	AB054878	T.....CT----------....--------A	-A..GAC.A.AAAT.ATTGGA
AG-1-IA	CS-Ka	Japan	AB000017	GA.....T.....CTTTTCTACC...---------A	---------...T.---------
AG-1-IB	SHIBA-1	Japan	AB000039	GA.....CTT----------....--------A	-A..CCCT..TTCC..TT.TG
AG-1-IC	PS-1	Japan	AB000029	GA.....T.....CT.TGGGG-....---------.	----------.....-----GGG
AG-3	#30	USA	AB000002	T.....C.----------....--------A	-A..GATGA.AA.T.ATTG.A
AG-4	AH-1	Japan	AB000012	T.....TC..TA----------....-------A-	--------.C.C--T.TTTGGA
AG-5	31Rs	Japan	AF354113	T.....-------.----T-G---AA	-A.GAAC.A.T..-TG.TGGA
AG-6	OHT-1-1	Japan	AF153779	T.....CT-------GA.TTTAA---TA	------T.A.T---TAAT.GA
AG-7	1556	Japan	AB000003	T.....CT-------TGATTCAT---TT	------GGACT---.--TCT.
AG-8	95399	Australia	AF153798	T.....TC.--------.---TTA-----A	------C-A.T---TATTGGA
AG-9	111Rs	USA	AF354108	T.....CT----------....--------CA	-A..G.C.A..A.TTATTGGA
AG-10	76107	USA	AB019026	T.....CT-------C...---------A	-A..GAC.A.AAATTATTGGA
AG-11	ZN667	Australia	AF153802	T.....-------..G.---------A	.A.GAAC.A.T..-TG.TGGA
AG-12	CH1	Australia	AF153803	T.....CTAAATGA-...TTTTAATTAA	----------.....ACT------

Figure 1. Alignment of partial sequences of the internal transcribed spacer 1 (ITS1) of the ribosomal DNA of various anastomosis groups of *Rhizoctonia solani* at the positions used to design the oligonucleotide primers G6-F2 and G6-R2 (nucleotides 54-75 and 177-196 in the ITS1-ITS4 sequence of the strain G6 of *R. solani* AG-2-2). Only nucleotides different from the sequence of the strain G6 are indicated. Dashes indicate sequence gaps.

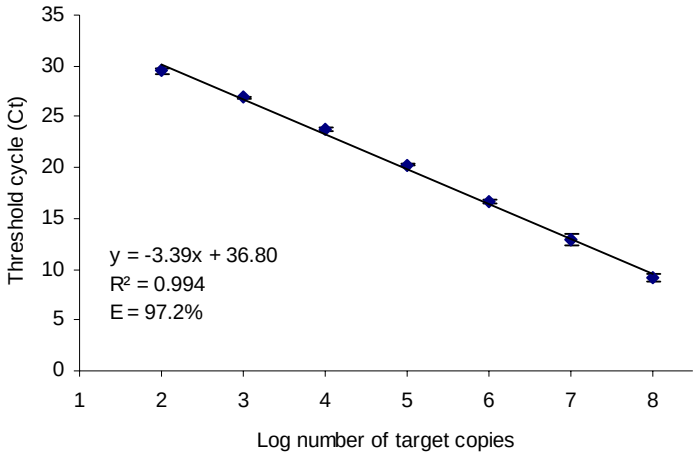

Figure 2. Standard curve generated using real-time PCR with primers G6-F2 and G6-R2 for the quantification of *Rhizoctonia solani* AG-2-2. Values are means ± standard error. The efficiency of the assay was calculated using the formula: $E = 10^{(-1/slope)}-1$.

Using real time PCR, *R. solani* AG-2-2 was detected in the soil originating from the diseased sugar beet field at both sampling dates. The quantity of target DNA in the field samples was found to be more than ten times higher in September ($3.2 \times 10^4 \pm 1.1 \times 10^4$ copies per g of dry soil) than in June ($2.8 \times 10^3 \pm 1.5 \times 10^3$ copies per g of dry soil). In contrast, real-time PCR yielded no amplification using the DNA from the plot where no disease was observed. The real-time PCR assay described in this study will allow rapid identification and direct detection of *R. solani* AG-2-2. The specific primers can also be used in conventional PCR assays in laboratories that are not equipped with real-time PCR detection systems. Using quantitative real-time PCR, it was possible to detect the pathogen in soil cultivated with sugar beets in June even before the appearance of the disease symptoms and to reveal the increase in the pathogen density in soil during the crop. Direct tracking and monitoring of *R. solani* AG-2-2 in soil using this rapid specific assay will permit investigations of the ecology of the pathogen and epidemiology of the disease in sugar beet fields.

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