Fungi in freshwaters: ecology, physiology and biochemical potential

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Abstract
Research on freshwater fungi has concentrated on their role in plant litter decomposition in streams. Higher fungi dominate over bacteria in terms of biomass, production and enzymatic substrate degradation. Microscopy-based studies suggest the prevalence of aquatic hyphomycetes, characterized by tetra- or sigmoid spores. Molecular studies have consistently demonstrated the presence of other fungal groups, whose contributions to decomposition are largely unknown. Molecular methods will allow quantification of these and other microorganisms. The ability of aquatic hyphomycetes to withstand or mitigate anthropogenic stresses is becoming increasingly important. Metal avoidance and tolerance in freshwater fungi implicate a sophisticated network of mechanisms involving external and intracellular detoxification. Examining adaptive responses under metal stress will unravel the dynamics of biochemical processes and their ecological consequences. Freshwater fungi can metabolize organic xenobiotics. For many such compounds, terrestrial fungal activity is characterized by cometabolic biotransformations involving initial attack by intracellular and extracellular oxidative enzymes, further metabolization of the primary oxidation products via conjugate formation and a considerable versatility as to the range of metabolized pollutants. The same capabilities occur in freshwater fungi. This suggests a largely ignored role of these organisms in attenuating pollutant loads in freshwaters and their potential use in environmental biotechnology.

Aquatic hyphomycetes are essential providers of ecosystem functions

What are aquatic hyphomycetes?

Aquatic hyphomycetes (also known as freshwater hyphomycetes, amphibious fungi or Ingoldian fungi) are a polyphyletic group of true fungi (Bärlocher, 1992a; Belliveau & Bärlocher, 2005). Their taxonomy and identification have traditionally been based on the morphology and development of asexually produced spores (mitosporic or conidia). There is no unambiguous definition of what makes a fungus an aquatic hyphomycete, but two crucial characteristics are the ability (1) to sporulate under water and (2) to thrive on deciduous leaves decaying in streams and rivers. In addition, a majority of aquatic hyphomycetes produce relatively large, multiradiate (often tetraradiate) or sigmoid spores (Ingold, 1975; Webster & Descals, 1981), whose tips may be covered with sticky mucilage (Read et al., 1992). These properties facilitate the attachment of the spore to leaves and other smooth surfaces (Webster, 1959; Dang et al., 2007; Kearns & Bärlocher, 2008). The major milestones in the discovery and taxonomic analysis of aquatic hyphomycetes and some useful keys are listed in Fig. 1 (for more details, see Bärlocher, 1992a) (Box 1).

Spore production

Ingold’s discovery of the main habitat of aquatic hyphomycetes has been called the starting point of a ‘minor mycological industry’ (Ainsworth, 1976). Kegel (1906) was the first to demonstrate that sporulation can be induced when an agar block overgrown with a pure culture is submerged in nutrient-poor water. Sporulation is considerably enhanced when submerged agar cultures are agitated with a stream of...
compressed air (Webster & Towfik, 1972). Reliance on spore counts and identifications have dominated research on aquatic hyphomycete community ecology: Iqbal & Webster (1973) introduced a filtering technique to follow seasonal changes in spore numbers and types suspended in the water column. The first major surveys of aquatic hyphomycete successions in streams were based on spores formed on leaves that were incubated in Petri plates (Bärlocher & Kendrick, 1974; Suberkropp & Klug, 1976). Subsequently, Bärlocher (1981) aerated leaves for 1–2 days before capturing newly formed spores on a membrane filter. This approach has been widely adopted to characterize the dynamics of fungal populations on leaves. Several studies have reported numbers of up to eight spores released per day per microgram of detrital dry mass (Gessner, 1997), which accounts for close to or greater than 50% of estimated fungal production on leaves (Suberkropp, 1984, 1991; Findlay & Arsuffi, 1989; Gessner & Chauvet, 1993; Sridhar & Bärlocher, 2000). Detailed information on useful methods to isolate and maintain pure cultures of aquatic hyphomycetes is provided by Descals (1997, 2005, 2008) and Marvanová (2005).

**Involvement in decomposition and food web**

While mycologists were busy investigating the taxonomy, biology and ecology of aquatic hyphomycetes and documenting their global occurrence, few limnologists seemed to be aware of these (or any other) fungi. This changed when the major contribution of allochthonous plant detritus to food webs in streams was rediscovered in the 1960s (for reviews, see Webster & Benfield, 1986; Allan & Castillo, 2007). In temperate streams, the most important source of this imported material is usually deciduous leaves. Kaushik & Hynes (1968) demonstrated that protein levels of some leaf species gradually increased when they were incubated in stream water. Because they did not observe a concurrent increase in bacterial numbers, they attributed protein accumulation to fungal growth. They confirmed this by showing that protein levels increased in the presence of antibacterial, but not antifungal antibiotics. Similar increases were also found on leaves decaying in streams (Kaushik & Hynes, 1971). In food choice experiments, several stream invertebrates clearly preferred ‘conditioned’ leaves, i.e. leaves that had been incubated for days to weeks under conditions

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**Fig. 1.** The involvement of fungi in litter breakdown (modified from Gessner et al., 1997; Zak et al., 2006). The pool of fungal species in the environment, other groups of organisms and internal and external variables determine the quantities and diversity of fungal mycelia on the substrate. The mycelia are agents regulating ecosystem processes, which we subdivide into core (major effects involving large quantities) and keystone (effect disproportionate to quantities involved). The evolutionary point of view puts individual mycelia at its center, and investigates how these units are able to maintain themselves by extracting nutrients in the litter, effectively allocating the acquired energy to growth and reproduction and by interacting with other organisms.
allowing fungal colonization, which resulted in increased protein levels. Triska (1970) observed the same feeding preferences in two detritivorous caddisfly larvae. Mackay & Kalf (1973) showed that fungal-conditioned leaves were much more palatable than bacterial-conditioned or sterile leaves. These early studies suggested that microbial community during leaf decomposition is dominated by fungi in terms of biomass and degradative activity, and that they play a greater role than bacteria in preparing the leaf litter for ingestion by detritus-feeders. It should be noted, however, that the studies generally did not distinguish between aquatic hyphomycetes and phylloplane fungi, which are common on terrestrial leaves and persist for weeks in the stream (Bärlocher & Kendrick, 1974; Gessner et al., 1997). Gessner et al. (2007) and Gulis et al. (2006) have recently reviewed the role of fungi and other microorganisms in leaf litter processing.

**Nutrition of stream invertebrates**

Kaushik & Hynes (1971) proposed that the leaf itself, consisting primarily of structural polysaccharides, is essentially an inert substrate for the accumulation of fungal cells, which provide the actual nutrition to the invertebrates. Numerous studies have confirmed that fungal growth (primarily, but not exclusively by aquatic hyphomycetes) on freshly shed leaves generally improves survival and growth of invertebrates, but is not an absolute requirement (for reviews, see Bärlocher, 1985; Suberkropp, 1992; Canhoto & Graça, 2008). Fungal mycelia generally provide a more concentrated and more efficiently digested food source than leaves; survival on fungal diets varied from excellent to poor. Survivorship on bacterial-conditioned food was lower than on fungal-conditioned food (Kostalos & Seymour, 1976). The contribution of fungal mycelia to the growth of various shredders typically varies between 1% and 59% (Suberkropp, 1992); in Pycnopodia helianthoides, it was sufficient to account for 100% of the observed growth of third instars, but only for 50% of fifth instars (Chung & Suberkropp, 2009). A second conditioning mechanism is the partial breakdown of plant polysaccharides by fungal exoenzymes into smaller units that are more easily digested by invertebrates (Bärlocher, 1982). It would be wrong, however, to conclude that fungal colonization inevitably improves the nutritional quality of the substrate: by inoculating a suitable fungus, the preference of an amphipod for certain leaf species could be reversed (Bärlocher & Kendrick, 1973). Caddisfly larvae differentiated between leaf patches colonized by different fungi (Arsuffi & Suberkropp, 1985).

**Biomass and growth**

Because microbial cells clearly contribute to invertebrate nutrition, accurate estimates of bacterial and fungal biomass and their production on decaying leaves are essential. In a first attempt, fungal biomass was estimated by measuring the length of stained fungal hyphae in decolorized leaves (Bärlocher & Kendrick, 1974). This is believed to result in considerable underestimates (Gessner & Newell, 2002). Nevertheless, direct counts suggested that fungal biomass typically contributed 90–95% to the total microbial biomass during early stages of leaf decay (Findlay & Arsuffi, 1989), confirming earlier conclusions on the relative importance of fungi vs. bacteria (Kaushik & Hynes, 1968, 1971).

More recently, indicator molecules such as ATP, chitin and ergosterol have been used to quantify fungal biomass (Suberkropp, 2008). ATP degrades rapidly after cell death, and therefore reflects living biomass, but it occurs in all organisms and does not allow differentiation between bacterial and fungal cells. Chitin occurs in many invertebrates, is refractory and persists long after fungal hyphae have died. Ergosterol has become the index molecule of choice for fungal biomass. It is the major membrane sterol in higher fungi and is rarely produced by other organisms (Gessner & Newell, 2002; Suberkropp, 2008). It is also believed to rapidly degrade upon cell death, although this has been disputed (Mille-Lindblom et al., 2004). Ideally, ergosterol levels should strictly correlate with fungal biomass, but, depending on species and environmental conditions, the ratio of ergosterol to biomass in pure cultures can
vary by a factor of up to 14 (Gessner & Chauvet, 1993; Charcosset & Chauvet, 2001; Raviraja et al., 2004). Estimates of fungal biomass based on ergosterol were up to three times higher than those calculated from ATP concentrations (Suberkropp et al., 1993); with other conversion factors, the discrepancy was less severe (Abelho, 2009). Clearly, ergosterol measurements from environmental samples have to be interpreted with caution. Nevertheless, they are useful for following the dynamics of fungal biomass during leaf decomposition and for comparing bacterial and fungal biomasses. Typically, ergosterol levels increase when deciduous leaves enter streams, reach a plateau and eventually decline. At its peak, fungal biomass (estimated by ergosterol level) may account for as much as 18–23% of detrital mass (Gessner, 1997; Methvin & Suberkropp, 2003). It is generally lower in floodplain ponds than in rapidly flowing river sections (Baldy et al., 2002). On leaves, fungal biomass consistently outweighs bacterial biomass (estimated by epifluorescence microscopy) by a factor of ≥10 (Baldy et al., 2002; Hieber & Gessner, 2002; Pascoal et al., 2005). At later stages of decay and on smaller organic particles, fungal biomass declines while bacterial biomass remains relatively constant (Findlay et al., 2002).

Microbial production can be determined by following biomass over time if losses due to death, conversion to spores, etc., are negligible. If they are not, direct estimates of growth rates are essential. For fungi, Newell & Fallon (1991) introduced a method based on 14C-acetate incorporation into ergosterol. Bacterial growth is generally estimated by measuring incorporation of leucine or thymidine into proteins and DNA, respectively. Application of these techniques to leaves in streams again demonstrated that production rates of fungi in most instances considerably exceeded those of bacteria (Findlay & Arsuffi, 1989; Weyers & Suberkropp, 1996; Baldy & Gessner, 1997; Baldy et al., 2002; Pascoal et al., 2005). Repeated measurements of fungal production rates and standing crops of leaf litter have been combined to estimate annual fungal production, which has ranged from 16 to 193 g m⁻² (Suberkropp, 1997; Methvin & Suberkropp, 2003; Carter & Suberkropp, 2004; Gulis et al., 2006).

While neither ergosterol nor acetate incorporation provide any information on the fungal taxa growing on leaves, ≥50% of this production is channeled into identifiable conidia of aquatic hyphomycetes (Suberkropp, 1984, 1991; Findlay & Arsuffi, 1989; Gessner & Chauvet, 1993; Sridhar & Bärlocher, 2000), which can be captured by filter-feeding invertebrates (Bärlocher & Brendelberger, 2004).

Enzymes

Vascular plant detritus consists primarily of cellulose, hemicelluloses, pectin and lignin (Webster & Benfield, 1986). Because leaf mass loss proceeded most quickly when fungal growth was possible (Kaushik & Hynes, 1968, 1971), fungi were assumed to actively degrade and use some of these polymers. Suberkropp et al. (1976) were the first to show significant declines in hemicellulose and cellulose during leaf processing in a stream. By contrast, there was an absolute increase in lignin, which the authors attributed to the complexing of nitrogenous compounds by lignin and polyphenols. Similar results were reported for conifer needles by Rosset et al. (1980). Single and mixed pure cultures of both aquatic hyphomycetes and common phyllosphere taxa such as Alternaria, Cladosporium and Aureobasidium caused weight loss of sterilized leaf disks, although at low incubation temperatures, aquatic hyphomycete species were generally more effective (Bärlocher & Kendrick, 1974). Mass loss of submerged leaves due to terrestrial fungi has also been reported by Godfrey (1983) and Graça & Ferreira (1995). Suberkropp & Klug (1980) reported specific enzymatic capabilities of selected aquatic hyphomycetes; due to strong pectinolyis, several species macerated intact leaves into fine particles, thus adding to the food supply of stream filter-feeders. Many other reports have since demonstrated that most aquatic hyphomycetes can degrade cellulose, various hemicelluloses and pectin (Chamier, 1985; Zemek et al., 1985; Abdullah & Taj-Aldeen, 1989; Chandrashekar & Kaveriappa, 1991; Abdel-Raheem & Ali, 2004). The breakdown of lignin is more complex and involves a variety of enzymes, among them monooxygenases, dioxygenases and peroxidases (Hendel et al., 2005). Several authors have reported limited activities of some of these enzymes in several aquatic hyphomycetes (Fisher et al., 1983; Zemek et al., 1985), and peroxidase activities on leaf litter in streams is generally low (Tank et al., 1998). Lignin decomposition in leaves is therefore likely to proceed slowly if at all, which partly explains the rise in lignin content reported by Suberkropp et al. (1976) and Rosset et al. (1980). As mentioned earlier, some of the increase may also be due to artifact lignins formed by microbial enzyme/tannin precipitates.

The significance of laccases or related enzymes extends beyond the breakdown of lignin: their activity in Clavariopsis aquatica correlated with the removal of technical nonylphenol (t-NP), which can disrupt endocrine function in vertebrates (Solé et al., 2008c).

Involvement of other fungi

Exponential decay rates of seven leaf species in a third-order stream in the French Pyrenees correlated strongly with maximum ergosterol concentration, net mycelial production and spore production by aquatic hyphomycetes (Gessner & Chauvet, 1994), suggesting that these fungi largely control this process. Similar correlations were found by Maharning & Bärlocher (1996), although the slopes of the
regression curves were significantly lower. While spore production is clearly connected to aquatic hyphomycetes, ergosterol occurs in other higher fungi, although it is absent in chytrids (Gessner & Newell, 2002). Because a substantial proportion of estimated fungal biomass on leaves is channeled into the production of aquatic hyphomycete conidia, it seems reasonable to assume that these fungi generally dominate leaf decay in streams, which is further supported by their enzymatic diversity. Nevertheless, several lines of evidence suggest that other groups cannot be ignored. Terrestrial fungi have consistently been observed or isolated from leaves decaying in streams (Bärlocher & Kendrick, 1974; Rossi et al., 1983). More recently, molecular analyses have revealed the ubiquitous presence of DNA from fungal taxa that have few or no aquatic hyphomycete representatives (Nikolcheva & Bärlocher, 2004; Bärlocher et al., 2008; Seena et al., 2008). The importance of these taxa may be greater when dissolved oxygen levels are low, and water temperatures and organic pollution high (Chergui & Pattée, 1988; Raviraja et al., 1996).

**Perspectives in research on aquatic hyphomycetes**

Aquatic hyphomycetes participate in the decomposition of plant remains, which has been defined as ‘the sum of biotic and abiotic transformations resulting in the formation of biomass (fungal, bacterial or animal), carbon dioxide and other mineral substances, dissolved organic matter (DOM) and fine particulate organic matter (FPOM)’ (Gessner et al., 1997). These transformations can be interpreted from a process or from a taxon oriented view (Fig. 1). The starting points for both are fungal mycelia on plant remains. Quantities and identities of these mycelia are involved in a feedback system with the local fungal species pool, external environmental variables (e.g. oxygen, pH, inorganic nutrients), leaf litter quality and quantity (chemistry, timing of production) and both microbial (primarily bacterial) and animal (primarily invertebrate) groups. Fungal mycelia perform core ecosystem functions such as regulating the rate of litter mass loss, providing food for leaf-shredders (accumulation of biomass) and for filter-feeders (production of conidia and fine leaf particles), and releasing inorganic nutrients. In addition, they are instrumental in keystone processes (processes with a disproportionate effect on the ecosystem relative to the quantities involved): they sequester metal ions and may break down estrogen mimics such as nonylphenol and other xenobiotics (see: Distinctive features of fungal organopollutant metabolism and metabolization of organic xenobiotics by freshwater fungi). However, even thorough knowledge of these processes and their rates provide only limited insights into mechanisms that govern the composition and persistence of observed fungal communities (Bärlocher, 2010). Topics in evolutionary ecology such as biodiversity, life history or adaptation to global climate change require more detailed knowledge of taxon-specific properties. Both structure (taxa) and function (processes) are considered essential in modern biodiversity research (Pascoal & Cássio, 2008).

**Freshwater fungal biodiversity is controlled by the environmental context and governs ecological functions**

Based on extensive collections on several continents, Ingold (1975) concluded that the diversity of aquatic hyphomycetes is highest in nonpolluted, relatively cool, well-aerated streams running through deciduous forests. It seems likely that their contribution to stream ecosystem functions also peaks under these conditions. While most mycologists and limnologists would probably agree with these statements, it has been surprisingly difficult to find unequivocal and consistent connections between natural or human-altered deviations from the ‘ideal’ stream and diversity and functions of aquatic hyphomycete communities. There is no doubt, moreover, that some aquatic hyphomycete species are remarkably resilient and occur, for example, in arctic or subarctic streams where terrestrial input is dominated by grasses (Engblom et al., 1986), in sulfur springs with temperatures reaching 36.5°C (Chandrashekar et al., 1991) and in surface water (Krauss et al., 2001), sediment (Sridhar et al., 2008) and groundwater (Krauss et al., 2003b, 2005) with extremely high levels of heavy metals.

**Riparian vegetation**

It has repeatedly been established that leaves of some tree species (e.g. *Alnus glutinosa*) are colonized more rapidly and by more species of aquatic hyphomycetes than others (e.g. *Fagus sylvatica*) (Bärlocher, 1992a, b). This is not due to strict substrate specificity by fungal species, but rather to shifts in their relative proportions (differential colonization is more pronounced when leaves are compared with grass or wood; Gulis, 2001). Assuming this is based on some degree of resource specialization, one would therefore expect a positive correlation between the diversity of riparian vegetation (which provides fungal substrates) and fungal diversity. Surprisingly, this has not always been observed. For example, Bärlocher & Rosset (1981) reported over twice as many fungal species from a stream running through a practically pure stand of *Picea abies* compared with two other streams bordered by mixed deciduous forest. In a more extensive study with 16 streams, no correlation was found between riparian tree diversity and fungal species richness (Wood-Egenschwiler & Bärlocher, 1983). By contrast, Fabre (1996) found a significant correlation between fungal species...
richness and richness and diversity of the riparian tree community. However, partial correlations showed that fungal and tree communities were structured simultaneously, but independently by environmental conditions summarized by elevation. Laitung & Chauvet (2005) and Lecerf et al. (2005) also found a significant correlation between leaf and fungal species richness in 10 streams.

The studies discussed above dealt with essentially naturally grown forests. Changes can also occur due to deliberate introductions (e.g. eucalypt in Portugal and Spain) or due to invasive species (e.g. Japanese knotweed in Europe, willow in Australia, saltcedar in the United States; for a review, see Lecerf & Chauvet, 2008). A meta-analysis of 16 studies revealed no consistent impact of afforestation on fungal diversity, although streams running through some Portuguese eucalypt plantations had a clearly reduced community (Bärlocher & Graça, 2002; Ferreira et al., 2006); in comparable Spanish streams, increased levels of inorganic nutrients may have negated this effect and similar numbers of fungal species colonized exposed leaves (Chauvet et al., 1997; Ferreira et al., 2006). Meta-analysis revealed no overall significant effect of altered forests on microbial breakdown rates or maximal mycelial biomass, but possibly a small stimulatory effect on spore production (Lecerf & Chauvet, 2008).

**pH and alkalinity**

Based on a number of studies, Bärlocher (1987) concluded that the number of aquatic hyphomycete species is generally high within a relatively broad pH region between 5 and 7 and declines below and above these values. Low pH values as such do not appear to be harmful to aquatic hyphomycetes: on solid media, 10 species grew best at values between 4 and 5 (in liquid media, the optimum was generally 1 U higher; Rosset & Bärlocher, 1985). Weight loss of maple leaves due to microbial activity peaked between 5.5 and 6.0 (Thompson & Bärlocher, 1989). However, anthropogenic acidification can raise aluminium concentration in the stream water, which severely depressed fungal richness and activity (Chamier, 1985; Chamier & Tipping, 1997; Baudoín et al., 2008). No difference in fungal richness was found between two humic streams in Sweden, both exposed to acid precipitation and one treated with lime (Bärlocher & Peterson, 1988).

Low alkalinity or levels of Ca$^{2+}$, which coincide with low pH values, make aquatic hyphomycetes more vulnerable to heavy metal exposure (Abel & Bärlocher, 1984). In a recent study (Cornut, 2010), anthropogenic acidification strongly depressed leaf mass loss on the stream bed surface and, to a lesser extent, in the hyporheic zone. This was attributed to the drastic impact of acidity on leaf-shredders and the reduced fine particle production by the impoverished fungal community. The latter may be connected to a reduced pectin lyase activity at lower pH values (Chamier, 1985). By contrast, fungal biomass showed no obvious trend and was in fact highest in the most acidic stream.

**Pollution by metals**

Mining pollution very consistently has a negative effect on fungal species richness and associated performance metrics such as maximum spore production and biomass and fungal leaf-processing rates (meta-analysis, Lecerf & Chauvet, 2008). This has been shown for effluents from mining for coal (e.g. Maltby & Booth, 1991; Bermingham et al., 1996; Schlief, 2004), gold (Lecerf & Chauvet, 2008), copper (Cu) (Sridhar et al., 2000, 2001; Krauss et al., 2001), zinc (Zn) (Niyogi et al., 2002, 2009) and uranium (Ferreira et al., 2010b). At moderate pollution, the effects are generally more severe on fungal diversity and less so on fungal biomass/growth and degradational activity (Duarte et al., 2008; Solé et al., 2008b), which may suggest some compensation by resistant strains or species. Exposure to higher levels drastically impoverishes fungal communities and reduces leaf decomposition (Krauss et al., 2001; Duarte et al., 2008; Solé et al., 2008b; Fernandes et al., 2009a).

Sridhar et al. (2005) demonstrated partial adaptation of some fungi to heavy metal pollution by transplantation experiments. They isolated two strains of Heliscus lugdunensis and one strain of Tetracladium marchalianum from two habitats differing in severity of heavy metal pollution. After precolonization in the laboratory, leaf disks were transferred to the two sites. Two out of three strains performed better at the site of their origin. The authors also observed a relatively swift recovery of the fungal community and metabolism upon removal from acute heavy metal exposure. Similar observations were recently made by Duarte et al. (2009).

Leaves incubated in a reference stream were exposed in microcosms supplemented with Cu or Zn, which reduced leaf mass loss, fungal reproduction and altered the fungal community. After release from metal stress, the structure and activity of the fungal community quickly recovered. Higher fungal diversity may mitigate the impact of heavy metal pollution on leaf decomposition in streams (Duarte et al., 2008).

**Eutrophication**

Lecerf & Chauvet (2008) reviewed the effects of eutrophication on fungal diversity and their growth and involvement in leaf breakdown. The only significant effect in their meta-analysis was declining fungal diversity, even though individual studies often found clear negative or positive effects. This inconsistency is not surprising, because eutrophication is a rather broad concept and can include both inorganic and organic sources. Especially in field studies, it is difficult to separate effects of the two and to consider potential
interactions with other factors. Nitrates and phosphates in particular are well known to stimulate fungal growth and leaf breakdown. In fact, extremely low levels of these nutrients may have been responsible for impoverished fungal communities in some streams studied by Suberkropp (1995). At higher concentrations, the positive correlation between nutrients and fungal diversity may reverse itself: Sridhar et al. (2009) found fast decay rates, but low fungal diversity in a more nutrient-enriched stream. Similarly, Raviraja et al. (1998) observed very low aquatic hyphomycete diversity in a eutrophied stream without significant impact on leaf breakdown rates. It seems unlikely that nutrients at the levels observed directly inhibited some species and led to their extinction (but see Solé et al., 2008b). Possibly, oxygen depletion on leaf surfaces due to sedimentation of fine particles or increased microbial respiration negatively affected aquatic hyphomycete metabolism and ultimately growth (Pascoal & Cassio, 2004; Medeiros et al., 2009). Or, increased levels of toxic organic and inorganic micropollutants typically associated with eutrophication may be responsible for reduced fungal diversity (Lecerf & Chauvet, 2008). Neither of these scenarios, however, explains the continued strong degradational activity, unless some species are resistant to low oxygen levels or to the postulated toxins, or other microbial groups take over their functions. Alternatively, increased nutrient levels may selectively stimulate some aquatic hyphomycete species, allowing them to displace weaker competitors (Bärlocher, 2005). This assumes a hump-shaped relationship between diversity and community productivity or growth, which is well documented for some plant-dominated systems (Gough et al., 2000).

Temperature and climate change

Temperature is generally considered the most important environmental factor affecting metabolic functions and ultimately growth and survival of microorganisms (Madi-gan et al., 2009). It is characterized by three cardinal points: \( T_{\text{min}} \), below which no growth is possible, \( T_{\text{opt}} \), at which growth is most rapid and \( T_{\text{max}} \) above which growth is not possible. The three temperatures usually differ for growth and reproduction.

Aquatic hyphomycete species in temperate regions are less inhibited by water temperatures close to 0°C than phylloplane fungi (Bärlocher & Kendrick, 1974). Their growth generally peaks between 15 and 25°C, while sporulation is highest at somewhat lower temperatures (Koske & Duncan, 1974; Webster et al., 1976; Suberkropp, 1984; Chauvet & Suberkropp, 1988; Sridhar & Bärlocher, 1993; Dang et al., 2009). Beyond minima and maxima, temperatures that kill the individual colony or propagule are important. For example, temperate species were shown to be more likely to survive freezing than tropical species (Sridhar & Bärlocher, 1993); the reverse may be true for survival at high temperatures. The occurrence and frequency of such extreme events may have a profound effect on fungal community composition and activities and override the impact of the overall trend.

Another point to consider is the fact that temperature rarely remains stationary. Growth averaged over a 24-h period is only identical with the growth at the average temperature during the same period if the relationship between growth and temperature is linear. If the curve is concave down (e.g. n-shaped), it is lower; if the curve is concave up (e.g. u-shaped), it is higher. Thus, temperature oscillations around 8°C accelerated decomposition by 18% (5 oscillation) and 31% (9 oscillation), respectively (Dang et al., 2009).

These responses to temperature can be expected to influence both seasonal and regional abundance of individual species and therefore fungal community composition, although the evidence is rarely straightforward and suggest that the ecological context (nutrients, oxygen, biological interactions) can strongly modulate temperature preferences (Bärlocher, 1992b). Thus, Flagellospora penicillioides and Lumulospora curvula do not grow below 5°C; not surprisingly, they disappear from streams during colder seasons (Webster et al., 1976; Suberkropp, 1984). In a Michigan stream, distinct winter and summer assemblages could be identified; however, temperature growth responses in cultures only partially explained their seasonal cycles (Suberkropp, 1984). On a larger scale, temperature preferences of individual species are broadly linked to geographical distribution (Bärlocher, 1992b), and species such as F. penicillioides, L. curvula, Lumulospora cymbiformis and Triscelophorus acuminatus are more common in subtropical or tropical streams (Wood-Eggeschwiler & Bärlocher, 1985; Goh, 1997; Santos-Flores & Betancourt-López, 1997).

The continued use of fossil fuels is projected to raise the atmospheric CO2 level from approximately 380 μL.L−1 to between 490 and 1060 μL.L−1 by the end of this century, which will contribute to an average global temperature increase of between 1 and 6°C (IPCC, 2007). Additionally, the frequency of extreme weather events (e.g. record high or low temperatures, floods, droughts) and overall variability are expected to increase. These changes will influence the activities of aquatic fungi, and by extension their distribution and occurrence, and the composition of fungal communities. Because of numerous and intimate fungal feed-back loops (Fig. 1) with resources (plant detritus), potential competitors (bacteria, invertebrates) and predators (invertebrates), predicting fungal responses to environmental change and the resulting effects on ecosystem processes are daunting challenges.

Because the water temperature in temperate streams is generally below the optimal temperature for most fungi (see
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due to climate change, retarded decomposition and caused example of an extreme event that may become more frequent treatment between stream and microcosm exposure, as an example of an extreme event that may become more frequent due to climate change, retarded decomposition and caused L. curvula, a typical summer species, to become extinct.

In a microcosm experiment with a mixture of six pure cultures, the rate of decomposition increased between 5 and 15 C (Ferreira & Chauvet, 2010). The rate of increase was significantly enhanced at the highest temperature, when nutrients (nitrate and phosphate) were added. Increased eutrophication is common in many scenarios of global change (IPCC, 2007).

Many studies have investigated the effects of the root cause of climate change, elevated CO2 levels, on the quality of leaf litter. Generally, this leads to greater leaf biomass, C/N ratio, concentrations of tannins and other phenolics and lower nitrogen levels (for reviews, see Norby et al., 2001; Stiling & Cornelissen, 2007; Ferreira et al., 2010a), with negative consequences for herbivores. However, in an extensive review of primarily terrestrial studies, Norby et al. (2001) failed to detect a consistently negative impact of these changes on short-term decay rates or respiration from decomposing litter.

Decomposition experiments in streams have also given inconsistent results. Leaves grown at elevated CO2 levels may decay more slowly (e.g. Rier et al., 2002), but fungal biomass can be as high as or higher than on control leaves (Rier et al., 2002; Ferreira et al., 2010a). Mosquito larvae had higher mortality rates and slower development rates when fed on leaves from elevated CO2 levels (Tuchman et al., 2003). Negative effects were also shown for Tipula abdominalis (Tuchman et al., 2002). The effects of elevated CO2-induced leaf quality on Sericostoma vittatum (Trichoptera: Sericostomatidae) were complex (Ferreira et al., 2010a). When offered at 10 C, leaves with the elevated treatment were preferred (no difference at 15 C). Growth and survival rates were generally higher at the lower temperature.

The ultimate goal of climate biology is to predict changes in stream ecosystems due to elevated CO2 and temperature. Several shortcomings and pitfalls of currently used approaches have been discussed in Bärlocher et al. (2008). To begin with, we cannot assume that increasing temperature and CO2 levels have the same effect and act independently (additive). For example, elevated CO2 increased allocation to phenolics in three deciduous trees, but elevated temperature had the opposite effect. In combination, the overall effect was nil (Veteli et al., 2007). The same principle applies to decomposition experiments: both temperature and CO2 levels should be elevated to simulate the effects of global change. Leaf chemistry influenced by CO2 levels and temperature during a feeding trial strongly interacted when its suitability as food to a detritus-feeder was studied (Ferreira et al., 2010a). Another potentially decisive point concerns the transition from current to projected conditions: a mycorrhizal community responded strongly to an abrupt doubling of CO2 concentration, but not to a gradual change of the same magnitude spread over 6 years (Klironomos et al., 2005). The anticipated climate changes will likely be spread out over several decades. In any given catchment area, members of the existing riparian plant community will become extinct unless they can adapt to the changing conditions. They may also be joined by invasive species (which, being microorganisms, may be ‘invisible’; Litchman, 2010). Local aquatic hyphomycetes in turn have to adapt to the changes in climate and resource chemistry and to invasive microbial competitors or become extinct. The next trophic levels, leaf-eating invertebrates, will have to deal with the changed leaf–fungus complex, climatic changes and invaders. The magnitude and directions of these interlocking feedback loops will depend on the initial context and evolutionary potential of local and neighboring species pools; the resulting cascades can become extremely complex and the outcome(s) difficult to predict. A large-scale simulation of the joint effects of temperature and flow regimes in Mediterranean streams suggested increases in instream metabolism and export of organic carbon (Acuña & Tockner, 2010).

In view of the ubiquitous occurrence of aquatic hyphomycetes on all continents, their ability to colonize essentially all types of plant litter and short life cycles facilitating rapid evolutionary change (Bärlocher, 1992b), extensive extinctions due to climate change seem unlikely. In the short term, however, an impoverished riparian vegetation may lower local fungal diversity, which in turn may lower the nutritional value of conditioned leaves (e.g. Lecerf et al., 2005).

Climate change over the last 55 years generally had a positive impact on forest productivity when water was not
limiting (Boisvenue & Running, 2006), and the fruiting period of forest fungi has been expanding since 1985 (Gange et al., 2007), suggesting increased mycelial growth and organic matter turnover in response to global warming.

**Connections between biodiversity and ecological functions**

Much of the concern about declining biodiversity is based on the fear that it will threaten ecosystem functions and services (Kinzig et al., 2001). Many observations and experiments in plant communities have indeed demonstrated an asymptotic relationship between species richness and production or accumulation of biomass (Loreau et al., 2001). Studies involving aquatic hyphomycetes have started only recently (Bärlocher & Corkum, 2003), are few in numbers (Pascoal & Cássio, 2008) and have involved relatively few species (up to eight, Dang et al., 2005). A problem unique to microbial systems is the difficulty of keeping track of individual species. While the composition of the initial inoculum can be controlled, it cannot be assumed that it will be maintained throughout the experiment. It is therefore uncertain whether the measured function is indeed the result of the initially established diversity. Estimates of the realized diversity throughout the experiment have been based on spore productions by the introduced species (Dang et al., 2005) or on intensities of denaturing gradient gel electrophoresis (DGGE) bands of their amplified DNA (Pascoal et al., 2010).

Investigated functions have most commonly included mass loss of leaves, followed by build-up of fungal biomass and release of conidia. Thus far, the evidence suggests that traits of certain species have a greater impact on these ecological functions than diversity *per se* (i.e. numbers of species present in a stream or microcosm; Pascoal & Cássio, 2008), and nutrient enrichment can overwhelm the often weak diversity effects (Bärlocher & Corkum, 2003). There appears to be sufficient redundancy in the fungal community to buffer its core functions against loss of biodiversity. However, the variability of decay rates increased at lower diversity (Dang et al., 2005), particularly under Zn stress (Pascoal et al., 2010), suggesting that the effects of fungal species loss on ecosystem stability may be exacerbated with increasing pollution. In addition, species-specific fungal traits may have a greater impact on higher trophic levels: food selection by leaf-eating invertebrates is often strongly influenced by the identity of fungi that have colonized the leaves (Bärlocher & Kendrick, 1973; Arsuffi & Suberkropp, 1985; Lecerf et al., 2005). Again, this raises the topic of keystone properties: even though all aquatic hyphomycete species tested appear to perform the same basic functions at similar rates (breakdown of structural polysaccharides, addition of mycelia, release of conidia), their effect on the ecosystem may be selectively amplified or attenuated at higher trophic levels.

**Fungal community structure**

Fungal communities on leaves decaying in streams have been detected and characterized by aerating substrates in nutrient-poor solutions (Bärlocher, 2005; see Aquatic hyphomycetes are essential providers of ecosystem functions). Clearly, this favors the discovery of aquatic hyphomycetes. The majority of the fungal biomass on decaying leaves, however, consists of nonreproductive mycelia (e.g. Bärlocher & Kendrick, 1974), which may or may not be able to form identifiable propagules under aeration. The traditional reliance on identifying newly formed spores to characterize dominant species therefore includes an element of circular reasoning (Bärlocher, 2005). In contrast, molecular methods, which isolate nucleic acids directly from environmental samples, facilitate the detection of fungal taxa regardless of life stage (i.e. reproducing or nonreproducing hyphae, mitospores and meiospores, dormant structures). Moreover, molecular methods are a powerful alternative when fungal biomass and sporulation are drastically reduced due to unfavorable environmental conditions (Solé et al., 2008a; Fig. 2). Isolated DNA is amplified with fungal group-specific primers (Nikolcheva et al., 2003, 2005; Nikolcheva & Bärlocher, 2004; Bärlocher, 2007; Das et al., 2007; Duarte et al., 2008; Seena et al., 2008; Harrop et al., 2009) and usually analyzed by fingerprint methods. Community fingerprinting techniques (e.g. terminal restriction fragment length polymorphism, DGGE, thermal gradient gel electrophoresis) have been successfully applied to study aquatic fungi in natural environments or in microcosms (Nikolcheva et al., 2003; Nikolcheva & Bärlocher, 2004; Raviraja et al., 2005; Das et al., 2007; Duarte et al., 2008; Solé et al., 2008a; Moreirinha et al., 2010).

Most of these studies resulted in estimates of fungal diversity in terms of operational taxonomic units or phyotypes, some of which did not correspond to fungal taxa dominating the spore production (Nikolcheva et al., 2005; Solé et al., 2008a). However, community fingerprints rarely allow unequivocal identification of all fungi present. Only the excision, cloning and sequencing of the ‘fingerprint bands’ can confirm or refute their phylogenetic affiliation to aquatic hyphomycetes, and, ideally, allow assigning them to described taxa. Alternatively, the initial mixture of amplicons with fungal-specific primers can directly be cloned and sequenced. Two such studies have revealed the dominance of ascomycete-affiliated taxa (most aquatic hyphomycetes are ascomycetes, but not all ascomycetes are
aquatic hyphomycetes). In both cases, however, substantial numbers of clones were assigned to fungal groups that rarely or never contain aquatic hyphomycetes (Bärlocher et al., 2008; Seena et al., 2008). A practical, standardized method for the identification of fungal structures at any life stage therefore remains a top priority. Currently, the most promising approach is the use of molecular barcodes. These are short DNA sequences from a specified region of the genome that are unique for each species (Hebert et al., 2003). Suitable barcodes should be sequences that can be reliably extracted and amplified, and are of low intraspecific and sufficient interspecific variability. For animals, a 648-bp fragment at the 5' end of the mitochondrial cytochrome c oxidase 1 gene (COX1 or CO1) has been recommended due to its high variability, low intron interference and robust amplification ability (Hebert et al., 2003). Identification is straightforward when a sequence is constant within one species and does not occur anywhere else. Failing this, intraspecific differences have to be much lower than those among species. Based on many studies in various animal groups, ranging from invertebrates to fish to birds, Hebert et al. (2004) suggest a threshold of a factor of 10 between sequence variation within and among species. For fungi, the entire internal transcribed spacer (ITS) sequence was recommended as a first step for species identification, with a second sequence added for more precise identification and phylogenetic placement (Fungi Barcode Initiative; Rossman, 2007). The suitability of this region to resolve the taxonomy of the aquatic hyphomycete genus Tetracladium was recently demonstrated by Letourneau et al. (2010). Seena et al. (2010) sequenced and compared the ITS site in 94 fungal isolates belonging to 19 species collected in Portuguese streams. The Portuguese isolates and sequences from GenBank exhibited taxonomic cohesiveness. All isolates grouped with their respective species; however, all Tricladium species did not group within the same genus.

Due to species-specific food preferences of leaf-eating invertebrates (Bärlocher & Kendrick, 1973; Arsuffi & Sukerkropp, 1985; Lecerf et al., 2005), quantitative measurements of biomass and enzyme activities of individual aquatic hyphomycete species in streams are of prime importance for documenting and understanding their ecological significance in food webs. As a first step, several studies attempted to assess the relative contribution of all fungi to aquatic microbial communities. Léfevre et al. (2010) developed a quantitative real-time PCR assay, based on 18S rRNA gene, to assess zoosporic fungi. Application to natural samples provided only semi-quantitative estimates, because the number of rRNA operons per cell is not well known. Quantitative real-time PCR was also used by Manerkar et al. (2008) to estimate fungal biomass in a stream during leaf decomposition. The data supported greater contribution of Fungi than Bacteria or Archaea to total microbial biomass as suggested by other methods (see Biomass and growth), but again, the data were only semi-quantitative.

Thus far, quantitative real-time PCR used in environmental studies have focussed on large taxonomic units

Fig. 2. Schematic diagram of molecular approaches linking diversity and structure of fungal communities involved in litter breakdown. Lac- cases are an example of functional genes puta- tively involved in ecological process. When based on phylogenetic genes, the scheme illustrates the absence of a direct connection of community diversity and structure to litter breakdown. In this case, only cDNA fingerprints might allow a more direct assessment of the ecological function (dashed arrow).

Community diversity (phyotypes, cloning, barcoding)

Community structure (DNA fingerprints)

Phylogenetic genes (18S, 28S, ITS, COI)

Genes expression (cDNA)

Functional genes / cDNA fingerprints (qPCR, pyrosequencing, RNA-SIP)

Ecological function (litter breakdown)
(zoosporic fungi, higher Fungi). It seems feasible to design primers for selective amplification of basidiomycetes or ascomycetes; selective primers for aquatic hypomycetes are unlikely due to the polyphyletic origin of this group (Belliveau & Bärlocher, 2005). Because quantitative real-time PCR works best when a limited number of species is targeted, this method nevertheless promises to be an excellent tool for microcosm experiments. It could be used to monitor the relative abundance of individual species within a fungal community (i.e. to determine ‘key-players’ for a defined ecosystem function), or to elucidate the role of selected taxa within simplified microbial food webs. More complete information would be provided by a cloning/sequencing approach targeting barcodes to establish relative frequencies of occurrence of the various species, or using new high-throughput pyrosequencing technology with barcodes. Such methods are becoming increasingly common in medical microbiology, and can undoubtedly be adapted to aquatic fungal ecology (Armougom & Raoult, 2008; Petrosino et al., 2009). This would overcome the current difficulty of assessing relative abundances of fungal species in natural communities, which remains one of the major limitations in functional ecology, because specific traits of some species often have a greater influence on ecosystem functioning than species number per se (Duarte et al., 2006).

**Ecological functions**

It is assumed that DNA in dead cells is rapidly broken down, so that the vast majority of amplified sequences are derived from living fungi or bacteria. This may generally be the case, but the presence of clay or colloids can prolong the integrity of environmental DNA (Pietramellara et al., 2009). Pure cultures of *Escherichia coli* cells killed by heat, acid, drying or starvation still provided positive PCR signals (Sheridan et al., 1998). On the other hand, RNAs were less stable. This is particularly true for mRNA, which rapidly turns over in living cells, and may be the best indicator of viability (Sheridan et al., 1998). These compounds can directly be isolated from environmental samples, reverse-transcribed into cDNA and amplified by PCR (RT-PCR). Pyrosequencing both genomic DNA and cDNA (from mRNA) allows simultaneous assessment of structure and (potential) functions of microbial communities (Urich et al., 2008; DeLong, 2009; Fig. 2). At least in principle, taxonomic composition and metabolic pathways can be connected, such that we can determine which taxa are present and whether or not they are actively transcribing specified genes. However, the correlation between mRNA and protein levels is often poor (Gygi et al., 1999; MacDonald et al., 2003). A realistic description of the actual processes taking place would therefore additionally require measuring synthesized proteins (enzymes) and their activities (e.g. using protocols by Sinsabaugh et al., 1994; Jackson et al., 1995, 2006).

Another approach to link function to specific taxa is stable isotope probing (DNA-SIP or RNA-SIP; Boschker et al., 1998; Boschker & Middelburg, 2002; Manefield et al., 2002; Fig. 2). This involves the incorporation of stable-isotope-labelled substrates into cellular biomarkers (e.g. specific DNA or RNA sequences) that can be used to identify organisms assimilating the substrate. A precondition, of course, is that no cross-feeding occurs, which may not necessarily apply when polysaccharides are digested in mixed cultures (e.g. Tiunov & Scheu, 2005; Schneider et al., 2010).

The interpretation of these complex data sets with DNA, RNA and proteins presents significant computational and statistical challenges (DeLong, 2009; Huttenhower & Hofmann, 2010). Potential pitfalls include biases in extraction of nucleic acids and proteins, and in amplification or activity measurements. In addition, these methods usually involve a homogenization step, which destroys the spatial arrangement of cells and enzymes. To reveal the distribution of mycelia belonging to different species, monoclonal antibodies (Bermingham et al., 1995, 1996, 2001; this method could also be applied to localize key enzymes) or FISH (McArthur et al., 2001; Baschien et al., 2008) may be useful. However, neither method has been sufficiently standardized for routine application.

We are not aware of any studies addressing fungal functional gene diversity in aquatic environments. However, primers have been designed to study the diversity of laccase genes in ascomycetes colonizing detritus in saltmarshes (Lyons et al., 2003). Ten species contained 39 distinct sequences, with several species yielding multiple distinct laccase types. Two laccase types from a natural-decay clone library were novel and did not match any of the sequences obtained from the pure cultures. Luis et al. (2005) compared RNA and DNA laccase of terrestrial basidiomycete sequences and showed that <30% of the laccase genes detected in a soil core were expressed. Solé et al. (2008c) identified two putative laccase gene fragments in the aquatic hyphomycete *C. aquatica*, and suggested the existence of additional laccase genes.

Fungal polyphenol oxidases such as laccases catalyze the oxidation of various substrates coupled to the reduction of oxygen to water. They are instrumental in the biodegradation of biopolymers such as lignin (Baldrian, 2006) and therefore play a major role in litter breakdown. Thus, the laccase gene family is a good candidate for attempts to link the structure and function of fungal communities. The results reinforce the point that in addition to studying functional genes, knowing their expression pattern is essential for characterizing fungal activities.

Schneider et al. (2010) investigated extracellular degradative enzymes in single and mixed cultures of the bacterium
Pectobacterium carotovorum and a terrestrial fungus, Aspergillus nidulans, growing on beech leaves. Semi-quantitative proteome analyses were combined with analyses of extracellular degradative enzyme activities (proteases, pectinases, cellulases and various hemicellulases). Most of the enzyme activity could be attributed to the fungus. Interestingly, the levels of cellulase and pectinase activities were very similar in pure fungal and bacterial/fungal co-cultures, suggesting comparable decompositional activities (actual mass loss of the leaves was not reported). However, ergosterol levels (indicator of fungal biomass) were less than half in the mixed culture, indicating that the bacterium benefited from the degradation products of A. nidulans at the expense of fungal growth. Antagonistic effects of bacteria on fungal growth in aquatic model systems have been reported before (e.g. Mille-Lindblom & Tranvik, 2003; Mille-Lindblom et al., 2006), while in mixtures of fungi both facilitative and antagonistic interactions have been documented (Bärlocher & Corkum, 2003; Tiunov & Scheu, 2005). These studies point to the potential disconnect between diversity and various ecological functions: a more diverse microbial community may well stimulate one aspect (e.g. mass loss rate) and inhibit another (e.g. fungal growth). It may therefore be futile to look for consistent rules connecting microbial diversity and ecological function. It must be remembered that communities are best understood as being structured by natural selection on individuals (or genes) pursuing their own interests (Bärlocher, 2010).

Future perspectives – transcriptome, proteome and metabolome analysis

Although fungal interactions with the biotic and abiotic environment usually involve multiple genes, gene expression studies frequently target just one or only a very few genes. The development of new tools such as cDNA microarrays has opened new perspectives for assessing microbial activities. cDNA microarrays offer the possibility of recording the whole transcriptome on a single chip and have been applied successfully to fungi, thus facilitating investigations of the exceedingly complex interactions among thousands of genes expressed simultaneously (Jacob et al., 2004; Doddapaneni & Yadav, 2005; Wright et al., 2005; Jakupović et al., 2006; Le Quéré et al., 2006). Adapting microarrays to environmental studies presents significant challenges (Zhou, 2003). However, microarrays appear promising for monitoring selected genes in the environment or for identifying genes involved in fungal interactions with the environment (Wu et al., 2001; Rhee et al., 2004). Rhee et al. (2004) demonstrated that in bacteria, microarray-based quantification was highly consistent with real-time PCR quantification. These results illustrate the potential of the method for qualitative (i.e. specific) and quantitative monitoring of microbial communities other than bacteria.

Like transcriptome analysis with cDNA microarrays, the analysis of the entire proteome (Blee et al., 2001; Vido et al., 2001) is a multivariate approach allowing investigations of complex interactions of fungi with their environments at the protein level, and has successfully been applied to fungal species (Kniemeyer et al., 2006; de Oliveira & de Graaff, 2011). Further identification of proteins may be achieved by sequencing with tandem MS, which nowadays is commonly performed using hyphenated techniques, for example through amino acid-coded mass tagging and liquid chromatography tandem MS as shown for Schizosaccharomyces pombe (Bae & Chen, 2004). While most proteome studies conducted in the past have focused on whole cell extracts, cytosolic proteins and fungal secretomes, fungal organelle proteomics are becoming increasingly important (de Oliveira & de Graaff, 2010). Careful sequential extraction or single-cell sampling methods may be used to obtain more information on membrane proteins, DNA-binding proteins and cell-to-cell signalling proteins.

GC and to an increasing extent liquid chromatography coupled with MS combine high sample throughput with acceptable measurement times and accurate metabolite identification (Moco et al., 2009). Parallel controlling of numerous metabolites in multiple samples may provide more detailed insights into dynamic metabolomic networks and their perturbations by specified environmental stresses as already shown for plants (Saïto et al., 2006). Fungi show less cellular differentiation than plants, but their complex metabolism produces a broad range of often bioactive metabolites (Turner & Aldrigde, 1983; Cole et al., 2003; Frisvad et al., 2004). Metabolite profiling, combined with genetic, proteomic and biochemical approaches (Reaves & Rabinowitz, 2010), can be expected to foster a comprehensive understanding of fungal metabolism especially under stress conditions. Analytical methods to study metabolic networks with respect to data acquisition, network statistics and biochemical interpretation have been extensively discussed and reviewed in the literature (e.g. Fiehn & Weckwerth, 2003; Goodacre et al., 2004; Reaves & Rabinowitz, 2010).

Physiological and biochemical versatility allows aquatic fungi to cope with environmental challenges

Response to metals

High concentrations of essential metals and presence of toxic metals cause major environmental and human health problems due to toxic effects in different biota, trophic transfer and biomagnification in food chains. Ecological and ecobiochemical research on heavy metals is an interdisciplinary effort considering both biotic and abiotic factors.
There is growing interest in understanding the ecological, physiological and biochemical properties that allow some fungi to colonize and live in heavy metal-polluted habitats. Together with bacteria, aquatic fungi are essential constituents of aquatic habitats (see Aquatic hyphomycetes are essential providers of ecosystem functions). The resources locked within substrates are efficiently released by mycelia, i.e. filamentous networks of hyphae. Filamentous fungi have a high surface area-to-volume ratio and therefore provide a large contact area with metals in the environment. They grow by extending the tips of hyphae to form an expanding mycelial network (Steinberg, 2007). Hyphae navigate around biological or abiotic surfaces. Their orientation is a vital aspect of orientated growth, morphogenesis and spatial ecology (Brand & Gow, 2009).

The speciation of heavy metals in the environment and their bioavailability have exerted selective pressures on prokaryotic and eukaryotic microorganisms, many of which have evolved mechanisms to withstand high concentrations of such pollutants (Nies & Silver, 2007; Krauss et al., 2008; Gadd, 2010). Terrestrial and aquatic fungi have developed stress responses to environmental changes: resistance [immediate response, e.g. response to reactive oxygen species (ROS), intracellular synthesis of chelating peptides and proteins] and tolerance (based on intrinsic biochemical and structural properties of fungal cells, e.g. changes in cell wall permeability, extracellular slimes and enzymes, metabolite excretion) (Krauss et al., 2008; Schlosser et al., 2008). Fungal tolerance mechanisms depend (1) on the metabolic and nutritional status of the fungus, which may induce an adaptive stress response, and (2) on the concentration of anions and metals in water, pH value and stability constants of specific extracellular complexes. Because they accumulate more biomass, filamentous fungi can sequester greater amounts of metals than bacteria (Massaccesi et al., 2002). Examining adaptive responses under metal stress will help us to explore the functioning and regulation of cellular metabolism in fungi and unravel the dynamics of metal-influenced biochemical processes and their ecological consequences. Metal avoidance and tolerance in freshwater fungi involve a sophisticated network of mechanisms involving both external and internal detoxification of metal ions.

**Extracellular metal precipitation**

Precipitation of metal ions onto or around hyphal cells represents a sink for metals. Metal-binding capacity is influenced by environmental pH, hyphal density and excretion of organic compounds (Gadd, 2007). Freshwater fungi were able to grow on and decompose alder leaves exposed in a small spring-fed stream in Central Germany (site H4) under heavy metal-precipitating conditions (Ehrman et al., 2008). The water of this site, a small spring-fed stream originating from a smelting waste dump, contained close to 3 g L⁻¹ of dissolved Zn, several mg L⁻¹ of other heavy metals and metalloids, and high levels of nitrate and sulfate (Krauss et al., 2001; Ehrman et al., 2008). The surface of exposed leaves quickly became covered with zincwoodwardite containing significant amounts of aluminum, sulfur, Cu and Zn (Ehrman et al., 2008). The growth of hyphae completely encased in the precipitate continued without marked loss of biomass. It appears that some properties of natural leaves are necessary for colonization because artificial substrates were not colonized to the same extent (Ehrman et al., 2008). It is possible, although unproven, that new filaments catalyze additional precipitation, and that fungal hyphae actively accelerate heavy metal precipitation. Regardless, the addition of inexpensive and environment-friendly leaves to contaminated water might be a valuable addition to chemical remediation methods.

More detailed studies of how aquatic fungi regulate adsorption of heavy metals may provide evidence as to the passive or active nature of this process. The interaction of fungi with toxic metals and xenobiotics in the habitat could be exploited to shield ecosystem functions and services, and to facilitate natural attenuation of pollutants (bioremediation).

**Biosorption to cell walls**

In terrestrial fungi, increased biosorption of heavy metals from soil has been attributed to the specific structure of the cell wall with chitin and chitosan as main constituents (Gadd, 2007). Ahrazem et al. (2001) identified glucose, galactose, mannose and glucuronic acid as the main components of the cell wall of *Sesquicillium* sp. (*Hypocreales*) and *Nectria cinnabarina*. These results were confirmed for two strains of *H. lugdunensis* (Teleomorph: *Nectria*) (G.-J. Krauss et al., unpublished data), an aquatic hyphomycete closely related to *N. cinnabarina*. The cell wall has the capacity to bind metal ions at negatively charged sites such as glucuronic acid. External biosorption of cadmium (Cd) in *H. lugdunensis* was up to 10 times greater than intracellular content (Jaecckel et al., 2005b; Braha et al., 2007). Similar characteristics were shown for Cu. In contrast, intracellular Zn levels were higher than sorptively bound Zn (Jaecckel et al., 2005b).

When exposed to metal ions, swollen hyphal cells were observed in aquatic hyphomycetes (Azevedo et al., 2007; Braha et al., 2007), similar to reports from the zygomycete *Mucor rouxii* for Cu (Gardea-Torresdey et al., 1996) and from *Fusarium* sp. and *Alternaria tenuis* for Cd (Gharieb, 2001). The function of these induced morphological changes is unknown. The response, however, does not appear to be specific. Other abiotic stresses (e.g. low pH, potassium or oxygen deficiency) can result in similar morphological changes (Gharieb, 2001).
Plasma membrane identity

When metals are taken up, the plasma membranes of hyphal cells are primary targets for metal action. Uptake and subsequent sequestration of metals to various subcellular localizations require transition metal transporters. Similar to plants, fungi take up heavy metals from the environment by transport systems of varying specificity, located in the plasma membrane (Clemens, 2006; Krämer et al., 2007). Saccharomyces cerevisiae has been used as model organism to study the processes of metal homoestasis and resistance in fungi, which include transport processes (Van Ho et al., 2002). These transport systems through the plasma membrane could be regulated in aquatic fungi such that only small amounts of toxic metals are taken up. Two strains of Heliscus submersus showed different metal uptake capacity for Cd and Cu (Braha et al., 2007). Distinct regulated transport systems might be involved in Cd and Cu uptake of the two strains isolated from differently contaminated sites. In the freshwater fungus Varicosporium elodeae and Heliscus submersus (Azevedo et al., 2007), Cu (up to 150 μM) induced a more severe disruption of plasma membrane integrity than Zn (up to 200 μM). It can be hypothesized that the redox-active nature of Cu and its ability to generate free radicals promote lipid peroxidation in plasma membranes and interruption of transport processes.

Heavy metal ATPases (HMAs) specifically transporting heavy metals (e.g., Cu, Cd, Zn, Pb, Mn) have been found in organisms ranging from bacteria to humans. Performing a phylogenetic analysis of fungal HMA, a remarkable diversity was found (Saitoh et al., 2009). HMAs can be divided into groups A, B and C. Group A is predicted to deliver Cu ions to Cu-containing proteins, while groups B and C are thought to function as cell membrane Cu-efflux pumps. While groups B and C contain fungal-specific HMAs, group A consists of fungal orthologues that have been well conserved in eukaryotes (Saitoh et al., 2009). A genomic assay is needed to determine the occurrence and nature of HMAs in freshwater fungi.

Intracellular changes of macromolecular complexes

Heavy metals elicit a set of fundamental metabolic responses. Stress can lead to the interruption of central metabolic processes, in particular those performed by macromolecular complexes such as spliceosomes. One study reports the effects of stress on the mechanisms of pre-mRNA splicing in ascomycetes (Bond, 2006). Using the knowledge of gene organization and transcriptome analysis in the aquatic chytridiomycete Blastocladiella emersonii, the inhibitory effect of Cd on intron splicing was documented (Georg & Gomes, 2007; Georg et al., 2009). Because this fungus has proteins with Zn-related domains involved in pre-mRNA processing, Cd might inhibit splicing by displacing Zn ions in metalloproteins, including those present in the spliceosome system. Global gene expression analysis of B. emersonii cells exposed to Cd revealed 189 upregulated and 110 downregulated genes (Georg & Gomes, 2007). Four genes encoding for heat shock proteins (HSP) 10, 20, 90 and 100 were upregulated threefold upon exposure to Cd. HSPs are ubiquitous tools for organisms to cope with stress-induced denaturation of cellular protein. Upon Cd exposure, HSP 70 synthesis was induced in a time- and dose-dependent manner in the aquatic hyphomycete H. lugdunensis (Miersch & Grancharov, 2008).

Modulation of Reactive Oxygen Species (ROS)

Several studies have indicated that exposure of fungi to a diverse array of metals elicits oxidative stress (Penninckx, 2002; Pocsi et al., 2004; Krauss et al., 2008). ROS play important roles in the pathways of cellular redox homeostasis based on gene expression and gene product regulation. Low molecular antioxidants [e.g. ascorbate, glutathione (GSH)] participate in redox signalling in a complex manner. Metal-induced formation of ROS together with changes in the cellular redox state elicit signals leading to alterations in the cellular pattern of redox-mediating enzymes and their substrates and cofactors.

Cu is of utmost significance for fungi. The occurrence of two oxidation states Cu(I) and Cu(II) allows the metal to function as a reducing or oxidizing agent in numerous biochemical reactions. This property also makes Cu potentially toxic. Exposure to Cu(II) can catalyze the formation of ROS in some aquatic hyphomycetes (Azevedo et al., 2007). In H. submersus both superoxide dismutase and catalase activity were stimulated under Cu stress, and catalase activity increased with increasing Cu(II) concentration. The magnitude of the increase in catalase activity was more pronounced in H. submersus than in V. elodeae, probably contributing to the higher tolerance of H. submersus to Cu and to its ability to survive in metal-polluted streams, such as the site where the fungus was isolated (Azevedo et al., 2007). In H. lugdunensis, Cu diminished the activity of glutathione reductase (Braha et al., 2007). One reason for decreased glutathione reductase could be a lack of redox equivalents such as NADPH. This effect might explain the inhibition of glucose-6-phosphate dehydrogenase after Cu exposure, limiting the essential production of NADPH as shown for V. elodeae, H. submersus (Azevedo et al., 2007) and H. lugdunensis (Braha et al., 2007).

In contrast, metals without redox-active properties (e.g. Cd and Zn) induce oxidative stress via indirect mechanisms such as interacting with the antioxidant defense system, for example by influencing the GSH pool. However, Cd, which is similar to Zn in many aspects, generally causes toxic effects in fungi (Pocsi et al., 2004; Baudouin-Cornu & Labarre, 2006; Krauss et al., 2008). The level of GSH in H.
lugdunensis was strain specific (Miersch et al., 1997, 2001; Jaeckel et al., 2005b; Braha et al., 2007). Under Cd exposure, GSH level increased drastically in two H. lugdunensis strains (Braha et al., 2007). The increased GSH to GSSG ratio suggests an increase in the GSH levels without drastic changes in GSSG concentrations. Varicosporium elodeae and T. marchalianum responded in a similar manner, while Cu exposure had no effect (Miersch et al., 1997, 2005). An increased production of thiol peptides in different freshwater fungi was found under exposure to Cd (Miersch et al., 1997, 2001; Guimarães-Soares et al., 2007) and Zn/Cu (Guimarães-Soares et al., 2007). In the zygomycete Mucor racemosus, Cd caused a decrease in GSH, but induced GSH-derived phytochelatins (Miersch et al., 2001). With Cd exposure, peroxidase activity increased in two H. lugdunensis strains, while dehydroascorbate reductase and glucose-6-phosphate dehydrogenase declined progressively (Braha et al., 2007). Exposure to Zn did not increase the GSH level (Jaeckel et al., 2005b). As shown for Zn, catalase activity was stimulated in both H. submersus and V. elodeae (Azevedo et al., 2007). The responses of superoxide dismutase in H. submersus and of catalase in V. elodeae were stronger when exposed to Cu/Zn mixtures than to Cu alone, suggesting a synergistic effect of metal mixtures (Azevedo et al., 2007).

Azevedo et al. (2009) described a link between metal-induced oxidative stress and the appearance of programmed cell death in freshwater fungi. Programmed cell death destroys cells that represent a threat to the organism as a whole. It is mediated by a complex intracellular program in freshwater fungi (Azevedo et al., 2009); both Cu and Zn exposure induce apoptotic events. The fungi most tolerant to Zn (V. elodeae) or Cu (H. submersus) exhibited higher levels of programmed cell death markers, suggesting that programmed cell death processes might be linked to fungal resistance/tolerance to metal stress. Exposure of V. elodeae to Cu promoted nuclear morphological alterations, caspase-like activity and DNA strand breaks, but did not lead to detectable intracellular ROS accumulation. Cu exposure of Flagellospora curta did not result in DNA strand breaks. No enhanced caspase-like activity was measured after Zn exposure of F. curta mycelia. The results reflect a high plasticity of the programmed cell death pathways allowing the sacrifice of certain hyphal cells for the benefit of the mycelium as a whole (Richie et al., 2007). The occurrence of programmed cell death in heavy metal-exposed freshwater fungi might be important for their acclimation in metal-polluted waters.

Thiol peptide stress response

Sulfate assimilation and Cd response in fungi are metabolically connected (Vido et al., 2001; Bae & Chen, 2004; Mendoza-Cozatl et al., 2005; Wysocki & Tamás, 2010). Like plants, fungi synthesize GSH from inorganic sulfate via reductive sulfate assimilation (Mendoza-Cozatl et al., 2005; Fig. 3). In H. lugdunensis, Cd causes an upregulation of mRNA for sulfite reductase (P. Nathan et al., unpublished data). Georg & Gomes (2007) described the upregulation of genes responsible for cysteine as well as GSH synthesis in response to Cd stress in the aquatic chytridiomycete B. emersonii. Cysteine, the final product of sulfate assimilation is metabolized to GSH in two consecutive ATP-dependent reactions via γ-glutamylcysteine (Mendoza-Cozatl et al., 2005). The levels of both GSH precursors increased under Cd exposure in H. lugdunensis (G.-J. Krauss et al., unpublished data).

Regarding enhanced synthesis of reduced GSH in Cd-stressed H. lugdunensis, Braha et al. (2007) hypothesized that a substantial proportion of this GSH pool was removed to detoxify the metal by intracellular chelation to bis(glutathionato)Cd complexes (CdGS2). In S. cerevisiae and S. pombe, Cd detoxification depends on binding by glutathione as CdG2 and the sequestration of these complexes into the vacuole mediated by the tonoplast-located ABC transporter ScYCF1 (Li et al., 1997), SpHTM1 (Preveral et al., 2009) and Abc2 (Mendoza-Cozatl et al., 2010, Fig. 3). Such translocation may contribute to the depletion of cytosolic GSH pools causing changes in cellular redox homeostasis. No CdG2 complexes were found in vacuoles of aquatic hyphomycetes. However, in T. marshallianum, Cd was present in vacuolar polyphosphate precipitates (Miersch et al., 2005; Fig. 3).

Phytochelatins differing in length were first isolated from fungi and described as cadystins (Kondo et al., 1984; Murasugi, 2008). They are GSH-derived peptides of the general structure (γ-Glu-Cys)n-C0Gly (Wesenberg et al., 2010) and are synthesized by phytochelatin synthase (PCS; Clemens & Persoh, 2009). However, the synthesis of metal-binding PC is not tied to PCS activity. The fungi S. cerevisiae and Neurospora crassa synthesize only phytochelatin2 (PC2) upon exposure to metal ions (Kneer et al., 1992). Saccharomyces cerevisiae lacks a PCS homologue. In this yeast, vacuolar serine carboxypeptidases are responsible for the synthesis of PC2 (Wünschmann et al., 2007). In H. lugdunensis, 100 μM Cd increased the GSH content and induced the synthesis of one phytochelatin (PC2, Jaeckel et al., 2005a). Because H. lugdunensis shows (1) a Cd-induced PC2 synthesis (Jaeckel et al., 2005a) and (2) does not have a PCS gene (D. Meissner et al. unpublished data), carboxypeptidases might catalyze the reaction in this fungus (Fig. 3). Phytochelatins in other Cd-stressed freshwater hyphomycetes have been postulated, but not identified (Guimarães-Soares et al., 2006). In the zygomycete M. racemosus, Cd caused an increase of the thiol pool with a simultaneous decrease of glutathione and induction of two phytochelatins (PC2, PC3), whereas Cu had no effect (Miersch et al., 2001). In contrast to S. pombe and S. cerevisiae (Kneer et al., 1992;
Ow et al., 1994; Murasugi, 2008), neither intracellular PC–metal complexes nor Cd-GS$_2$- or phytochelatin-encapped CdS crystallites have so far been detected in freshwater fungi.

Cd exposure at a concentration of 50 μM raised cellular soluble sulfide levels in *T. marchalianum* strains between three- and sevenfold, while Cu and Zn had no effect (Miersch et al., 2005). Sulfide was not detectable in the culture (Miersch et al., 2005). Probably due to the upregulation of sulfite reductase in *H. lugdunensis*, high concentrations of soluble sulfide were found in Cd-exposed cells (Nathan et al., unpublished data). *Heliscus lugdunensis* seems to be able to detoxify Cd extracellularly by the formation of CdS crystallites, probably by initiation of a matrix-mediated mineralization process (J. Miersch et al., unpublished data, Fig. 3).

*Heliscus lugdunensis* seems to be the first organism described that under Cd stress synthesized a novel small metallothionein (MT) MT1-NECLU together with a PC (Jaeckel et al., 2005a, Fig. 3). This was accompanied by increased GSH levels (Jaeckel et al., 2005a). Metallothioneins are gene-encoded, Cys-rich metal-binding proteins of low molecular mass. They have been found in fungi and representatives of other kingdoms (Blindauer & Leszczyszyn, 2010). Metallothioneins chelate heavy metals through clusters of thiolate bonds. Fungal metallothioneins are also involved in other intracellular reactions, for example redox regulation, and developmental processes (Singh & Ashby, 1999; Clemens & Simm, 2003; Tucker et al., 2004). Generally, fungal metallothioneins are induced by Cu. Thus, the ascomycete *N. crassa* synthesizes a small metallothionein (Lerch, 1991; Cobine et al., 2004; Kumar et al., 2005), which belongs to family 8 of the metallothionein classes (http://www.expasy.ch/cgi-bin/lists/metallo.txt). Small Cu-binding cysteine-rich (30%) metallothioneins were isolated from the ectomycorrhizal fungi *Laccaria laccata* and *Paxillus involutus*, and characterized by their molecular masses and spectroscopic features (Howe et al., 1997). In addition to Cu, Cd induced the expression of a metallothionein gene in the fungus *Colletotrichum gloeosporioides* (Hwang & Kolattukudy, 1995). *Candida glabrata* produces a metallothionein in response to high concentrations of Cu, but under Cd exposition it mainly synthesizes phytochelatins (Mehra et al., 1988, 1989).

The synthesis of MT1_NECLU (molecular mass 2330 Da) from *H. lugdunensis* can be induced by Cd (Jaeckel et al., 2005a), but not by other heavy metals (G.-J. Krauss et al., unpublished data). Due to similarities in amino acid sequence of other small metallothioneins from the ascomycete *N. crassa* (Münger et al., 1985) and the basidiomycete *Agaricus bisporus* (Münger & Lerch, 1985), the novel metallothionein can be categorized as a member of family 8 of the metallothionein classes (Jaeckel et al., 2005a). The Cd metallated metallothionein has a loop-like structure
(Meissner et al., unpublished data). In another H. lugdunensis strain isolated from a highly polluted habitat, Cd induced another small metallothionein, which slightly differs from MT1_NECLU (Meissner et al., unpublished data). This could indicate that the two H. lugdunensis strains represent distinct ecotypes, which is supported by other metabolic differences (Braha et al., 2007). Due to the lack of structure identification, the proposed occurrence of high molecular metallothioneins in metal-exposed freshwater fungi remains speculative (Guimarães-Soares et al., 2006; Miersch & Grancharov, 2008).

Despite growing knowledge regarding responses to high amounts of essential metals or to toxic metals and their detoxification, information on metal sensing, intracellular signal transduction and metabolic changes in fungal cells is limited. More details are needed for understanding the impact of these compounds on the metabolic network (Wesenberg et al., 2010). Biochemical single-cell analysis performed by laser microdissection after harvesting the cells might allow the description of the initial metabolic impact of metals on hyphal tips, but needs a chamber allowing the growth of single filaments and cutting hyphal cells in a standardized manner (B. Leyh et al., unpublished data).

**Effects of xenobiotics on freshwater fungi**

Several studies have addressed the effects of organic pollutants on aquatic hyphomycetes in the field and the laboratory, with pesticides being most frequently investigated among pollutants with defined chemical structures. Here, we briefly present only effects of major relevance because among pollutants with defined chemical structures. Here, toxicity, with pesticides being most frequently investigated zenyne, styrene and toluene can be used as sole sources of carbon and energy for growth of filamentous fungi (Gerasimova et al., 1975; Murphy & Perry, 1984; Fedorak & Westlake, 1986; Weber et al., 1995; Qi & Moe, 2002) and yeasts (Jigami et al., 1974; Cox et al., 1993, 1996; Craft et al., 2003). Mitosporic fungi (Hofrichter & Scheibner, 1993; Jones et al., 1993, 1994; Garcia-Peña, 2007) and yeasts (Middelhoven & Spaaij, 1997; Middelhoven et al., 2004) are able to use phenol, o-cresol, m-cresol, p-cresol and 4-ethylphenol as growth substrates.

However, apart from the aforementioned examples and in contrast to bacteria, fungi cometabolize the majority of aromatic environmental pollutants, resulting in the formation of organic biotransformation products or in mineralization to CO₂ (Prenafeta-Boldú et al., 2006) (Fig. 4). The initial intracellular oxidative attack on organic pollutants commonly involves hydroxylation, resulting from the activation of dioxygen and the insertion of one oxygen atom into the substrate. Cytochrome P450 monoxygenase systems are frequently responsible for such reactions in both ligninolytic and nonligninolytic terrestrial fungi (Cerniglia & Sutherland, 2001; Matsuzaki & Wariishi, 2004; Hiratsuka et al., 2005; Prenafeta-Boldú et al., 2006; Yadav et al., 2006) (Fig. 4). Evidence for the involvement of cytochrome P450
monooxygenases in the metabolism of xenobiotics by freshwater fungi has not yet been provided. Nevertheless, cytochrome P450 reactions can be inferred from hydroxylated metabolites produced during intracellular biotransformation of environmentally relevant micropollutants such as the endocrine-disrupting chemical t-NP (Corvini et al., 2006) and the polycyclic musk compounds HHCB and AHTN (Martin et al., 2007) by aquatic hyphomycetes and other mitosporic freshwater isolates (Fig. 4). Primary fungal pollutant oxidation is often followed by additional oxidation steps, leading to the excretion of biotransformation products at various stages of oxidation. Intracellular t-NP metabolism by the aquatic hyphomycete *C. aquatica*, a species frequently observed in rivers and streams (Baldy et al., 2002; Nikolcheva et al., 2003), and another mitosporic freshwater isolate oxidation of the differently branched side chains of the nonylphenol isomers contained in t-NP, whereas their phenolic moieties remained unchanged (Junghans et al., 2005; Martin et al., 2009). Metabolites of t-NP detected in *C. aquatica* indicated nonyl chain hydroxylation, further oxidation into keto or aldehyde compounds and the subsequent formation of carboxylic acid derivatives (Martin et al., 2009).

Pollutant attack by aquatic hyphomycetes via reductive reactions is suggested by the reported formation of the metabolite 1,1-dichloro-2,2-bis(4′-chlorophenyl)ethane (DDD) from DDT. Within 330 h, *H. submersus* converted DDT into DDD at 27% and into 1,1-dichloro-2,2-bis(4′-chlorophenyl)ethylene (DDE) at 8%, whereas *C. aquatica* transformed 6% of DDT into these metabolites (Hodkinson, 1976). Reductive dechlorination is well known from anaerobic bacteria, but has also been reported for terrestrial fungi (Reddy & Gold, 1999).

Phase II reactions such as methylation or conjugate formation with glucose, xylose, glucuronic acid or sulfate involve polar functional groups of pollutant metabolites or of the pollutants themselves, leading to the excretion of products that are more water-soluble than their parent compounds and usually not amenable to further metabolism (Cerniglia et al., 1992; Cerniglia & Sutherland, 2001; Gadd, 2004; Zhang et al., 2004; Prenafeta-Boldú et al., 2006) (Fig. 4). Conjugate formation is well known from the metabolism of pollutants by terrestrial fungi (Reddy et al., 1997; Hundt et al., 2000; Cerniglia & Sutherland, 2001; Hammer et al., 2001; Gesell et al., 2004), but has also been reported for aquatic hyphomycetes (Augustin et al.,

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**Fig. 4.** Major reactions and enzymes involved in fungal metabolism of xenobiotics. Reactions and enzymes described for freshwater fungi according to Augustin et al. (2006), Junghans et al. (2005) and Martin et al. (2007, 2009) are highlighted in blue and italics. Figure modified from Schlosser et al. (2008).
tic hyphomycetes and other mitosporic freshwater isolates transformed the pesticide and polyyclic aromatic hydrocarbon (PAH) metabolite 1-naphthol to 1,4-naphthoquinone, 1-methoxynaphthalene and 1-naphthylsulfate, indicating the involvement of intracellular phase II enzymes such as methyl- and sulfotransferases (Augustin et al., 2006). *Heliscus lugdunensis* was consistently found among the top-ranked fungal species identified at different polluted sites related to Cu shale mining (Krauss et al., 2001; Sridhar et al., 2005), which suggests a role of aquatic hyphomycetes in metabolizing polyaromatic hydrocarbon byproducts of Cu shale smelting processes. The formation of methyl conjugates from t-NP and HHCB biotransformation metabolites was implicated in *C. aquatica* (Martin et al., 2007, 2009).

Terrestrial wood- and soil litter-decomposing white-rot basidiomycetes possess an outstanding potential for mineralizing many xenobiotic compounds to CO$_2$. This is attributed to a complex and nonspecific cometabolic degradation mechanism also active during lignin degradation (Bumpus et al., 1985; Field et al., 1993; Hammel, 1995; Reddy, 1995; Sack et al., 1997; Scheibner et al., 1997; Mester & Tien, 2000; Cerniglia & Sutherland, 2001; Steffen et al., 2002). Extracellular radical-generating enzymes such as laccases (EC 1.10.3.2) and certain lignin-modifying peroxidases are involved in lignin degradation (Hofrichter, 2002; Martínez, 2002; Baldrian, 2006; Hammel & Cullen, 2008; Ruiz-Duenas et al., 2009; Giardina et al., 2010), but also oxidatively attack many xenobiotics, for example PAHs (Johannes & Marchezczyk, 2000; Hofrichter, 2002), chlorophenols (Hofrichter et al., 1998; Hofrichter, 2002), nitroaromatic compounds (Hofrichter et al., 1998; Hofrichter, 2002), dyes (Heinfling et al., 1998; Mayer & Staples, 2000; Wesenberg et al., 2003; Camarero et al., 2005), herbicides, pesticides (Mayer & Staples, 2002; Davila-Vazquez et al., 2005) and endocrine disruptors (Tsutsumi et al., 2001; Dubroca et al., 2005; Cabana et al., 2007). Manganese peroxidase (EC 1.11.1.13) can even cleave aromatic rings and catalyze the mineralization of xenobiotics to CO$_2$ in the absence of cells (Hofrichter et al., 1998; Hofrichter, 2002). Concerning the occurrence of lignin-modifying enzymes in fungi from aquatic environments, laccases have repeatedly been demonstrated in aquatic hyphomycetes and other mitosporic freshwater isolates (Abdel-Raheem & Ali, 2004; Junghanns et al., 2005, 2009; Martin et al., 2007, 2009). Sound evidence for lignin-modifying peroxidases in aquatic fungi at this point is limited to marine fungi (Raghukumar et al., 1999, 2008). Besides intracellular metabolism of t-NP, the biotransformation of this compound by *C. aquatica* and another freshwater ascomycete anamorph involved concomitant extracellular t-NP oxidation by laccase, leading to the formation of oxidative coupling products in the range of di- to pentamers (Junghanns et al., 2005; Martin et al., 2009) (Fig. 4). In *C. aquatica*, evidence for a nonspecific oxidation of the various nonylphenol isomers contained in t-NP by laccase was provided. Such reactions contrast with the selective degradation of t-NP isomers by bacteria and could prevent the enrichment of highly estrogenic isomers in remaining t-NP. In contrast to laccase reactions, intracellular fungal biotransformation was much more isomer-specific and caused a significant shift in the isomeric composition of remaining t-NP. As a result, certain t-NP constituents related to more estrogenic isomers were less efficiently degraded than others (Martin et al., 2009). Likewise, the polycyclic musk fragrances HHCB and AHTN were oxidized by extracellular laccases from aquatic hyphomycetes and mitosporic freshwater isolates, leading to the identification of the environmental metabolite HHCB-lactone as a product of HHCB laccase oxidation (Martin et al., 2007). As for t-NP, these results suggest the involvement of both extra-(laccase) and intracellular reactions in the biotransformation of HHCB and AHTN by freshwater fungi (Martin et al., 2007) (Fig. 4). More recently, aquatic hyphomycetes and other mitosporic freshwater isolates were shown to metabolize several anthraquinone and azo dyes typical of textile industry effluents (Junghanns et al., 2008). The demonstrated ability of laccases from freshwater ascomycete anamorphs to oxidize synthetic dyes (Junghanns et al., 2009) may indicate their involvement in dye biotransformation.

Evidence for the mineralization of xenobiotic organic pollutants in freshwater fungi has not yet been provided (Fig. 4). The further metabolic fates of metabolites formed from various xenobiotics by aquatic hyphomycetes and other mitosporic freshwater isolates (Hodkinson, 1976; Augustin et al., 2006; Martin et al., 2007, 2009; Junghanns et al., 2008) remain open.

The demonstrated capability of terrestrial species (Bumpus et al., 1985; Reddy, 1995; Pointing, 2001) and of freshwater isolates (Junghanns et al., 2005, 2008; Martin et al., 2007) to metabolize representatives of more than one particular chemical class of xenobiotics considerably differs from bacterial degradation, which generally seems to be much more species- and compound-specific. In addition to aquatic hyphomycetes occurring predominantly in streams, ubiquitous mitosporic fungi and zygomycetes such as *Penicillium, Aspergillus, Fusarium, Paecilomyces, Mucor* and *Rhizopus* spp., as well as yeasts, can be found in freshwater environments (Niewolak, 1975; Tóthová, 1999; Amaral Zettler et al., 2002). Members of these genera degrade several organic environmental pollutants such as PAHs (Cerniglia & Sutherland, 2001; Verdin et al., 2004), fungicides (Geisel et al., 2001) and chlorophenols (Hofrichter et al., 1994), suggesting that they may also contribute to the degradation of other organic contaminants in aquatic environments.
Outlook

The broad concepts of the taxonomy and ecology of aquatic hyphomycetes were worked out several decades ago, and have largely been confirmed, even though many details remain to be filled in. Major new insights concerning their phylogeny and biochemistry, however, can be expected from the increased use of molecular methods (Bärlocher, 2010). These new approaches have already revealed the consistent presence of other fungal groups and are shedding light on how aquatic hyphomycetes and other microorganisms deal with heavy metals and xenobiotics. The responses of this vital group of stream xenobiotics to these and other anthropogenic stresses is one of the most pressing concerns today. A first step may be identifying species or communities, whose presence/absence indicates a specified stress. Solé et al. (2008b) studied aquatic hyphomycete communities in rivers and aquifers in the Mansfeld region (Central Germany), which has long been the site of intensive mining and numerous chemical industries. Eleven sites were investigated, with between 1 and 13 species of aquatic hyphomycetes (which is considerably lower than what would be expected in pristine streams). Within this set, stress factors such as heavy metals, sulfate, nitrate and low oxygen concentrations were connected to reduced fungal diversity and biomass. Redundancy analysis indicated that 86.2% of the changes in fungal community structure was due to variations in water chemistry. The observed species could be divided into two groups based on their correlation with DOC/TOC (dissolved organic carbon/total organic carbon), Cl− and Eh. The first group (T. marchalianum, T. setigerum, Cylindrocarpon sp., Tricladium angulatum, Lemonniera centrostrophaera, Alatospora acuminata and Alatospora flagellata) were not correlated, while the second group (Anguillolspora longissima, Clavatospora longibrachiata, C. aquatica, Flagellospora curvula, H. lugdunensis, Tumularia aquatica and Lemonniera aquatica) were strongly negatively correlated to these parameters. Clearly, more analyses incorporating other regions are required to confirm the consistency of these associations. The study nevertheless points to the potential use of aquatic hyphomycete communities as bioindicators of anthropogenic stresses. This approach will be useful, but it does not provide much insight into mechanisms that allow certain strains or species to persist, maybe even flourish, in the face of severe anthropogenic stress. It is unlikely that they are based on ‘core’ enzymatic properties, such as the ability to use plant polysaccharides, which seems to be nearly universal among aquatic hyphomycetes (Chamier, 1985). More likely they are based on ‘keystone’ genes (Fig. 1) that allow, for example, sequestration and detoxification of heavy metals or other toxins. If this is the case, screening aquatic fungal communities for such genes (PCR with specific primers) or their products (RT-PCR to produce mRNA, or proteome analyses) may facilitate predictions of their resilience when challenged by selected anthropogenic stressors. Of these, factors associated with climate change have attracted a huge amount of interest. A commonly expected response of affected species is a migration toward greater altitude and latitude. Ingold once claimed that if he were ‘transported to a part of the world without knowledge of its locality’, he could ‘make a very rough guess as to its latitude by examining the spora of a suitable stream’ (Money, 2010). If the aquatic hyphomycete species in a given region respond in a similar manner, spore samples would provide an easy and cheap approach to evaluate the progress of climate change and its impact on stream communities.

References


