Chapter 9
Linking Microbial Decomposer Diversity to Plant Litter Decomposition and Associated Processes in Streams

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Abstract The physiology, biochemistry and diversity of aquatic microbial decomposers have been largely investigated in low-order streams. However, some aspects still need further attention to better ascertain how microbial decomposer diversity can ensure ecosystem processes and services, particularly under the challenges posed by global environmental change. Aquatic microbial decomposers play a key role in processing plant litter in streams by degrading the most recalcitrant compounds and facilitating nutrient and energy transfer to higher trophic levels. Among microbial decomposers, fungi, particularly aquatic hyphomycetes, play a fundamental role at the early stages of plant litter decomposition, while the relevance of bacteria increases at the late stage of the decomposition. High-throughput sequencing and metagenomic techniques open new avenues towards a more comprehensive understanding of microbial decomposer ecology. This chapter provides a general overview

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of aquatic microbial diversity and activity on decomposing plant litter. Attention will be paid to the relationships between microbial diversity and their ecological functions under the major threats posed by the ongoing global environmental change to provide the response patterns of microbial decomposers to maintain nutrient and energy fluxes in streams.

9.1 An Introduction to Microbial Decomposers in Freshwaters

In forest streams, filamentous fungi, mainly aquatic hyphomycetes, are the key microbial decomposers of plant litter entering streams from the riparian vegetation (Bärlocher, 1992; Gessner & Chauvet, 1994; Hieber & Gessner, 2002; Pascoal & Cássio, 2004). The role of bacteria becomes evident once leaf litter has been partially broken down by fungi (Baldy et al., 1995; Pascoal et al., 2005). The diversity of aquatic hyphomycetes associated with decomposing litter evaluated from spore morphology is well documented, particularly in Europe, Asia and North America (Duarte et al., 2016), but the identity of bacterial plant litter decomposers has been rarely investigated either through cultivable taxa or by counting of morphotypes (Baldy et al., 2002; Suberkropp & Klug, 1976). Moreover, many microbial decomposers are not cultivable, impairing their identification (Bärlocher, 2007, 2010). On the contrary, molecular methods do not depend on the reproductive status of microbes or pure cultures and have the potential to holistically assess microbial decomposer diversity (Bärlocher, 2007, 2010). Molecular methods are being developed allowing disentangling the contribution of each species to the overall community composition. Combining this information with microbial activity measurements might open new research avenues for disentangling the respective roles of different microbial taxa in the decomposition process and to further clarify the missing links between microbial decomposers diversity and the processes they drive in freshwater ecosystems. Indeed, microbes play a key role in biogeochemical cycles, ensuring several ecosystem functions and services, but the impacts of biodiversity losses in microbial communities associated with decomposing plant detritus in freshwaters have been overlooked.

9.2 Profiling Microbial Decomposers to Unravel Microbial Diversity and Functions in Freshwaters

A major challenge to understand the role of microbial decomposers in ecosystem processes is to accurately detect their identity and activity in trophic relationships. The development of molecular approaches either to identify species or their functions in decomposing leaves has had a profound impact on unraveling the diversity

(Baschien et al., 2013; Duarte et al., 2015; Seena et al., 2019) and ecology of microbial decomposers (Andrade et al., 2016; Fernandes et al., 2011; Hayer et al., 2016).

9.2.1 Identification of Aquatic Hyphomycetes

Species identification and quantification is a crucial step in biodiversity assessment. Traditionally, aquatic hyphomycetes are identified microscopically based on the size and shape of asexually produced spores (conidia), typically tetraradiate or filiform (Gulis et al., 2020) (Fig. 9.1). Aquatic hyphomycetes conidia can be found in foam (Pascoal et al., 2005; Sridhar & Bärlocher, 1994), suspended in stream water (Bärlocher, 2000; Bärlocher & Graça, 2002; Pascoal et al., 2005), or released from decomposing leaves (Fernandes et al., 2015; Pascoal & Cássio, 2004). However, the identification of aquatic hyphomycetes based on conidium morphology has drawbacks: some conidia are morphologically similar demanding taxonomic expertise and fungal sporulation is achieved only under particular conditions (Bärlocher, 2007). DNA barcoding offers an opportunity to identify aquatic hyphomycetes accurately. This technique uses short DNA sequences linked to individual species (Letourneau et al., 2010; Seena et al., 2010). The internal transcribed spacer (ITS) gene region is considered to be the most relevant barcode for identifying aquatic hyphomycetes (Seena et al., 2010). Approximately 26% of the described aquatic hyphomycetes species are connected to their ITS barcode in databases (Duarte et al., 2014). However, the major hurdle in DNA barcoding of aquatic hyphomycetes is the low number of DNA sequences derived from voucher specimens thereby lacking reliable sequences in the public database (e.g., National Center for Biotechnology Information—NCBI 2009–2019), which often leads to misidentifications. In a recent study to identify fungi through DNA barcoding using the basic GenBank local alignment search tool program (BLAST), around 30% error was detected in linking the number of ITS sequences to taxon (Hofstetter et al., 2019). Nevertheless, DNA based approaches open opportunities to circumvent the current constrains when assessing aquatic hyphomycete diversity in streams.

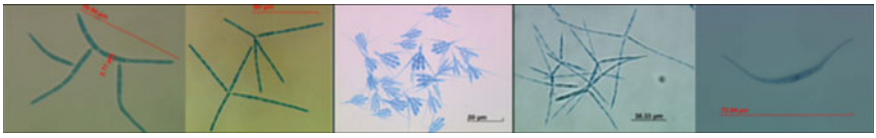

Fig. 9.1 Conidia of aquatic hyphomycete species. From left to right: *Varicosporium elodeae*, *Geniculospora grandis*, *Tetracladium setigerum, Articulospora tetracladia* and *Lunulospora curvula*

9.2.2 Genetic diversity

Genetic diversity, which includes inter and intraspecific variability, is a critical feature of natural populations and is considered crucial for ensuring ecosystem functions and stability in a changing environment (Duarte et al., 2019; Fernandes et al., 2011; Seena et al., 2019). Genetic diversity was first explored within the population of two dominant sporulating aquatic hyphomycete species, *Neonectria lugdunensis* (former *Heliscus lugdunensis*) and *Articulospora tetracladia*, suggesting a high degree of genetic variation within the strains from a single foam patch as determined by random amplified polymorphic DNA (RAPD) analyses (Peláez et al., 1996). Later, the population genetic structure of *Tetrachaetum elegans,* a common aquatic hyphomycete found during the initial stages of leaf litter decomposition, was explored by amplified fragment length polymorphism (AFLP) multilocus fingerprints; significant genetic differentiation of this fungus was found within a limited geographic area (Laitung et al., 2004). Furthermore, genetic variation of *T. elegans,* evaluated via RAPD analysis, suggested substrate preferences among the genotypes of aquatic hyphomycetes (Charcosset & Gardes, 1999). Another aquatic fungus, *Tetracladium marchalianum*, was genotyped at eight microsatellite loci and genotypic diversity of this fungus was found to be very large and well connected at local scales (Anderson & Shearer, 2011). Thus, it can be speculated that genetic differentiation is mainly controlled by the distances between streams and dispersal barriers. Conversely, genotypes of *A. tetracladia*, discriminated through ITS barcodes, were generally geographically widespread regardless of sampling time, sites or substrates (Seena et al., 2012). This suggests that the drivers that lead to intraspecific diversity of aquatic fungi and the links to their ecological functions are largely unknown.

9.2.3 Phylogeny and Diversity

Because morphological variations of conidia evolved as a consequence of selective pressure, they do not relate to their phylogeny (Belliveau & Bärlocher, 2005; Seena et al., 2018). Therefore, the strategy to investigate the phylogenetic relationship between microbes is based on DNA sequence analyses of stable markers. Nuclear rDNA sections are reputed to be optimal for the study of fungal phylogeny, e.g., 18S rDNA, ITS and 28S rDNA (Bärlocher, 2007). However, to improve the phylogenetic signal, multi loci comparisons are advocated (Duarte et al., 2013). Phylogenetic studies of aquatic hyphomycetes using DNA sequences date back almost two decades (Nikolcheva & Bärlocher, 2002): complete sequencing of 18S rDNA regions of 5 representative species of the genus *Tetracladium* supported that they are part of a monophyletic group, as suggested by morphology-based taxonomy. The comparison based on 18S rDNA and other rDNA sequences confirmed the polyphyletic origin of aquatic hyphomycetes (Baschien et al., 2006, 2013; Belliveau & Bärlocher, 2005; Campbell et al., 2006, 2009). Moreover, phylogenetic studies allow the establishment

of anamorphs and teleomorphs connections of aquatic hyphomycetes (e.g., anamorph *Jaculispora submersa* and teleomorph *Classicula fluitans*; Bauer et al., 2003), provide evidence for their occurrence in several ecological niches (Seena & Monroy, 2016) and show relatives of terrestrial origin (Baschien et al., 2013).

9.2.4 Leaf Litter Associated Microbial Communities

Molecular characterization of the fungal community on decomposing leaves in streams began with terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) targeting a section of 18S rRNA (Nikolcheva et al., 2003). It was concluded that species richness and community evenness decreased with the advancement of litter decomposition, probably due to an increase in substrates homogeneity and a decrease in the nutritional value of the substrate. Moreover, ITS primers with enhanced specificity for particular fungal groups, revealed strikingly high fungal diversity on leaf litter compared to that found based on spore morphology (Nikolcheva & Bärlocher, 2004). Additionally, these analyses showed a clear fungal succession pattern during plant litter decomposition: the role of terrestrial fungi tends to decrease after few days of plant litter entering in streams, while the contribution of aquatic hyphomycetes progressively increases.

The diversity of microbial decomposers in specific habitats and the contribution of different microbial groups to plant litter decomposition have been also addressed. Clone libraries were successfully used to determine fungal diversity in hyporheic zones (Bärlocher et al., 2008) and on decomposing leaf litter (Seena et al., 2008). The relative amount of DNA of Archaea, Bacteria and Fungi during decomposition of leaf litter was assessed for the first-time using quantitative polymerase chain reaction (qPCR) (Manerkar et al., 2008). Moreover, Das and collaborators (2007) were able to uncover the phylotypes associated with actinomycetes on decomposing leaves. The dynamics of yeast populations associated with decomposing leaves have rarely been investigated (but see Sampaio et al., 2004, 2007). Further research has addressed shifts in the structure of fungal, bacterial and ciliate communities associated with plant litter of different species alone or in mixtures using DGGE (Fernandes et al., 2013), but most studies have focused on assessing the shifts in fungal and bacterial communities under exposure to several anthropogenic stressors (e.g., metals, Duarte et al., 2008; eutrophication, Duarte et al., 2009; nanoparticles, Pradhan et al., 2011; Chapters 16, 17, 18).

More recently, metabarcoding, an outcome of the fusion of DNA barcoding with high-throughput or next-generation sequencing (HTS or NGS), has become one of the most relevant tools in biodiversity assessment. By using this method, the genetic diversity of microbial communities can be acquired at a high-resolution scale (e.g., Duarte et al., 2015; Seena et al., 2019a). Pyrosequencing was used to characterize fungal communities on leaves and particulate organic matter by targeting the 18S ribosomal gene region (Wurzbacher et al., 2015) or the ITS gene region (Duarte et al., 2015). Both studies revealed a very high fungal diversity even under eutrophication

(Duarte et al., 2015). Further, Duarte et al. (2017) suggested that larger leaf areas tended to harbor a more diverse and active fungal community, as revealed by Illumina MiSeq analysis based on ITS region from RNA.

9.2.5 Microbial Biomass Accrual and Reproduction

Traditionally, fungal activity has been assessed by measuring biomass build-up and reproduction on decomposing leaves. Fungal biomass on leaf litter is difficult to determine since fungal hyphae penetrate the substrate they are decomposing. To overcome this problem, ergosterol, a fungal membrane constituent (not present in bacteria or plant material) that suffers rapid oxidation after cell death, has been used as a measure of living fungal biomass on leaves (Gessner & Newell, 2002). However, some studies showed that ergosterol could persist at appreciable concentrations and for a considerable time in the absence of living fungi (Mille-Lindblom et al., 2004). Moreover, the mycelial ergosterol concentration can vary with the fungal species (Gessner & Chauvet, 1993), their nutritional status (Charcosset & Chauvet, 2001), growth phase (Barajas-Aceves et al., 2002) and/or the presence of stressors (e.g., fungicide zineb, Barajas-Aceves et al., 2002; fungicide tebuconazole, Baudy et al., 2020). In addition, ergosterol is unable to distinguish individual species biomass in a complex community. Despite these limitations, ergosterol is still the most widely used fungal biomass indicator today (e.g., Bergmann & Graça, 2020; Pimentão et al., 2020).

In aquatic hyphomycetes, a significant proportion of carbon is used in the formation of conidia, which are released from fungi on decomposing leaves (Gessner & Chauvet, 1997; Suberkropp, 1991). Sporulation by aquatic hyphomycetes can be induced by incubating pure cultures or naturally-colonized plant substrates in deionized water or filtered stream water under aeration (Bärlocher, 2020). By identifying and counting these conidia, sporulation rates can be estimated, and so the reproductive potential of individual fungal species (Bärlocher, 1982, 2009). Fungal reproduction is very sensitive to a panoply of abiotic and biotic factors, such as nutrient concentration in water (Abelho & Graça, 2006; Gulis & Suberkropp, 2004), temperature (Chauvet & Suberkropp, 1998; Fernandes et al., 2009; Suberkropp, 1984), pollutants (Pereira et al., 2016; Pradhan et al., 2011; Sridhar & Bärlocher, 2011), oxygen concentration (Medeiros et al., 2009), current velocity (Ferreira & Graça, 2006), litter quality (Fernandes et al., 2012; Pérez et al., 2014), or fungal species competitive interactions (Treton et al., 2004). Fungal reproduction is frequently one of the most sensitive parameters to stressors (e.g., metals, Duarte et al., 2008; eutrophication, Duarte et al., 2009; nanomaterials, Pradhan et al., 2011), making aquatic hyphomycetes potential bioindicators of anthropogenic stress (Solé et al., 2008).

Since bacteria are also important players in the decomposition of plant litter in streams and rivers (Baldy et al., 1995; Duarte et al., 2010), it is important to accurately assess their abundance and biomass. The commonly used approach to obtain such estimates is to detach bacterial from plant litter by sonication, pass the bacterial

suspension through a membrane filter, stain the trapped cells with a fluorescent dye and count them under an epifluorescence microscope (Buesing & Gessner, 2020). This technique presents limitations, namely observer bias, requiring strictly standardized counting procedures and thorough cross-calibration among individuals to ensure reproducible results, and it is extremely time-consuming (Frossard et al., 2016). An alternative to epifluorescence microscopy is flow cytometry (Frossard et al., 2016). This technique has been successfully used to assess bacterial biomass associated with decomposing plant litter after chronic exposure to silver nanoparticles (Batista et al., 2020; Tlili et al., 2017) and to compare the effects of litter quality and litter standing stocks on microbial biomass associated with decomposing litter (Frossard et al., 2013). Nevertheless, information on cell size and shape, which can be obtained by epifluorescence microscopy, is mostly lost when using flow cytometry (Frossard et al., 2016).

A limitation of the above described methods to asses microbial biomass accrual is the difficulty in discriminating between living and non-living microbial biomass. A more dynamic measure of microbial activity can be given by estimating fungal or bacterial productivity, from incorporation rates of radioactive labelled acetate or leucine into ergosterol or protein, respectively (Baldy et al., 1995; Pascoal & Cássio, 2004; Suberkropp et al., 2020), that reflect the specific fungal or bacterial growth rate on leaf litter. This has permitted us to conclude that fungal production represents more than 90% of the total microbial production (Baldy et al., 1995; Pascoal & Cássio, 2004), supporting a major role of fungi in leaf litter decomposition in streams, at least during the first stages of the process.

ATP is present in living cells and disappears rapidly in dead cells. The ease of ATP measurement has fostered its use as an indicator of living and active microbial biomass associated with decomposing leaves in streams (Abelho, 2020, 2009; Sales et al., 2015; Suberkropp, 1991). A limitation of this technique is the inability to discriminate between microbial communities (e.g., fungi, bacteria, protists).

9.2.6 Catabolic Reactions and Enzymatic Activity

Community respiration is a measure of biological activity, reflecting the microbial use of organic matter and, therefore, the functional significance of microbes in decomposition (Graça & Abelho, 2020). Respiration rates of microorganisms associated with decomposing leaves are generally determined by measuring oxygen consumption (e.g., Carlisle & Clements, 2005; Stelzer et al., 2003). Alternatively, microbial respiration can be measured using the MicroResp™ method (Tlili et al., 2017), a colorimetric assay based on the color change of a pH indicator dye caused by the release of CO_2 by heterotrophic communities.

Another measure of microbial activity on plant litter can be obtained by determining extracellular enzymatic activities (Romaní et al., 2006). This can be done by incubating decomposing plant litter with fluorescent-linked artificial substrates, specific for each enzyme (Romaní et al., 2006). In a study comparing growth and

patterns of degradative enzymes expressed by communities of bacteria and fungi grown separately and in coexistence, enzyme activities were in general low when bacteria grew alone, and the activity of key enzymes in the degradation of lignin and cellulose was undetectable, while fungi growing alone had a high capacity for the decomposition of lignin, cellulose and hemicelluloses (Romaní et al., 2006). Most studies, however, measure the overall enzyme activity that does not discriminate fungal and bacterial contribution (Artigas et al., 2012; Mora-Gómez et al., 2020).

9.2.7 Discriminating Individual Species Performances Within Communities

Discriminating the growth of individual fungal or bacterial species within a community is still a challenge but is critical to better understand the role of microbial decomposers diversity in maintaining ecological processes. Monoclonal antibodies were used in several assays, such as the enzyme-linked immunosorbent assay (ELISA), which allows the identification and quantification of mycelium, and immunofluorescence (IMF) for visualization of mycelium on leaf material (Bermingham et al., 1995, 2001). This allowed the identification and biomass quantification of *Anguillospora longissima*, *Alatospora acuminata*, *T. marchalianum* and *N. lugdunensis* (Bermingham et al., 1995, 2001).

Fluorescent in situ hybridization (FISH) can be an alternative to monoclonal antibodies. It uses short DNA sequences provided with a fluorescent tag complementary to the *taxon* of interest (Baschien et al., 2001; McArthur et al., 2001). However, the autofluorescence of hyphae and colonized substrates, and the weak probe-conferred signals due to low probe permeabilization may lead to confounding results (Baschien, 2003).

DNA-based quantitative stable isotope probing (qSIP) can identify growing microorganisms in environmental samples (Hayer et al., 2016; Hungate et al., 2015). Briefly, organisms are exposed to an isotopically-labelled substrate and those that assimilate the substrate incorporate the isotope into their DNA, increasing their buoyant density (Hayer et al., 2016). The heavy DNA can be separated from non-labelled DNA through isopycnic centrifugation and analyzed, using metabarcoding and qPCR, to identify growing microorganisms (Hayer et al., 2016). Using this technique, Hayer and collaborators (2016) showed that a large proportion of the bacterial taxa associated with decomposing leaf litter grew slowly, and several less abundant taxa were highly frequent, indicating that rare organisms may be important for the decomposition of leaf litter in streams.

The relative intensity of each band (phylotype) in DGGE gels can give semi-quantitative estimates of the relative biomass of each fungal species within the community (Nikolcheva & Bärlocher, 2005). However, caution is needed since techniques relying on PCR like DGGE, can be biased because of preferential amplification (Kanagawa, 2003). PCR can be limited by inhibitors of the polymerase reaction,

reagent limitation, or accumulation of pyrophosphate molecules, being the reaction no longer at an exponential rate and so generating more products in some reactions than in others (Ginzinger, 2002).

The qPCR technique allows measurements of the amount of PCR product when the reaction is still at the exponential phase, by determining a fluorescence signal threshold at which all samples can be compared (Ginzinger, 2002). The fractional number of PCR cycles required to generate enough fluorescent signal to reach the threshold (Ct value) is directly proportionate to the amount of starting template (Ginzinger, 2002), allowing accurate quantification of species DNA. The qPCR was successfully used to discriminate the relative abundance of specific microbial groups, Archaea, Bacteria, and Fungi on leaves decomposing in streams (Manerkar et al., 2008) or bacterial and archaeal ammonia oxidizers and nitrogen-fixing bacteria in leaf and sediment samples (Rico et al., 2014). Also, qPCR allowed explaining the putative mechanisms underlying biodiversity effects on leaf decomposition under stress by discriminating the contribution of different aquatic hyphomycete ecotypes to the total fungal biomass produced in multicultures (Fernandes et al., 2011). This approach allowed evidencing the importance of species traits in modulating biodiversity effects under stress (Fernandes et al., 2011). The expression profiles of the functional marker gene also helped to discriminate ecotypes from polluted and nonpolluted habitats (Seena et al., 2020).

Recently, TaqMan® probe-based qPCR assays targeting aquatic hyphomycete species common in temperate regions were designed and validated to detect and quantify species within communities (Baudy et al., 2019; Feckler et al., 2017). In addition, qPCR-obtained DNA levels showed a positive correlation with ergosterol concentrations, confirming that DNA levels are a suitable species-specific biomass proxy (Baudy et al., 2019). However, aquatic hyphomycetes DNA concentrations were found to vary upon exposure to the fungicide tebuconazole (Baudy et al., 2020). This highlights the need to further develop and test this technique to assess its reliability to accurately disentangle single species contribution to the overall community biomass. Nevertheless, molecular techniques open new avenues to gain deeper insights into the ecological role of aquatic hyphomycetes and other microorganisms in freshwaters and to address biodiversity and ecosystem functioning relationships (Baudy et al., 2019; Fernandes et al., 2011).

9.3 Microbial Metabolism and Stoichiometry

Microbial decomposers use the substrate they are inhabiting (e.g., leaf litter) leading to large changes in resource chemical quality through time accompanied by a progressive disappearance of their growth substrate (Suberkropp et al., 1976). Moreover, aquatic microbial communities can acquire nutrients and carbon both from their decomposing substrate and from the water, either in dissolved organic or mineral forms (Cheever et al., 2012). Therefore, the microbial decomposer´s metabolism is

largely dependent on detrital resources availability and quality and on the physical and chemical characteristics of their surrounding environment.

9.3.1 Carbon Quality and Priming Effect on Litter Decomposition

While most stoichiometric studies have considered carbon as a single pool, carbon quality has long been acknowledged as a factor modulating leaf litter decomposition. The abundance of lignin, in particular, has been shown as a major factor regulating microbial decomposition (Fernandes et al., 2012; Melillo et al., 1982). For example, the nutrient demand of microorganisms for ensuring leaf litter decomposition depends largely upon carbon recalcitrance, indicating that in nature, nutrient limitations of microbial growth might occur at different nutrient levels depending on the carbon quality of litter (Sinsabaugh & Follstad Shah, 2011). Considering carbon recalcitrance into stoichiometric investigation would permit us to refine stoichiometric predictions about microbial decomposer´s activity and the consequences for ecosystem functioning (including carbon respiration and nutrient remineralization). In particular, splitting leaf litter carbon into distinct pools of different quality permits to consider that, in some specific cases, leaf litter decomposers might be limited by the availability of labile carbon. Stimulatory effects of labile carbon inputs (e.g., through the release of algal exudates) on leaf litter decomposition have been shown in several studies (Danger et al., 2013; Kuehn et al., 2014; Pope et al., 2020), a process called aquatic priming effect (Guenet et al., 2010). These labile carbon additions can also result in opposite trends, i.e., reductions in leaf litter decomposition due to shifts in carbon substrate use (Halvorson et al., 2019). To date, the influence of microbial community structure on priming effect occurrence and intensity has not been deeply investigated. Yet, microbial diversity involving different microorganisms with distinct traits seems to control this phenomenon in soils (Fontaine & Barot, 2005). Thus, a better understanding of leaf litter decomposition budgets should include information on the links between microbial diversity and the relative availability of different carbon sources in water and leaf litter.

9.3.2 Microbial Leaf Litter Decomposition Budgets

Microbial leaf litter decomposition budgets confirm that aquatic fungi are the main microbial contributors to leaf mass loss in freshwaters (Baldy et al., 1995; Komínková et al., 2000; Suberkropp, 1991). Laboratory studies have shown that fungal growth efficiency (i.e., the proportion of leaf organic matter assimilated by fungi channelled for biomass and conidial production) ranged from 24 to 46% (35% on average),

with the remaining assimilated matter being respired. Based on such growth efficiency values, aquatic fungi can account for 42 to 65% of the overall carbon loss from leaf litter of diverse deciduous tree species during elevated fungal activity (Baldy et al., 1995; Gessner & Chauvet, 1997). Nevertheless, such budgets might be largely impacted by environmental conditions, such as nutrient availability (Gulis & Suberkropp, 2003; Pascoal & Cássio, 2004; Pascoal et al., 2005).

Since leaf litter is generally a nutrient-poor but carbon-rich substrate, microbial decomposers' activity is often considered as nutrient limited in stream ecosystems (Cross et al., 2005; Enríquez et al., 1993; Güsewell & Gessner, 2009). Elevated nutrient availabilities have thus long been associated with fast microbial litter decomposition. For example, litter rich in nitrogen (N) or phosphorus (P) has been shown to decompose faster than litter exhibiting lower N or P concentrations (Hladyz et al., 2009; Melillo et al., 1982). Similarly, most laboratory (e.g., Gulis et al., 2017) or field studies on nutrient enrichment have shown a stimulatory effect of dissolved N and/or P concentrations (see Ferreira et al., 2015 for a meta-analysis; Rosemond et al., 2015). Overall microbial decomposer activity increases asymptotically (Michaelis–Menten kinetics) with N or P concentrations ($0.09 < \text{N-NO3} < 3.5$ mg L^{-1}) (Ferreira et al., 2006; Gulis et al., 2006; Fernandes et al., 2014). This stimulatory effect of nutrients is modulated by litter recalcitrance, with an increase in dissolved N from 0.1 to 3.0 mg N L^{-1} accelerating the decomposition of lignin-poor litter (e.g., < 10% of lignin, 2.9 × increase) more strongly than that of litter rich in lignin (e.g., > 15% of lignin, 1.4 × increase) (Jabiol et al., 2019). The response to nutrient enrichment might also differ between groups of microbial decomposers. For example, fungal biomass, but not necessarily diversity, tends to increase from the most oligotrophic to moderate eutrophic streams and decrease under hypertrophic conditions (Duarte et al., 2009; Dunck et al., 2015; Pascoal & Cássio, 2004; Pereira et al., 2016). In contrast, bacterial biomass increases monotonically from oligotrophic to hypertrophic streams (Duarte et al., 2009; Pascoal et al., 2005). Moreover, higher temperatures appear to reduce litter recalcitrance (lignin) effects on microbes that mediated litter decomposition (Fernandes et al., 2012). Future research on microbial diversity responses to nutrients (see Sect. 9.5. below) might permit to identify nutrient preferences of the different taxa, and ultimately help to predict the responses of leaf litter decomposition to nutrient variations in ecosystems.

9.3.3 Microbial Stoichiometry and Carbon-Use Efficiency

One convenient way to predict microbial decomposer´s responses to nutrient availability is the ecological stoichiometry framework. Ecological stoichiometry specifically investigates consumers elemental requirements (generally focusing on their C:N:P ratios) and the relative availability of the same elements in their resources (see Chapter 3, for further details). The intensity of elemental imbalance permits to estimate which element limits consumers' activity as well as the functional consequences in terms of nutrient recycling (Sterner & Elser, 2002). To optimally decompose leaf

litter, microorganisms must balance their carbon and nutrient acquisition with their stoichiometric requirements. Both bacteria and fungi have been shown to be non-homeostatic, at least at the community level (Danger et al., 2008), i.e., microbial biomass exhibit variable elemental composition (Danger et al., 2016). While stoichiometric data are still scarce for aquatic fungi, the few available results suggest differences between the elemental plasticity of different fungal strains (Danger & Chauvet, 2013). For example, in P-depleted liquid cultures, *Lemonniera terrestris* reached higher C:P ratios than *Articulospora tetracladia* and *Tricladium chaetocladium* (Danger & Chauvet, 2013). However, on average, microbial decomposers have lower and less variable carbon-to-nutrient ratios than what can be found in the substrates they colonize (Cleveland & Liptzin, 2007; Manzoni et al., 2010; Sardans et al., 2012). In addition, bacteria have lower carbon-to-nutrient ratios than fungi, which renders bacteria more susceptible to nutrient limitations than fungi. Conversely, fungi generally have higher carbon demand and are able to decompose detritus with higher carbon-to-nutrient ratios than bacteria (Keiblinger et al., 2010).

Carbon Use Efficiency (CUE) corresponds to the ratio of microbial growth to total microbial carbon assimilation (i.e., including microbial respiration) and gives information on the conversion efficiency of detritus into microbial biomass as well as indications on the potential carbon storage in ecosystems (Sinsabaugh et al., 2013). CUE can be modeled from the C:N:P ratios of decomposing material (Manzoni et al., 2010). The application of such models demonstrated that decomposers could adapt to low-nutrient conditions by reducing their CUE.

An alternative way to investigate nutrient effects on microbial activity is to search for optimal stoichiometric ratios, i.e., nutrient ratios that will maximize their development and/or activity. For other groups, like metazoans, these ratios can be approached for each taxon by evaluating their Threshold Elemental Ratios (TER) that correspond to the ratios at which limitation shifts from one element to another (Frost et al., 2006). The large immobilization capacities of microorganisms complexify this approach. Immobilization allows microorganisms to balance their nutrient and carbon requirements even for extremely nutrient-poor substrates (Sinsabaugh et al., 2013). Several studies investigated microbial stoichiometric requirements in terrestrial contexts (e.g., Mooshammer et al., 2014), far less in aquatic ecosystems. For aquatic microbial communities, dissolved N:P ratios maximizing cellulose decomposition varied from 1.7 to 45 depending on the overall nutrient supply (Güsewell & Gessner, 2009). The next step for better understanding the mechanisms at play would involve investigating microbial taxa independently; however, most approaches are generally carried out with complex microbial communities (often including fungi and bacteria).

9.4 Substrate Diversity and Quality for Microbial Decomposers

Microbial decomposers are likely to be sensitive to the benthic litter standing stock's characteristics, which reflects the type of riparian vegetation. It could be anticipated that higher diversity of riparian trees (major contributors to litter inputs in forest streams), and consequently, of benthic organic matter, would support a higher diversity of aquatic microbes ('niche complementarity hypothesis'). Positive correlations have been found between aquatic hyphomycete species richness in stream water and benthic leaf litter species richness (Laitung & Chauvet, 2005; Lecerf et al., 2005) or riparian tree species richness (Ferreira et al., 2016; Rajashekhar & Kaveriappa, 2003) in undisturbed streams. Similar positive relationships have been found between aquatic hyphomycete conidium concentration in stream water and benthic leaf litter species richness and amount (Ferreira et al., 2016; Laitung et al., 2002).

Human-mediated decreases in riparian tree diversity may have adverse effects on aquatic hyphomycete diversity. Aquatic hyphomycete species richness associated with decomposing oak (*Quercus robur*) leaves was lower in streams flowing through commercial beech (*Fagus sylvatica*) forests than in streams flowing through mixed deciduous forests in southwestern France (Lecerf et al., 2005). In central Portugal, aquatic hyphomycete species richness in stream water and associated with decomposing alder (*Alnus glutinosa*) and oak leaves was lower in streams in eucalypt (*Eucalyptus globulus*) monocultures than in streams in mixed deciduous forests (Bärlocher & Graça, 2002; Ferreira et al., 2006). In the Azores islands, aquatic hyphomycete species richness associated with decomposing holly (*Ilex perado*) leaves was lower in streams in cryptomeria (*Cryptomeria japonica*) monocultures than in streams with native laurel forests (Ferreira et al., 2017). However, the replacement of native forests by tree plantations not always led to decreases in aquatic hyphomycete species richness if a riparian strip of native tree species is maintained or native tree species are allowed to grow within the plantation, thus ensuring a diverse litter input to streams. In northern Spain, aquatic hyphomycete species richness associated with decomposing alder and pine (*Pinus radiata*) leaves did not significantly differ between streams in deciduous forests and pine plantations, where a riparian strip of native species is maintained (Martínez et al., 2013). Also, in northern Spain, the humid climate allows for the development of an understory of native species in eucalypt plantations; thus, aquatic hyphomycete species richness associated with decomposing leaves did not significantly differ between streams in deciduous forests and those in plantations (Chauvet et al., 1997), or it was even higher in the latter streams (Ferreira et al., 2006).

It could also be expected that higher amounts of benthic litter standing stocks could support higher aquatic hyphomycete species richness and biomass ('productivity hypothesis'). Indeed, higher species richness and reproductive output (i.e., conidium production) have been found where the amount of benthic organic matter is high (Ferreira et al., 2016; Laitung et al., 2002).

Aquatic hyphomycete species richness and community composition also vary with the identity and type of decomposing organic matter since different leaf species and plant parts (e.g., leaves *vs.* woody substrates) differ in physical and chemical characteristics. Although aquatic hyphomycete species are not apparently excluded from any particular substrate, they seem to have substrate preferences (Canhoto & Graça, 1996; Chauvet et al., 1997; Gulis, 2001; Ferreira et al., 2006; Ferreira & Graça, 2016) likely driven by differences in toughness, nutrient concentration and concentration of structural and secondary compounds among substrates. In a given stream, aquatic hyphomycete species richness is generally lower on woody substrates and conifer needles than on deciduous leaves (Ferreira et al., 2006, 2017; Gonçalves et al., 2007; Martínez et al., 2013), which can be attributed to the higher toughness and lignin:nutrients ratio of the former litter types than of leaves. Differences in the aquatic hyphomycete species richness and community composition may also occur among deciduous leaf species, although these are generally smaller than between leaves and woody substrates (Canhoto & Graça, 1996; Ferreira & Graça, 2016; Gonçalves et al., 2013; Gulis, 2001).

Plant-litter decomposition also varies with the substrate diversity: a compilation of manipulative studies showed that 44% of litter mixtures decomposed faster than predicted from the sum of single litter species and 39% of litter mixtures decomposed slower than expected from individual species decomposition (Lecerf et al., 2011). Moreover, effects of leaf-litter quality and diversity on stream ecosystem functioning may vary with the environmental context: synergistic effects of leaf species number on leaf decomposition were found in oligotrophic but not in eutrophic streams (Lima-Fernandes et al., 2015). This suggests that oligotrophic streams are more dependent on the number of leaf species than eutrophic streams. If so, riparian plant diversity should be preserved in oligotrophic systems to maintain leaf-litter decomposition. On the other hand, the positive effects of leaf-litter quality (leaf N) on leaf-litter decomposition were strengthened by moderate increases in nutrient concentrations in the stream water (Lima-Fernandes et al., 2015), suggesting that leaf-litter decomposition depends more on the quality than the number of leaf species in eutrophic streams. These findings support that eutrophication modulates leaf diversity effects on leaf decomposition with potential implications for ecosystem management.

9.5 Microbial Diversity and Litter Decomposition Under Global Change

Many factors regulating microbial diversity and their ecological functions are currently affected by human activities. Such impacts can occur at large spatial scales and represent an important component of human-induced global change. Examples include increases in the concentration of dissolved nutrients in stream water, as a result of atmospheric nitrogen deposition and agriculture; increase in water temperature resulting from global warming, removal of riparian vegetation and urbanization;

impoverishment and homogenization of the riparian vegetation, as a result from tree monocultures and invasions by exotic species; water stress as a result from increases in human needs for freshwater and decreases in precipitation; and contamination by pharmaceuticals, agrochemicals, healthcare products, nanoparticles and plastics. This non-exhaustive list of changes has been addressed in other chapters of this book. Here, we will focus on the effects of increases in the concentration of dissolved nutrients and water temperature, two of the most widespread environmental changes (Woodward et al., 2012; IPCC, 2014).

The number of aquatic hyphomycete species in stream water and associated with decomposing leaf litter on a given sampling date is generally higher under moderate nutrient enrichment (Ferreira et al., 2006; Gulis & Suberkropp, 2003, 2004), which can be explained by the increased availability of resources (i.e., nutrients) not yet confounded by deleterious changes (e.g., sedimentation, pesticides, hypoxia) ('productivity hypothesis' and 'intermediate stress hypothesis'). Nutrient enrichment of stream water can also result in changes in the relative contribution of aquatic hyphomycete species to conidial production, although it is difficult to associate a given species to a given nutrient status across studies (Artigas et al., 2008; Ferreira et al., 2006; Gulis & Suberkropp, 2003, 2004). The reproductive potential of individual fungal species (based on conidia numbers) may also change with nutrient enrichment, with some species being over benefited by increased resource availability (Gulis & Suberkropp, 2003). Since aquatic hyphomycete species have different decomposing capability and palatability to shredders, changes in species richness and relative abundance may affect litter decomposition. Indeed, litter decomposition is generally higher under moderate nutrient enrichment (Ferreira et al., 2015; Gulis & Suberkropp, 2003; Gulis et al., 2019; Rosemond et al., 2015; Woodward et al., 2012), but this is more likely attributed to stimulated microbial activities than to changes in aquatic hyphomycete species richness or community composition (Ferreira, 2020; Chapter 16).

In laboratory experiments, increases in temperature by 5ºC (with maximum temperature $\leq$ 15ºC) led to changes in aquatic hyphomycete community structure associated with decomposing alder leaves (Dang et al., 2009; Ferreira & Chauvet, 2011a, b). On the contrary, an increase in temperature from 16ºC to 24ºC generally led to decreases in aquatic hyphomycete species richness and shifts in species dominance (Fernandes et al., 2012). Similarly, Gonçalves and collaborators (2013) found higher aquatic hyphomycete species richness associated with alder and oak leaves at 10ºC than at colder (5ºC) or warmer (15ºC and 20ºC) temperatures. Also, increases in temperature induced changes in the species dominance pattern. Moreover, aquatic hyphomycete community structure responded to temperature more strongly on alder than on oak; however, they were affected first by litter species (resource quality) and then by temperature (Gonçalves et al., 2013).

In a manipulative experiment in a springbrook in a mixed forest in Canada, a 4ºC temperature increase led to a rise in aquatic hyphomycete species richness on leaf litter, but not on other substrate types (e.g., needles, grass and wood) (Bärlocher et al., 2008). In a similar experiment in a forest stream in central Portugal, a ~ 3ºC temperature increase did not change aquatic hyphomycetes species richness

or community structure associated with oak and chestnut (*Castanea sativa*) leaves during winter (Ferreira et al., 2015). Additionally, season (spring *vs.* autumn) played a greater role in structuring aquatic hyphomycete communities associated with oak leaves than experimental warming (Duarte et al., 2016). This suggests that the major factor structuring aquatic hyphomycete communities may not be temperature, but other seasonally changing factors, such as substrate availability (Gossiaux et al., 2019). However, on the global scale, water temperature has been described as the prime factor ruling aquatic hyphomycete community composition associated with alder leaf litter, independently of biogeographic realms (Seena et al., 2019a).

Different effects of warming on aquatic hyphomycete communities may partially depend on community composition. Aquatic hyphomycete species have distinct thermal preferences, with thermal optimum generally between 10–30ºC across species (reviewed by Ferreira et al., 2014). Effects may also depend on the ambient water temperature and the magnitude of the increase. Increases in water temperature when the ambient temperature is already high (>15ºC) may have more severe effects than when the ambient temperature is low (<15ºC) since species may be already near their thermal optimum (Ferreira et al., 2014).

Increases in temperature have been found to stimulate litter decomposition (Amani et al., 2019; Boyero et al., 2011), but, as for nutrient enrichment, effects of warming are likely mediated by stimulation of microbial activities than by changes in species richness and community structure (Ferreira, 2020).

9.6 Functional Consequences of Microbial Biodiversity Loss

Over the last two centuries, the intensification of human activities on our planet has led to a massive species extinction (Chapin III et al., 2000), with freshwaters being one of the most endangered ecosystems (Dudgeon et al., 2006). This has motivated a bloom of studies on the relationship between biodiversity and ecosystem functioning (BEF, Hooper et al., 2012). BEF research has focused primarily on terrestrial ecosystems, while aquatic ecosystems have received increased attention only over the last decade; BEF studies focusing on microbial communities are even scarcer (Daam et al., 2019; Pascoal & Cássio, 2008). Traditionally, species richness has been used as a biodiversity measure in BEF studies targeting microbial decomposer communities (e.g., Bärlocher & Corkum, 2003; Duarte et al., 2006; Pascoal et al., 2010). Studies in which aquatic hyphomycete species were manipulated point to a positive relationship between fungal diversity and leaf decomposition (Bärlocher & Corkum, 2003; Duarte et al., 2006; Fernandes et al., 2011; Pascoal et al., 2010; Raviraja et al., 2006; Treton et al., 2004). Conversely, other studies failed to detect the effects of fungal diversity on leaf mass loss, pointing to considerable functional redundancy among fungi (Andrade et al., 2016; Dang et al., 2005; Geraldes et al., 2012). However, such studies showed that higher diversity decreases the variability of

process rates, probably increasing ecosystem stability (portfolio effect or statistical average effect, see Doak et al., 1998). Fungal diversity had positive effects on other microbial functions such as fungal reproduction (Geraldes et al., 2012) or biomass build-up (Andrade et al., 2016), despite having no effect on leaf decomposition, supporting the importance of considering multiple functions when addressing BEF relationships. In addition, diversity effects of aquatic fungi have also been attributed to species identity, indicating that certain species´ traits may have a greater impact on ecosystem processes than species diversity *per se* (reviewed in Pascoal & Cássio, 2008). Actually, the structure and function of leaf-associated microorganisms can be decoupled under anthropogenic pressure: microbially-mediated leaf litter decomposition remaining stable, increasing or exhibiting a U-shaped response as structural metrics (e.g., taxonomic diversity) change gradually (Feckler & Bundschuh, 2020).

Few studies have addressed other biodiversity measures, like intraspecific diversity considering species background (polluted *vs.* unpolluted stream) (Duarte et al., 2019; Fernandes et al., 2011) or genetic diversity based on species genetic divergence of the ITS1-5.8S-ITS2 rRNA genes (Andrade et al., 2016). Fernandes et al. (2011) demonstrated that positive diversity effects were maintained under metal stress when an ecotype (fungal strain from one species which is adapted to specific environmental conditions) of the worldwide distributed species *A. tetracladia* from a metal-polluted site was incorporated in the assemblage, but these effects were lost when it was replaced by the other ecotype from an unpolluted site. Species or strains that have a redundant role in an ecological process may exhibit noticeable traits when exposed to different environmental contexts (Fernandes et al., 2011). Different environmental contexts have been shown to modulate the impacts of fungal diversity on litter decomposition or decomposer´s activity (nutrients, Bärlocher & Corkum, 2003; metal stress, Pascoal et al., 2010; Fernandes et al., 2011; Duarte et al., 2019; warming, Geraldes et al., 2012).

Diversity effects can result from mechanisms of complementarity and/or selection, whose relative contribution can be quantified if individual species performances in multicultures are determined (partitioning model, Loreau & Hector, 2001). However, most studies examining the relationships between microbial diversity and ecological processes have been limited by difficulties in tracking individual species performances within assemblages (e.g., Bärlocher & Corkum, 2003; Duarte et al., 2006). For instance, Fernandes et al. (2011) successfully determined the contribution of each fungal species to the total biomass produced in multicultures by qPCR. In the absence of metal, positive diversity effects were observed for fungal biomass and leaf decomposition as a result of species complementarity; but, under metal stress, the dominance effect maintained positive diversity effects in assemblages containing the ecotype from the metal-polluted site (Fernandes et al., 2011).

9.7 Outlook

Although the role microbial decomposers play in aquatic detritus-based food webs is acknowledged, there are still gaps that need to be further addressed. Freshwater systems harbor high microbial diversity (Schloss et al., 2016), but data are still scarce compared to other organisms (Balian et al., 2008; Debroas et al., 2017). This lack of knowledge about microbial diversity has limited our deeper understanding of the role that microbial decomposers play in ensuring several ecosystem services, such as nutrient recycling, water purification and carbon sequestration (Ducklow, 2008).

Several gaps that need to be clarified on the relationships between biodiversity and ecosystem functioning in aquatic systems have been identified (Daam et al., 2019) and should be considered when envisaging the functional role of aquatic microbial decomposer´s diversity, namely: (i) looking at multiple ecosystem functions, (ii) studying the role of rare species and focusing on realistic species losses; (iii) integrating different biodiversity metrics (intraspecific diversity, genetic diversity, phylogenetic diversity), (iv) integrating various trophic components (taxonomic groups, trophic composition, trophic interactions), (v) testing different environmental conditions, (vi) targeting larger spatial and temporal scales, (vii) integrating trait-based approaches, and (viii) applying ecological modelling to BEF relationships.

Nowadays, relevant advances to unravel microbial diversity are associated with metabarcoding or environmental DNA techniques. DNA sequencing of environmental samples is increasing in public databases (e.g., NCBI), which facilitate the knowledge of microbial diversity. Moreover, omics are a relevant approach to discover functional traits that can help to explain microbial functional diversity. Further links have to be established between taxonomic, genetic and functional diversity, particularly if we aim to understand how microbial decomposer communities and plant litter decomposition respond to multiple threats derived from global change (Fig. 9.2), such as warming, drought events, eutrophication, persistent and emergent contaminants. Some data already exist on the role of fungal ecotypes to maintain ecological functions under stress (e.g., metal stress, Fernandes et al., 2011) and the biological mechanisms underlying stress responses based on proteomics (e.g., metal nanoparticles, Barros et al., 2020). These data suggest that microbial populations and communities can adapt to stressors following the pollution-induced community tolerance (Tlili et al., 2016). It is conceivable that in nature, these microbes may be able to evolve at relatively short times due to their high replication time. So, they can be crucial to ensure multiple ecosystem functions and services under the ongoing global change (Fig. 9.2).

Another important issue to be further addressed is how fungal assemblages on leaf litter are a food resource for invertebrate detritivores. Beyond the ability of fungi to degrade complex carbon sources from plant litter to easier assimilable food sources due to their enzymatic capabilities (Romaní et al., 2006), it has been claimed that invertebrate shredders have a preference for leaf litter colonized by certain species of fungi (e.g., Arsuffi & Suberkropp, 1984, 1985, 1986). However, no clear explanations have been provided so far for such evidence. Increasing the knowledge on fungal traits

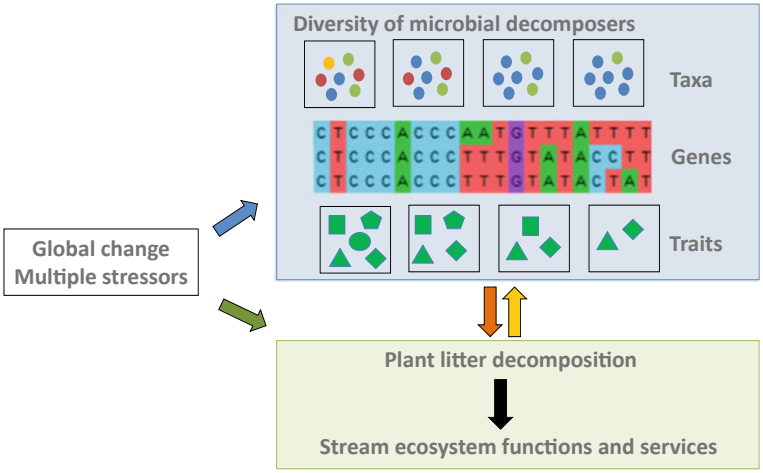

Fig. 9.2 Diagram with possible effects of multiple stressors on different dimensions of diversity (taxonomic, genetic and functional diversity) of microbial decomposers and plant litter decomposition with impacts on stream ecosystem functioning and services. Blue arrow, direct effects of stressors on diversity; green arrow, direct effects of stressors on functions; orange arrow, effects on functions mediated by changes in diversity; yellow arrow; effects on diversity mediated by changes in functions; black arrow, impacts on ecosystem services

could help to elucidate these aspects related to the availability of certain nutrients, such as nitrogen, vitamins or fatty acids (Arce Funck et al., 2015).

How fungal community assembly on plant litter in streams is another aspect that is poorly understood but can be important to ensure plant litter decomposition, particularly under stressful abiotic and biotic conditions. It is recognized that there is a colonization succession with some species of fungi appearing to be early colonizers, while others appear to occur later (e.g., Sridhar et al., 2009). However, the relative importance of stochastic versus deterministic processes in microbial community assembly has been poorly investigated (Chase & Myers, 2011; Stegen et al., 2012). Although stochastic processes, in which communities would be randomly assembled through birth–death, drift, and speciation (neutral theory), are believed to play a role in shaping community structure, most studies focus on deterministic processes considering the selection imposed by biotic interactions and environmental filtering (Vellend, 2010). Anyway, if key early colonizers are lost, it may compromise species succession and, consequently, plant litter decomposition. Therefore, powerful predictive models will contribute to better understand microbial community dynamics and the interactions between plant litter and the micro- and macro-organisms that shape stream ecosystem functioning under global change.

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