# Fungicide effects on the structure and functioning of leaf-associated aquatic fungal communities

# Effekte von Fungiziden auf die Struktur und Funktion laubassoziierter aquatischer Pilzgemeinschaften

by
Patrick Baudy-Groh (né Baudy)
from Albersweiler, Germany

Accepted Dissertation thesis for the partial fulfillment of the requirements for a
Doctor of Natural Sciences
Fachbereich 7: Natur- und Umweltwissenschaften
Universität Koblenz-Landau

Thesis examiners:
Jun.-Prof. Dr. Mirco Bundschuh, University of Koblenz-Landau
Prof. Dr. Ralf Schulz, University of Koblenz-Landau

Date of the oral examination: July 08, 2022

**DECLARATION**

I hereby declare that I independently conducted the work presented in this thesis entitled “Fungicide effects on the structure and functioning of leaf-associated aquatic fungal communities”. All used assistances are mentioned and involved contributors are either co-authors of or are acknowledged in the respective publication. In all cases, I designed and planed the studies, conducted the experiments, performed the associated analyses, evaluated the data, and wrote the respective publication – with support of the named persons. This thesis has never been submitted elsewhere for an examination, as a thesis or for evaluation in a similar context to any department of this university or any scientific institution. I am aware that a violation of the aforementioned conditions can have legal consequences.

Albersweiler, August 17, 2022

Place, Date **Patrick Baudy-Groh**

The following parts of this thesis are published in peer-reviewed international journals:

**APPENDIX A.1**: Baudy, P., Zubrod, J.P., Röder, N., Baschien, C., Feckler, A., Schulz, R., Bundschuh, M., 2019. A glance into the black box: Novel species-specific quantitative real-time PCR assays to disentangle aquatic hyphomycete community composition. Fungal Ecology 42, 100858.

**APPENDIX A.2**: Baudy, P., Konschak, M., Sakpal, H., Baschien, C., Schulz, R., Bundschuh, M., Zubrod, J.P., 2020. The fungicide tebuconazole confounds concentrations of molecular biomarkers estimating fungal biomass. Bulletin of Environmental Contamination and Toxicology 105, 620–625.

**APPENDIX A.3**: Baudy, P., Zubrod, J.P., Konschak, M., Kolbenschlag, S., Pollitt, A., Baschien, C., Schulz, R., Bundschuh, M., 2021. Fungal-fungal and fungal-bacterial interactions in aquatic decomposer communities: bacteria promote fungal diversity. Ecology 102, e03471.

**APPENDIX A.4**: Baudy, P., Zubrod, J.P., Konschak, M., Röder, N., Nguyen, T.H., Schreiner, V.C., Baschien, C., Schulz, R., Bundschuh, M., 2021. Environmentally relevant fungicide levels modify fungal community composition and interactions but not functioning. Environmental Pollution 285, 117234.

**ACKNOWLEDGEMENT**

This thesis would not have been possible without the support of many great people.

First of all, I would like to express my sincere gratitude to Prof. Dr. Mirco Bundschuh and Prof. Dr. Ralf Schulz for their supervision, continuous support, and trust in me and my skills.

The German Research Foundation (Deutsche Forschungsgemeinschaft; DFG) is acknowledged for funding this project.

Through the dedicated support of my friends and colleagues, the busy phases of this thesis turned into one of the best and funniest times of my life, with lots of laughing and swearing. Many thanks to Dr. Dominic Englert, Dr. Alexander Feckler, Lisa Friedrichs, Sara Kolbenschlag, Dr. Marco Konschak, Dr. Simon Lüderwald, Frederik Meyer, Sebastian Pietz, Nina Röder, Dr. Ricki R. Rosenfeldt, Dr. Frank Seitz, Tobias Schmitt, and Dr. Jochen P. Zubrod.

Further, I would like to express my sincere thanks to all other members of Landau's Institute for Environmental Sciences, who supported me in the lab (Therese Bürgi, Andreas Hirsch, Dr. Verena C. Schreiner, and Britta Wahl-Ermel) as well as bureaucratic issues (Jone Kammerer, Christina Krämer, and Franziska Wollnik).

My sincere thanks go to PD Dr. Christiane Baschien, from the Leibniz Institute DSMZ, for sharing her vast knowledge of genetics and microbiology.

I am also grateful for the help by a number of students, who assisted me during my lab work. Particular thanks go to Thu Huyen Nguyen, Annika Pollitt, and Harshada Sakpal.

Dr. Dominic Englert and Dr. Jochen P. Zubrod are acknowledged for proofreading this thesis.

Last but not least and from the bottom of my heart, I want to thank my wife Hanna and my entire family for the overwhelming support they have given me in so many ways.

## TABLE OF CONTENTS

1. ABSTRACT ..... 1
2. ZUSAMMENFASSUNG ..... 2
3. INTRODUCTION ..... 4
3.1 BACKGROUND ..... 4
3.2 OBJECTIVES ..... 8
4. THESIS LAYOUT AND METHOD OVERVIEW ..... 9
4.1 MODEL ORGANISMS ..... 9
4.2 PHASE 1: DESIGN AND VALIDATION OF FUNGAL SPECIES-SPECIFIC BIOMASS PROXIES ..... 11
4.3 PHASE 2: VALIDATION OF FUNGAL BIOMASS PROXIES UNDER FUNGICIDE EXPOSURE ..... 12
4.4 PHASE 3: CHARACTERIZATION OF FUNGAL B-EF RELATIONSHIPS ..... 14
4.5 PHASE 4: FUNGICIDE IMPACTS ON FUNGAL B-EF RELATIONSHIPS ..... 15
5. RESULTS ..... 17
5.1 DESIGN AND VALIDATION OF FUNGAL SPECIES-SPECIFIC BIOMASS PROXIES ..... 17
5.2 VALIDATION OF FUNGAL BIOMASS PROXIES UNDER FUNGICIDE EXPOSURE ..... 21
5.3 CHARACTERIZATION OF FUNGAL B-EF RELATIONSHIPS ..... 23
5.4 FUNGICIDE IMPACTS ON FUNGAL B-EF RELATIONSHIPS ..... 29
6. DISCUSSION ..... 34
6.1 RQ 1: DESIGN AND VALIDATION OF FUNGAL SPECIES-SPECIFIC BIOMASS PROXIES FOR ECO(TOXICO)LOGICAL APPLICATION ..... 34
6.2 RQ 2: CHARACTERIZATION OF FUNGAL B-EF RELATIONSHIPS ..... 36
6.3 RQ 3: FUNGICIDE IMPACTS ON FUNGAL B-EF RELATIONSHIPS ..... 39
7. CONCLUSION AND OUTLOOK ..... 41
8. REFERENCES ..... 44
APPENDIX ..... 55
APPENDIX A.1 ..... 56
APPENDIX A.2 ..... 88
APPENDIX A.3 ..... 101
APPENDIX A.4 ..... 150
APPENDIX A.5 ..... 182

## 1. ABSTRACT

Aquatic hyphomycetes are a polyphyletic group of saprotrophic fungi growing abundantly on submerged leaf litter. In stream ecosystems shaped by allochthonous leaf litter inputs, they play a central functional role as decomposers and food source for other organisms. Fungicides pose a threat to aquatic hyphomycetes and their functions, since these substances are inherently toxic to fungi and contaminate surface waters around the world due to their widespread use in agricultural and urban landscapes. While fungicides' potential to reduce fungal diversity are discerned, the extent of impacts on biodiversity-ecosystem functioning relationships (B-EF) remains unclear. This is partly attributed to methodological constraints in the detection and quantification of single aquatic hyphomycete species within microbial leaf-associated communities. The primary aim of this thesis was, therefore, (1) to assess the ecotoxicological impacts of fungicides on B-EF relationships in aquatic hyphomycete communities. To facilitate this, subordinate aims were to (2) develop DNA-based biomolecular tools (i.e., qPCR assays) to detect and to quantify the biomass of different aquatic hyphomycete species in mixed cultures and (3) to investigate the mechanisms underlying B-EF relationships in the absence of chemical stressors.

In the course of this thesis, qPCR assays were developed for detection and species-specific biomass quantification of ten common aquatic hyphomycete species and successfully validated for application in eco(-toxico-)logical microcosm experiments. Via a systematic manipulation of fungal diversity, these assays allow the examination of B-EF relationships by assessments of deviations between observed and (monoculture-based) predicted activities in fungal mixed cultures. Taking advantage of these tools in a microcosm experiment, it was uncovered that leaf decomposition results from the additive activity of community members, even though functionally distinct species were present. Colonization dynamics are characterized by complex interactions. Colonization success of aquatic hyphomycetes is higher if co-occurring species are genetically and functionally distinct (i.e., complementary interactions). However, the co-occurrence of aquatic hyphomycete species does not necessarily result in a greater colonization success compared to monocultures, unless bacteria are present. Accordingly, the presence of other microbial groups such as bacteria may induce new fungal diversity-based feedback loops, which ultimately enable coexistence of aquatic hyphomycete species in the environment. Exposure to fungicides revealed substantial differences in sensitivities among aquatic hyphomycetes. The most productive species were able to cope with extremely high fungicide concentrations up to the mg/L-range. In assemblages containing these species, leaf decomposition was maintained under fungicide exposure. Yet, already at environmentally relevant fungicide concentrations, tolerant species displaced more sensitive ones, potentially affecting leaves' nutritional quality for consumers. This thesis thus indicates that fungicide exposure poses a risk to stream food webs rather than the microbial leaf decomposition process per se.

## 2. ZUSAMMENFASSUNG

Aquatische Hyphomyceten sind eine polyphyletische Gruppe saprotropher Pilze, die bekannt dafür ist submerse Laubstreu zu besiedeln. In Fließgewässerökosystemen, die durch allochthone Einträge von Laubstreu geprägt sind, spielen sie eine zentrale Rolle als Zersetzer und Nahrungsquelle für andere Organismen. Fungizide stellen eine Bedrohung für aquatische Hyphomyceten und ihre Funktionen dar, da diese Substanzen inhärent toxisch für Pilze sind und aufgrund ihres weit verbreiteten Einsatzes in der Landwirtschaft und in städtischen Gebieten Oberflächengewässer auf der ganzen Welt kontaminieren. Während das Potenzial von Fungiziden, die Pilzdiversität zu verringern, erkannt wurde, bleibt das Ausmaß der Auswirkungen auf die Beziehungen zwischen Biodiversität und Ökosystemfunktionen (B-EF) unklar. Dies ist zum Teil auf methodische Einschränkungen beim Nachweis und der Quantifizierung einzelner aquatischer Hyphomycetenarten in mikrobiellen laubassoziierten Gemeinschaften zurückzuführen. Das Hauptziel dieser Arbeit war daher (1) die Bewertung der ökotoxikologischen Auswirkungen von Fungiziden auf die B-EF Beziehungen in aquatischen Hyphomycetengemeinschaften. Um dies zu ermöglichen, wurden (2) DNA-basierte biomolekulare Methoden (d.h. qPCR-Tests) entwickelt, um die Biomasse verschiedener aquatischer Hyphomycetenarten nachzuweisen und zu quantifizieren, und (3) die Mechanismen zu untersuchen, die den B-EF-Beziehungen in Abwesenheit von chemischen Stressoren zugrunde liegen.

Im Rahmen dieser Arbeit wurden qPCR-Tests zum Nachweis und zur artspezifischen Quantifizierung der Biomasse von zehn häufig vorkommenden aquatischen Hyphomycetenarten entwickelt und für den Einsatz in öko(-toxiko-)logischen Mikrokosmosexperimenten erfolgreich validiert. Durch eine systematische Manipulation der Pilzdiversität ermöglichen diese Tests die Untersuchung von Biodiversitätseffekten, welche durch Abweichungen zwischen beobachteter und (monokulturbasierter) vorhergesagter Aktivitäten in Pilzmischkulturen angezeigt werden. In einem Mikrokosmos-Experiment wurde mit Hilfe der qPCR-Tests aufgedeckt, dass die Zersetzung von Laub aus der additiven Aktivität aller Arten in einer Mischkultur hervorgeht, obgleich diese sich funktionell unterscheiden. Die Besiedlungsdynamik ist durch komplexe Interaktionen gekennzeichnet. Der Besiedlungserfolg aquatischer Hyphomyceten ist höher, wenn die gemeinsam vorkommenden Arten genetisch und funktionell unterschiedlich sind (d.h. komplementäre Interaktionen). Co-Kultivierung von aquatischen Hyphomycetenarten führt jedoch nicht zwangsläufig zu einem größeren Besiedlungserfolg im Vergleich zu Monokulturen, außer in Anwesenheit von Bakterien. Dementsprechend kann die Anwesenheit anderer mikrobieller Gruppen wie Bakterien neue, auf der Pilzdiversität basierende Rückkopplungsschleifen auslösen, die letztlich die Koexistenz aquatischer Hyphomycetenarten in der Umwelt ermöglichen. Bei der Exposition gegenüber Fungiziden zeigten sich erhebliche Unterschiede in der Sensitivität der aquatischen Hyphomycetenarten. Die produktivsten Arten waren in der Lage sehr hohe Fungizidkonzentrationen bis in den mg/L-Bereich zu tolerieren. In Mischkulturen, die diese Arten enthielten, blieb die Laubabbaufunktion unter Fungizidexposition erhalten. Doch bereits bei umweltrelevanten Fungizidkonzentrationen

verdrängten die toleranten Arten die sensitiveren, was sich möglicherweise auf die Nahrungsqualität des Laubs für detritivore Organismen auswirkt. Diese Arbeit deutet also darauf hin, dass Fungizidexpositionen eher ein Risiko für Nahrungsnetze in Fließgewässern als für den mikrobiellen Laubabbauprozess an sich darstellen.

## 3. INTRODUCTION

### 3.1 BACKGROUND

Aquatic hyphomycetes are a cosmopolitan, polyphyletic group of over 300 described species of true fungi, which are mainly composed of ascomycetes (besides few basidiomycetes; Baschien et al., 2013; Duarte et al., 2016; Gulis et al., 2020). They can be broadly defined as anamorphic fungi colonizing plant substrates in aquatic habitats (Shearer et al., 2007). Members of this group grow abundantly on submerged allochthonous leaf litter in running waters and disperse via conspicuously shaped conidia (i.e., multiradiate, variously branched, or sigmoid) adapted to this niche (Webster, 1959; Webster and Descals, 1981). Due to their adaptations, they play a leading role in detritus-based stream food webs primarily fueled by allochthonous leaf litter, such as woodland streams, which are also home to a wide variety of other microbes and higher organisms (Petersen and Cummins, 1974; Vannote et al., 1980; Wallace, 1997). While other leaf-associated microbes such as bacteria vastly outnumber aquatic hyphomycetes in terms of species diversity (hundreds vs. up to ~20 species per leaf; e.g., Garnett et al., 2000; Newman et al., 2015), the latter dominate in terms of biomass throughout various stages of leaf decay (Das et al., 2007; Duarte et al., 2010) and accomplish two major functions: first, the initiation of the biological decomposition of leaves, catalyzed by various fungal extracellular enzymes cleaving specific plant structures (Evans and Hedger, 2001), and the concurrent release of bound nutrients and energy (Gessner et al., 2007; Hieber and Gessner, 2002); and second, the chemical and physical transformation of leaves during colonization (i.e., conditioning), improving the nutritional quality of leaves as food source for detritivorous (in-)vertebrates (Bärlocher, 1985; Bundschuh et al., 2021; Danger et al., 2016).

Virtually all ecosystems around the world are subject to degradation driven by various types of human activity, whereby the ecological status of freshwaters is deteriorating faster than in most other habitats (Millennium Ecosystem Assessment, 2005a, 2005b). In running waters, chemical pollution is one of the major pressures threatening aquatic life (Di Lorenzo et al., 2020; Schäfer et al., 2016; Vörösmarty et al., 2010). Among a long list of surface water pollutants (e.g., Amoatey and Baawain, 2019), the group of fungicides (sc., synthetic, organic fungicides) poses a particular threat to aquatic hyphomycetes and the functions they provide. This concern is justified by these substances' toxicity and occurrence in the environment (Halbach et

al., 2021; Zubrod et al., 2019). Obviously, fungicides are inherently toxic to fungi, as their modes of action are not specific to the fungal pathogens targeted (Fungicide Resistance Action Committee, 2020; Stenersen, 2004). At the same time, fungicides occur worldwide in a vast number of running waters (Casado et al., 2019; Zubrod et al., 2019) and chronic (year-long) exposures to mixtures of different fungicides, comprising multiple modes of action, are common in streams with urban or agricultural catchments (Manoli et al., 2019; Zubrod et al., 2019). Yet, in the latter, exposures are typically more pronounced in terms of the number of detected substances and measured sum concentrations during base flow (up to ~1 µg/L) and runoff events (up to dozens of µg/L; Zubrod et al., 2019).

Aquatic hyphomycetes are proposed for use as sensitive bioindicators of water quality (Solé et al., 2008) and have already been the subject of numerous ecotoxicological studies on surface water pollutants. However, only a minority of these studies is dedicated to fungicides, and, in this context, research on these substances is still fragmented (Krauss et al., 2011; Maltby et al., 2009; Zubrod et al., 2019). In the first studies, Sridhar and Kaveriappa (1986) and Chandrashekar and Kaveriappa (1989, 1994) investigated the potential of dithiocarbamate, phthalimide, morpholine, and benzimidazole fungicides to impair the dispersal of aquatic hyphomycetes (i.e., mycelial growth, sporulation, and germination) in laboratory experiments. They observed considerable differences in sensitivities among species, while, overall, effect concentrations were found to be in the mg/L-range. A series of recent laboratory and (semi-)field studies, however, indicates much lower effect concentrations in the µg/L-range for single substances and mixtures of currently used fungicides (e.g., Dimitrov et al., 2014; Fernández et al., 2015; Zubrod et al., 2015a). Generally, effects on the most commonly investigated functional parameters, namely leaf decomposition and total fungal biomass production, tend to occur at higher fungicide concentrations (i.e., beyond field relevant levels; e.g., Pimentão et al., 2020; Zubrod et al., 2019) or are only transient at lower field-relevant concentrations (e.g., Schreiner et al., 2018). In comparison, effects on community structure (i.e., species diversity and community composition) were already observed in the field or rather at concentrations representative of environmentally relevant exposure scenarios (Fernández et al., 2015; Pimentão et al., 2020; Schreiner et al., 2018; Zubrod et al., 2015a). The differences in sensitivity between functional and structural responses can be explained by a displacement of sensitive by more tolerant aquatic

hyphomycete species in combination with functional redundancy (i.e., functions are performed equally well by sensitive and tolerant species; Pascoal et al., 2005). Yet, functional redundancy may be less pronounced in other functions such as leaf conditioning. Besides fungal biomass per se, aquatic hyphomycete community composition is a critical determinant in the conditioning of leaves and decisive for leaves' nutritional quality for consumers (Danger et al., 2016). Accordingly, it has been shown that detritivorous consumers feeding on leaves conditioned in the presence of fungicides are subject to physiological implications, which are suspected to be triggered by shifts in aquatic hyphomycete community composition rather than direct toxic effects (Bundschuh et al., 2021; Konschak et al., 2019; Zubrod et al., 2015b). Since our knowledge of the mechanistic links between aquatic hyphomycete community structure and functioning (i.e., biodiversity-ecosystem functioning relationships; B-EF) is still superficial (Grossart and Rojas-Jimenez, 2016), ecosystem-level impacts of fungicide exposures (and, more generally, human activity) cannot be predicted with certainty (Wright et al., 2017).

To address these ecotoxicological questions, it is first necessary to understand the ecological mechanisms underlying B-EF relationships. A major challenge is to shed light on interactions occurring between different aquatic hyphomycete species and between aquatic hyphomycetes and other leaf-associated microorganisms such as bacteria as well as consequential implications for functioning (Grossart and Rojas-Jimenez, 2016). Investigating these relationships necessitates the characterization of functional traits of individual aquatic hyphomycete species (e.g., specific decomposition rates or enzyme activity profiles) and the ability to track their biomass species-specifically within complex communities across different successional stages (Bärlocher and Corkum, 2003; Duarte et al., 2006). Particularly the latter condition poses a major obstacle, as conventional methods do not or only insufficiently satisfy this demand. The most common quantification tool for aquatic hyphomycetes, namely estimating fungal biomass using the fungal membrane molecule ergosterol, does not allow a distinction between species and solely serves as indicator of total biomass of a fungal community (Gessner, 2020). Its further breakdown into aquatic hyphomycete community composition is widely accomplished via morphological identification and quantification of characteristically shaped conidia under the microscope (Gulis et al., 2020). This method, however, relies on reproductive mycelia and is subject to considerable uncertainties: species are not always unequivocally identifiable due to a

convergent evolution in conidial morphology (Baschien et al., 2013). In addition, the release rate of conidia is species-dependent and responsive to environmental conditions (e.g., nutrients and pollution stress), which is why conidial counts neither provide proof of species' absence nor do they necessarily reflect species' biomass (Bermingham et al., 1996; Bermingham et al., 1997; Fernandes et al., 2015).

As an alternative method circumventing these obstacles, the development and use of quantitative real-time polymerase chain reaction (qPCR) assays targeting deoxyribonucleic acid (DNA) sequence motifs is highly recommended (Grossart and Rojas-Jimenez, 2016; Krauss et al., 2011; Zubrod et al., 2019). Accordingly, qPCR assays may be designed to detect and quantify species-specific sequence motifs correlating with hyphal biomass. The internal transcribed spacer (ITS) region of the ribosomal DNA operon, which is the most frequently used barcoding region for fungi (Schoch et al., 2012), is considered to fulfill this demand (Raidl et al., 2005). Moreover, compared to other loci (e.g., the translation elongation factor 1-α or β-tubulin genes), the ITS region offers the most reference sequences deposited in publicly accessible databases (e.g., INSDC; https://www.insdc.org). Using ITS as target region, in a recent proof-of-concept study by Feckler et al. (2017), it was successfully demonstrated that a qPCR assay based on TaqMan® chemistry can species-specifically detect the aquatic hyphomycete *Alatospora pulchella* and appears suitable to reliably quantify DNA concentrations of this species in the leaf matrix. This promising tool, however, needs to be expanded to allow the identification and quantification of further aquatic hyphomycetes, which will enable the assessment of complex (near-)natural communities typically composed of up to ~20 species and dominated by up to ~6 species per leaf (Bärlocher, 1991; Carl et al., 2022; Garnett et al., 2000; Gulis and Suberkropp, 2003). Finally, these tools may facilitate a detailed assessment of the ecotoxicological impacts of fungicides on B-EF relationships in aquatic hyphomycete communities.

## 3.2 OBJECTIVES

Given the knowledge gaps identified above, the primary aim of this thesis is the assessment of the ecotoxicological impacts of fungicides on B-EF relationships in aquatic hyphomycete communities. As a part of this endeavor, the subordinate objectives are the development of qPCR assays to investigate community composition and the investigation of ecological mechanisms underlying B-EF relationships both within aquatic hyphomycete communities and between these and leaf-associated bacteria. Therefore, this thesis aimed at answering the following three research questions:

1. Are ITS-based qPCR assays suitable species-specific biomass proxies for application in eco(-toxico-)logical microcosm experiments?
2. What are the major ecological mechanisms driving B-EF relationships in aquatic hyphomycete communities?
3. Do environmentally relevant fungicide concentrations affect B-EF relationships in aquatic hyphomycete communities?

## 4. THESIS LAYOUT AND METHOD OVERVIEW

This thesis was subdivided into two ecological and two ecotoxicological experimental phases (Fig. 4.1) to answer the research questions outlined above (see chapter 3.2).

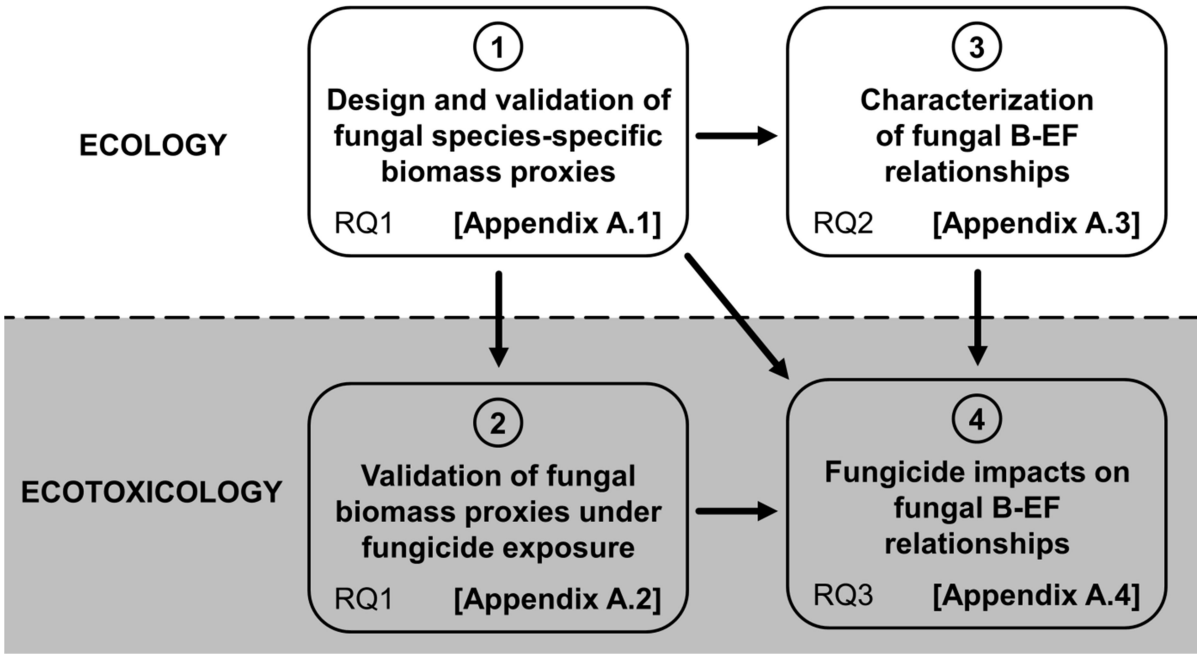



Fig. 4.1. Scheme displaying the four phases (encircled numbers) of this thesis including their main objective (box title), the research questions (RQs) that were tackled (see chapter 3.2) and the corresponding publications **[Appendix A.1-A.4]**.

### 4.1 MODEL ORGANISMS

To answer the research questions outlined above, ten aquatic hyphomycete species, representative of microbial decomposer communities in streams of the temperate latitudes were chosen as model organisms. This selection includes *Alatospora acuminata*, *Articulospora tetracladia*, *Clavariopsis aquatica*, *Clavatospora longibrachiata*, *Flagellospora curvula*, *Heliscella stellata*, *Lemonniera terrestris*, *Neonectria lugdunensis*, *Tetracladium marchalianum*, and *Tricladium angulatum*. Aquatic hyphomycete strains were obtained by cultivation of single spores, which were isolated from streams in Germany (following the method described in Descals, 2005). To this end, in total, 81 aquatic hyphomycete strains (comprising at least 26 species) were cultivated and subcultures were deposited in the open collection of the Leibniz Institute DSMZ (German Collection for Microorganisms and Cell Cultures, Germany). For all strains, ITS sequences were determined, which were used for the design and validation of qPCR assays **[Appendix A.1]**. Further details on sampling

data, specifications, and sequence accession numbers of all isolated strains as well as methods on DNA extraction and sequencing can be found in the supporting information of **Appendix A.1**. Specifications for the ten strains used for experimental purposes **[Appendix A.1-A.4]** are shown in Table 4.1. Leaves of *Alnus glutinosa* (L.) GAERTN. (black alder), a common riparian tree species (Copolovici et al., 2014), were used as model substrate **[Appendix A.1, A.3, and A.4]**. The leaves were handpicked from trees near Landau, Germany (49.20116 °N; 8.09331 °E) shortly before abscission in 2015/2016 and stored frozen until further use. Genetic material used for preparation of DNA standards or sample analysis was extracted from mycelia of pure cultures or (conditioned) leaf substrate using the FastDNA® Spin Kit for Soil in conjunction with the FastPrep™-24 5G Instrument (MP Biomedicals, Germany) as per manufacturer's instructions.

Table 4.1. List of species, associated classes, strain specification, and deposited ITS sequences. Table modified from **Appendix A.2**.

| Species | Class | Representative strain [1)] | ITS sequence [2)] |
|---|---|---|---|
| *Alatospora acuminata* Ingold | Leotiomycetes | DSM 104360 [3), 4)] | MH930815 |
| *Articulospora tetracladia* Ingold | Leotiomycetes | DSM 104345 [3)] | MH930816 |
| *Clavariopsis aquatica* De Wild. | Dothideomycetes | DSM 104362 [3)] | MH930817 |
| *Clavatospora longibrachiata* (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson | Sordariomycetes | DSM 104365 [3)] | MH930818 |
| *Flagellospora curvula* Ingold | Leotiomycetes | DSM 104334 [3)] | MH930819 |
| *Heliscella stellata* (Ingold & V.J. Cox) Marvanová | Dothideomycetes incertae sedis | DSM 104386 [3), 4)] | MH930820 |
| *Lemonniera terrestris* Tubaki | Leotiomycetes | DSM 104344 [3)] | MH930821 |
| *Neonectria lugdunensis* (Saccardo & Therry) L. Lombard & Crous | Sordariomycetes | DSM 104361 [3), 4)] | MH930822 |
| *Tetracladium marchalianum* De Wild. | Leotiomycetes | DSM 104373 [3), 4)] | MH930823 |
| *Tricladium angulatum* Ingold | Leotiomycetes | DSM 104374 [3)] | MH930824 |

[1)] DSM accession number: Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany.
[2)] GenBank accession number (https://www.ncbi.nlm.nih.gov/genbank/)
[3)] Strain used in **Appendix A.1 and A.2**
[4)] Strain used in **Appendix A.3 and A.4**

## 4.2 PHASE 1: DESIGN AND VALIDATION OF FUNGAL SPECIES-SPECIFIC BIOMASS PROXIES

The first phase aimed at the design of species-specific qPCR assays for the ten model fungi (Table 4.1) as well as the *in silico* and experimental evaluation of these assays' species-specificity and built the basis for the following experimental phases **[Appendix A2-A4]**. Therefore, the ITS region (including the subregions ITS1, 5.8S, and ITS2) and the adjacent D1/D2 domain of the 28S large subunit (LSU) of representative target strains (Table 4.1) were used as template. Primers and probe of each assay were manually designed using the software Primer Express (version 2.0, Applied Biosystems, USA). For each assay, primer and probe sequences were validated *in silico* using sequences of target and closely related non-target species identified via GenBank's "basic local alignment search tool" algorithm (BLAST) in the International Nucleotide Sequence Database (INSD) and all sequences produced in this thesis. A detailed list of sequence accession numbers can be found in the supporting information of **Appendix A.1**. Once suitable primer and probe binding regions were visually identified, TaqMan® assays (primer and probe mix) were synthesized by Applied Biosystems (Germany). qPCR reactions were performed (in duplicate) in a Mastercycler® ep gradient S (Eppendorf, Germany) with a reaction volume of 10 µL. Each reaction consisted of 2 µL of sample, standard, or $ddH_2O$ and 8 µL of master mix. The latter was composed of 4 µL of 2x Environmental Mastermix 2.0 (Applied Biosystems, Germany), 3.48 µL of $ddH_2O$, and 0.52 µL of primer and probe mix. The qPCR cycling conditions, as recommended by the assay manufacturer, were as follows: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95 C and 1 min at 60 C. Fluorescence intensity was measured at the end of each cycle. Further details on the calculation of DNA concentrations can be found in **Appendix A.1**. For each assay, specificity for target DNA and PCR inhibition due to the presence of non-target DNA (from non-target fungal species and leaves) was experimentally cross-validated. Therefore, qPCR reactions were prepared with different combinations of target and non-target DNA standards (Fig. 4.2). In a final step, using the newly developed qPCR assays, intra- and inter-specific variability of DNA and ergosterol concentrations was compared in lyophilized mycelium of the ten model fungi (Table 4.1). Extraction and analysis of ergosterol in mycelium followed procedures described in Gessner and Chauvet (1993) and Gessner (2005). Ergosterol was extracted in alkaline methanol, purified via solid-phase extraction

(Sep-Pak® Vac RC tC18 500 mg sorbent, Waters, USA), and quantified via high-performance liquid chromatography (HPLC; 1200 Series, Agilent Technologies, USA). Both analytical methods applied during this phase were used to estimate fungal biomass in lyophilized mycelium and leaf samples in the remainder of this thesis.

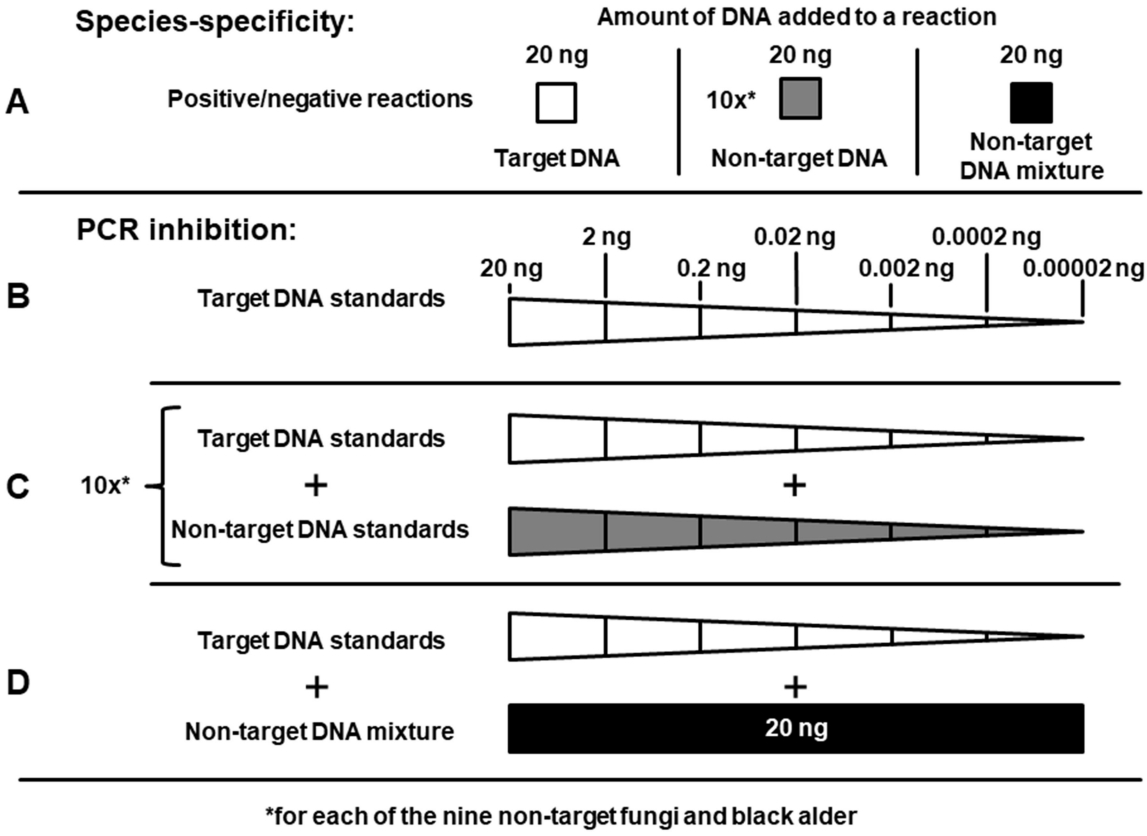



Fig. 4.2. Scheme illustrating the experimental design used in the first phase of this thesis **[Appendix A.1]**.

## 4.3 PHASE 2: VALIDATION OF FUNGAL BIOMASS PROXIES UNDER FUNGICIDE EXPOSURE

In the second phase, it was investigated whether fungal molecular biomarkers provide reliable biomass estimates for use in conjunction with test systems subjected to substances specifically targeting molecular processes involved in the membrane formation of fungal cells. Therefore, the demethylase inhibitor (DMI) tebuconazole, targeting an enzyme involved in the sterol biosynthesis pathway (i.e., sterol 14α-demethylase; ERG11; Fungicide Resistance Action Committee, 2020), was used as model substance. The ten model fungi (Table 4.1) were incubated in absence and

presence of a tebuconazole concentration of 35 µg/L (Fig. 4.3), potentially triggering effects on fungal biomarker levels, but at the same time not completely inhibiting fungal growth (as identified in a preliminary experiment; see supporting information of **Appendix A.2**). Each liquid culture consisted of 50-mL Erlenmeyer flasks filled with 30 mL of nutrient medium (i.e., malt extract solution). The culture flasks were added 0.5 mL of homogenized inoculum, prepared from colonized agar plugs and nutrient medium, and the respective tebuconazole concentration (i.e., 0 and 35 µg/L). The liquid cultures were incubated on a rotary shaker (model KS 15, Edmund Bühler GmbH, Germany) at 120 rpm, at 16°C, in darkness for 7 to 24 days (depending on the species' growth rate). After incubation, mycelium was sampled, lyophilized, and DNA and ergosterol concentrations were analyzed. Additionally, initial tebuconazole concentrations were verified by analysis of tebuconazole stock solutions and fungicide-free medium via a liquid chromatography/mass spectrometry system (LC/MS; Thermo Fisher Scientific, Germany). To assess overall and species-specific effects of tebuconazole, separate random-effects meta-analyses were performed on DNA and ergosterol data.

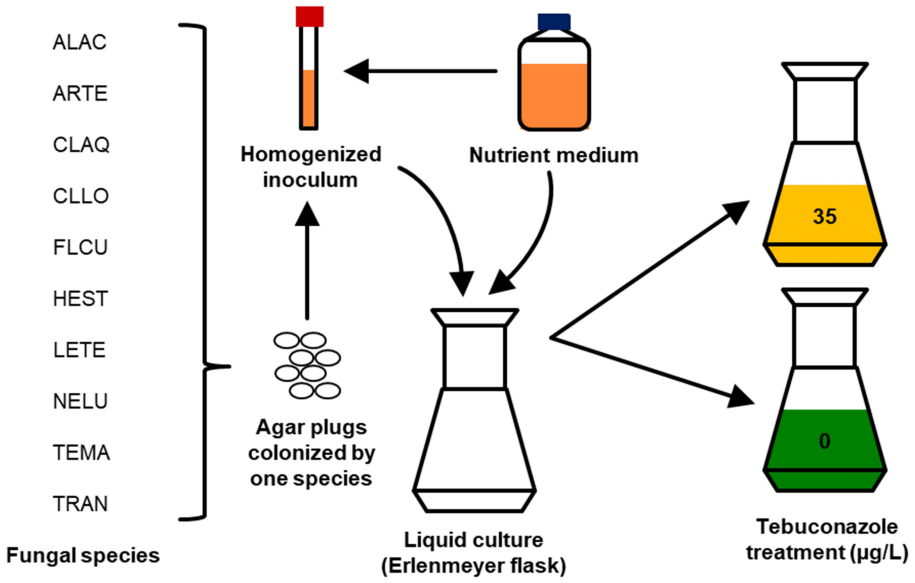



Fig. 4.3. Scheme illustrating the experimental design used in the second phase of this thesis (ALAC: *A. acuminata*, ARTE: *A. tetracladia*, CLAQ: *C. aquatica*, CLLO: *C. longibrachiata*, FLCU: *F. curvula*, HEST: *H. stellata*, LETE: *L. terrestris*, NELU: *N. lugdunensis*, TEMA: *T. marchalianum* and TRAN: *T. angulatum*). Image modified from **Appendix A.4**.

## 4.4 PHASE 3: CHARACTERIZATION OF FUNGAL B-EF RELATIONSHIPS

Via a laboratory microcosm experiment, the third phase aimed at unraveling B-EF relationships in fungal decomposer communities comprised of the four model fungi *A. acuminata*, *H. stellata*, *N. lugdunensis*, and *T. marchalianum* and a natural community of stream bacteria co-occurring with aquatic hyphomycetes in the environment. The bacterial inoculum consisted of size-fractionated stream water (≤ 1.2 µm), sampled from the near-natural stream Hainbach, Germany (49.24202 °N; 8.04586 °E). Fungal and bacterial inoculation of the microcosms largely followed methods described in Andrade et al. (2016) and Danger et al. (2013), respectively. Microcosms consisted of 50-mL Erlenmeyer flasks filled with nutrient medium (i.e., buffered mineral salt solution; Dang et al., 2005). In the microcosms, leaf disks were incubated in the presence of different combinations of the model fungi (colonized agar plugs covering 1, 2, or 4 species) and in absence (sterilized bacterial inoculum) or presence of stream bacteria (bacterial inoculum; Fig. 4.4). The flasks were maintained on a horizontal shaker (model VKS 75 B control; Edmund Bühler GmbH, Germany) at 110 rpm, at 16°C, in darkness for 14 days. The nutrient medium was renewed after 7 days. After incubation, leaves were sampled, lyophilized, leaf mass loss was determined, and DNA and ergosterol concentrations were analyzed. In microcosms incubated in presence of bacteria, bacterial densities were determined via epifluorescence microscopy according to Buesing (2005). To gain insight on the functional composition of the communities, activities of the hydrolytic enzymes phosphatase, α-1,4-glucosidase, β-1,4-glucosidase, cellobiohydrolase, β-1,4-xylosidase and the oxidative enzymes peroxidase and phenol oxidase were analyzed as described in DeForest (2009) modified for leaf samples (a detailed overview of the modifications is highlighted in the supporting information of **Appendix A.3**). To identify biodiversity effects (i.e., synergistic or antagonistic interactions), observations in monocultures were used to predict outcomes of mixed cultures, which were compared to actual observations in mixed cultures. In particular, the test design allowed for assessing mechanisms underlying biodiversity effects, using DNA concentrations in monoculture and mixed cultures in combination with a modified Price equation *sensu* Fox (2005; i.e., tripartite partition) **[Appendix A.3 and A.4]**.

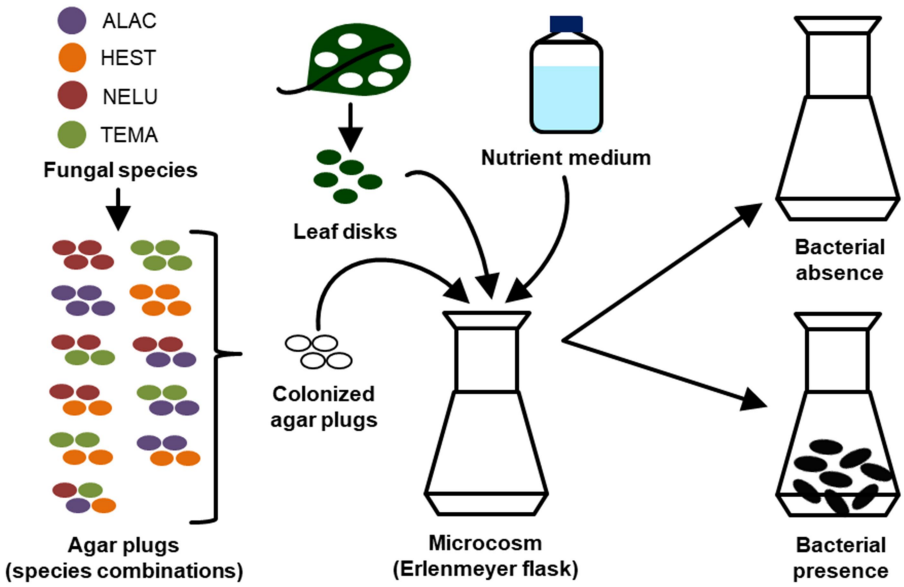



Fig. 4.4. Scheme illustrating the experimental design used in the third phase of this thesis (ALAC: *A. acuminata*, HEST: *H. stellata*, NELU: *N. lugdunensis* and TEMA: *T. marchalianum*). Image modified from **Appendix A.4**.

## 4.5 PHASE 4: FUNGICIDE IMPACTS ON FUNGAL B-EF RELATIONSHIPS

The fourth experimental phase of this thesis aimed at assessing fungicide impacts on B-EF relationships in fungal decomposer communities, which were identified in phase 3 of this thesis. Therefore, the experimental setup (except for the presenece of bacteria) and analytical methods developed for phase 3 were employed in a second microcosm experiment (Fig. 4.5). In this phase, the different combinations of the model fungi were exposed to a mixture of the fungicides with differing modes of action (i.e., azoxystrobin, carbendazim, cyprodinil, quinoxyfen, and tebuconazole) and a fungicide-free control. The applied fungicide concentrations covered environmentally relevant (5 and 50 µg/L) and worst-case exposure scenarios (500 and 2500 µg/L; Zubrod et al., 2019) to achieve a gradient from slight to pronounced effects on the fungal community functioning and structure (Zubrod et al., 2015a). Fungicide concentrations were measured in fresh and 7-day old medium via LC/MS. Biodiversity effects were evaluated as done in phase 3 of this thesis and observations in fungicide-treated cultures were compared to the respective fungicide-free control.

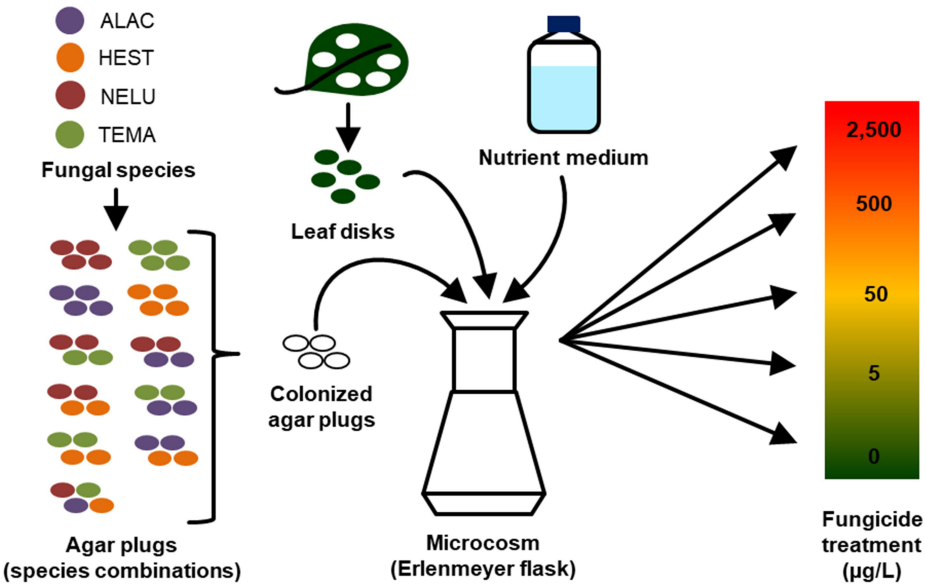



Fig. 4.5. Scheme illustrating the experimental design used in the fourth phase of this thesis (ALAC: *A. acuminata*, HEST: *H. stellata*, NELU: *N. lugdunensis* and TEMA: *T. marchalianum*). Image modified from **Appendix A.4**.

## 5. RESULTS

The results gained during the four experimental phases, which were part of this cumulative thesis, were documented in detail in four manuscripts and submitted to peer-reviewed journals (Fig. 4.1) **[Appendix A.1-A.4]**.

### 5.1 DESIGN AND VALIDATION OF FUNGAL SPECIES-SPECIFIC BIOMASS PROXIES

The qPCR assays were designed to bind to the ITS2 region, partially including the flanking 5.8S and LSU regions. Details on assay performances, including hybridization properties, efficiency, sensitivity, repeatability, and precision, are shown in Table 5.1. The *in silico* validation indicated species-specificity of qPCR assays for *A. acuminata*, *A. tetracladia*, *C. aquatica*, *C. longibrachiata*, *H. stellata*, and *T. angulatum* (Table 5.2). Other assays matched at least with one sequence of a non-target species (Table 5.2). Overall, qPCR assays matched approximately 90% to 100% of target species sequences deposited in the INSD (including sequences produced in this study), except of assays for *A. acuminata*, *N. lugdunensis*, and T. marchalianum (28%, 75%, and 74%, respectively; Table 5.2).

The experimental validation (setup is shown in Fig. 4.2) indicated that the qPCR assays are target-specific (reactions with non-target DNA mixtures were negative) and are not interfered by the presence of non-target DNA (amplification efficiency of target DNA is not affected by the presence of non-target DNA). Only the qPCR assay for *L. terrestris* was slightly affected by the presence of high DNA concentrations of *A. tetracladia*. Further details on the results of the experimental validation can be found in the supporting information of **Appendix A.1**.

Measured ergosterol and DNA concentrations in mycelium of the model fungi varied among the species by factors of approximately two and five, respectively, and inter- and average intraspecific variability in DNA concentrations was approximately twice as high as in ergosterol concentrations (Table 5.3). Yet, concentrations of both biomarkers were positively correlated among all fungal species (Fig. 5.1).

Table 5.1. Information on qPCR assay designations, targeted species, including the used model strain and template sequences as well as technical properties including melting temperature, guanine-cytosine content, binding region, amplicon length, efficiency parameters, inter- as well as intra-assay coefficients of variation (CV), limits of detection as well as quantification (expressed as cycle of quantification ($C_q$) and DNA concentration per PCR), and quantitative ranges covered by DNA standards **[Appendix A.1]**.

| qPCR assay design-nation | Target species | Target strain (DSM number) | Template ITS sequence (GenBank accession number) | Primer/ probe | Sequence (5'-3') | Primer/ probe length (bp) | Melting tem-perature (°C) | Guanine-cytosine content (%) | Binding region | Ampli-con length (bp) | Slope | $R^2$ | Ampl ifi-catio n effici ency (%) | Overall inter-assay variatio n and range (CV in %; *n*=7) | Overall intra-assay variatio n and range (CV in %; *n*=5) | Limit of detection | | Limit of quantification | | Quantitative standards covered by the assay (ng/µL) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | | | $C_q$ value | fg/ PCR | $C_q$ value | fg/ PCR | |
| ALAC-tqmn | *Alatospora acuminata* | DSM 104360 | **MH930815** | Forward | CGTAGTAATTTTCTCGCTTTGGAGA | 21 | 59.0 | 52 | ITS2 | 82 | -3.599 | 1.000 | 90 | 0.9 (0.6-1.1) | 0.4 (0.2-1.0) | 39.04 | 3.57 | 34.50 | 65.22 | $10\text{-}10^{-5}$ |
| | | | | Reverse | CCTGATCCGAGGTCAACCTTT | 21 | 59.0 | 52 | ITS2/LSU | | | | | | | | | | | |
| | | | | Probe | 6 FAM-TTTGCCAACAACCC-MGBNFQ | 14 | 68.0 | 50 | ITS2 | | | | | | | | | | | |
| ARTE-tqmn | *Articulospora tetracladia* | DSM 104345 | **MH930816** | Forward | GCCCTGTGGTATTCCGCA | 18 | 59.0 | 61 | 5.8S | 77 | -4.267 | 0.999 | 72 | 0.8 (0.2-1.6) | 0.7 (0.4-1.0) | 44.44 | 38.54 | 41.15 | 227.47 | $10\text{-}10^{-4}$ |
| | | | | Reverse | AGGCCCCAATACCAAGCG | 18 | 59.5 | 61 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-TCATTTCAACCCATCAAG-MGBNFQ | 18 | 68.0 | 39 | 5.8S/ITS2 | | | | | | | | | | | |
| CLAQ-tqmn | *Clavariopsis aquatica* | DSM 104362 | **MH930817** | Forward | AATTCATTGGCAGCCGGTAA | 20 | 59.0 | 45 | ITS2 | 82 | -3.435 | 1.000 | 95 | 0.6 (0.4-1.1) | 0.6 (0.2-0.9) | 40.08 | 0.65 | 35.79 | 11.56 | $10\text{-}10^{-5}$ |
| | | | | Reverse | AAGAGGCTTGATGGAAGGAGGTA | 23 | 59.2 | 48 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-TGTGTCGCGATCCAGT-MBGNFQ | 16 | 70.0 | 56 | ITS2 | | | | | | | | | | | |
| CLLO-tqmn | *Clavatospora longibrachiata* | DSM 104365 | **MH930818** | Forward | GTTTGACCAAGACACAGCAAAATG | 24 | 59.6 | 42 | ITS2 | 89 | -3.450 | 1.000 | 95 | 0.4 (0.2-0.6) | 0.9 (0.3-2.0) | 41.55 | 3.26 | 37.09 | 63.99 | $10\text{-}10^{-5}$ |
| | | | | Reverse | CTACGTTTCTCTTACGAAAAACTGTTTGT | 29 | 59.6 | 34 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-CTGGAAATTTGAACTATTTG-MGBNFQ | 20 | 69.0 | 30 | ITS2 | | | | | | | | | | | |
| FLCU-tqmn | *Flagellospora curvula* | DSM 104334 | **MH930819** | Forward | CTCAAGCTCTGCTTGGTATTGG | 22 | 57.8 | 50 | ITS2 | 108 | -3.339 | 1.000 | 99 | 0.7 (0.6-0.9) | 1.0 (0.4-2.2) | 39.16 | 0.72 | 35.03 | 12.33 | $10\text{-}10^{-5}$ |
| | | | | Reverse | GGTCCTCCAGAGCGAGAAGA | 20 | 58.1 | 60 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-CGCCTTAAAGACAGTGGC-MGBNFQ | 18 | 70.0 | 56 | ITS2 | | | | | | | | | | | |
| HEST-tqmn | *Heliscella stellata* | DSM 104386 | **MH930820** | Forward | GCGTCGTAGTTTGACCAAGACA | 22 | 58.9 | 50 | ITS2 | 79 | -3.445 | 0.999 | 95 | 0.8 (0.5-1.2) | 0.5 (0.1-1.2) | 41.41 | 7.71 | 37.05 | 142.13 | $10\text{-}10^{-4}$ |
| | | | | Reverse | AAACAGATGATTCAAGGCTAATCGT | 25 | 58.3 | 36 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-CAATGTGAAACTAAGTCAGATAA-MGBNFQ | 23 | 70.0 | 30 | ITS2 | | | | | | | | | | | |
| LETE-tqmn | *Lemonniera terrestris* | DSM 104344 | **MH930821** | Forward | AGCGTCATTACAACCCCTCAAG | 22 | 59.1 | 50 | 5.8S/ITS2 | 81 | -3.498 | 1.000 | 93 | 0.6 (0.4-1.2) | 0.6 (0.1-1.6) | 40.58 | 2.01 | 36.13 | 37.55 | $10\text{-}10^{-5}$ |
| | | | | Reverse | ATGGCACCGCCACTGAGT | 18 | 58.6 | 61 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-TGGTATTGGAGCATGCG-MGBNFQ | 17 | 70.0 | 53 | ITS2 | | | | | | | | | | | |
| NELU-tqmn | *Neonectria lugdunensis* | DSM 104361 | **MH930822** | Forward | TGTAGCTTCCTCTGCGTAGTAGCA | 24 | 59.0 | 50 | ITS2 | 90 | -3.431 | 1.000 | 96 | 0.9 (0.5-1.8) | 0.5 (0.1-1.3) | 38.02 | 3.52 | 33.73 | 62.56 | $10\text{-}10^{-5}$ |
| | | | | Reverse | CCGAGGTCAACCTTTCAGAAGT | 22 | 58.0 | 50 | ITS2/LSU | | | | | | | | | | | |
| | | | | Probe | 6 FAM-TCGCACTGGAAAGC-MGBNFQ | 14 | 69.0 | 57 | ITS2 | | | | | | | | | | | |
| TEMA-tqmn | *Tetracladium marchalianum* | DSM 104373 | **MH930823** | Forward | GCTGTCAGGCTCTAAGCGTAGTAA | 24 | 58.0 | 50 | ITS2 | 64 | -3.484 | 1.000 | 94 | 0.6 (0.3-1.2) | 0.5 (0.1-0.9) | 39.11 | 2.03 | 34.75 | 36.16 | $10\text{-}10^{-5}$ |
| | | | | Reverse | GTTCTGGCGAGTGTCCATCA | 20 | 58.0 | 55 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-CTCTCTCGCTACAGACAC-MGBNFQ | 18 | 69.0 | 56 | ITS2 | | | | | | | | | | | |
| TRAN-tqmn | *Tricladium angulatum* | DSM 104374 | **MH930824** | Forward | CCTGTTCGAGCGTCATCAAA | 20 | 58.5 | 50 | 5.8S/ITS2 | 129 | -3.523 | 1.000 | 92 | 0.6 (0.3-1.1) | 0.5 (0.2-1.8) | 42.16 | 0.72 | 37.68 | 13.36 | $10\text{-}10^{-5}$ |
| | | | | Reverse | CCGACGTCTATAGCGAGAAGAATT | 24 | 59.0 | 46 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-CCTGGCAGCCCTTA-MGBNFQ | 14 | 68.0 | 64 | ITS2 | | | | | | | | | | | |

Table 5.2. List of assay compatible species identified during the *in silico* validation as well as numbers of related INSD entries covering the full assay target region and the number of entries being 100% compatible with the respective assay. Species targeted by the respective assay are printed in bold. Values in parentheses indicate the proportion of sequences produced in this study, included in the number of INSD sequences outside the parentheses. For a complete list of INSD entries used for the *in silico* validation, see **Appendix A.1** Table S2 **[Appendix A.1]**.

| | | INSD entries | | |
|---|---|---|---|---|
| qPCR assay designation | Species | Number of entries covering the full assay target region | Number of entries showing 100% similarity with all assay sequences | Entry coverage (%) |
| ALAC-tqmn | ***Alatospora acuminata*** | 18 (8) | 5 (2) | 28 |
| ARTE-tqmn | ***Articulospora tetracladia*** | 172 (6) | 161 (5) | 94 |
| CLAQ-tqmn | ***Clavariopsis aquatica*** | 13 (3) | 13 (3) | 100 |
| CLLO-tqmn | ***Clavatospora longibrachiata*** | 6 (4) | 6 (4) | 100 |
| FLCU-tqmn | ***Flagellospora curvula*** | 8 (3) | 7 (3) | 88 |
| | *F. fusarioides* | 1 | 1 | 100 |
| HEST-tqmn | ***Heliscella stellata*** | 2 (2) | 2 (2) | 100 |
| LETE-tqmn | *Lemonniera aquatica* | 9 (3) | 8 (3) | 89 |
| | *L. centrosphaera* | 3 (1) | 3 (1) | 100 |
| | *L. cornuta* | 2 | 2 | 100 |
| | ***L. terrestris*** | 7 (3) | 7 (3) | 100 |
| | *Margaritispora aquatica* | 2 (2) | 2 (2) | 100 |
| NELU-tqmn | *Cylindrocarpon obtusisporum* | 12 | 6 | 50 |
| | ***Neonectria lugdunensis*** | 36 (14) | 27 (6) | 75 |
| | *Phaeoacremonium vibratile* | 1 | 1 | 100 |
| TEMA-tqmn | *Tetracladium breve* | 7 (1) | 1 (0) | 14 |
| | ***T. marchalianum*** | 77 (6) | 57 (5) | 74 |
| TRAN-tqmn | ***Tricladium angulatum*** | 11 (3) | 11 (3) | 100 |

Table 5.3. Incubation time and mean ergosterol as well as DNA concentrations (with 95% confidence intervals and coefficients of variation (CV)) in freeze-dried liquid cultures of the target species **[Appendix A.1]**.

| | Liquid culture incubation time (d) | Ergosterol | | DNA | |
|---|---|---|---|---|---|
| Species | | Concentration (µg/mg) | CV (%) | Concentration (ng/mg) | CV (%) |
| *Alatospora acuminata* | 18 | 5.75 (4.88 - 6.63) | 12 | 548.93 (475.62 - 622.25) | 11 |
| *Articulospora tetracladia* | 17 | 3.28 (3.05 - 3.52) | 5 | 112.70 (41.70 - 183.72) | 40 |
| *Clavariopsis aquatica* | 24 | 6.10 (5.48 - 6.71) | 8 | 227.20 (169.23 - 285.18) | 21 |
| *Clavatospora longibrachiata* | 17 | 5.69 (5.25 - 6.14) | 6 | 527.14 (418.78 - 635.50) | 17 |
| *Flagellospora curvula* | 17 | 5.86 (4.35 - 7.37) | 21 | 481.28 (239.20 - 723.37) | 41 |
| *Heliscella stellata* | 18 | 4.41 (3.60 - 5.22) | 15 | 164.71 (129.46 - 199.95) | 17 |
| *Lemonniera terrestris* | 20 | 3.18 (2.89 - 3.47) | 7 | 114.73 (61.56 - 167.90) | 37 |
| *Neonectria lugdunensis* | 7 | 2.95 (2.45 - 3.45) | 14 | 381.60 (232.04 - 531.16) | 32 |
| *Tetracladium marchalianum* | 14 | 2.67 (0.80 - 4.55) | 44 | 212.39 (133.95 - 290.84) | 23 |
| *Tricladium angulatum* | 17 | 3.83 (3.38 - 4.27) | 9 | 171.24 (128.95 - 213.53) | 20 |

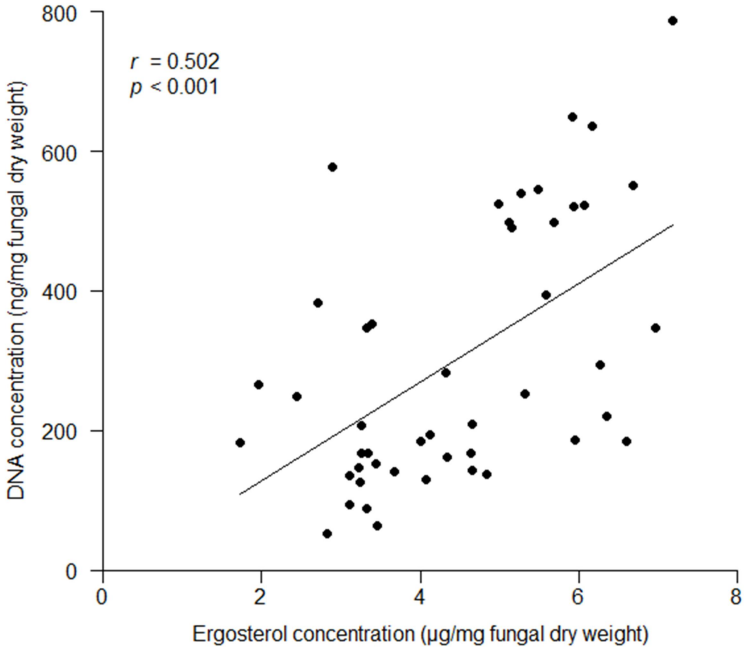



Fig. 5.1. Correlation between ergosterol (µg/mg of fungal dry weight) and DNA concentrations (ng/mg of fungal dry weight) in the tested strains with Spearman's correlation coefficient (*r*) and *p*-value. The regression line was added to visualize the positive relationship between the variables. Note that the initial number of replicates was reduced to *N* = 48 due to the loss of two replicates through contamination **[Appendix A.1]**.

## 5.2 VALIDATION OF FUNGAL BIOMASS PROXIES UNDER FUNGICIDE EXPOSURE

Exposure to 35 µg/L tebuconazole on average reduced ergosterol concentrations in fungal mycelium by 13% (Fig. 5.2a) and increased DNA concentrations by 13% (Fig. 5.2b), compared to fungicide-free controls. These effects were, however, species-dependent (Table 5.4). Contrary to the overall effects, tebuconazole exposure lead to increased ergosterol concentrations in *F. curvula*

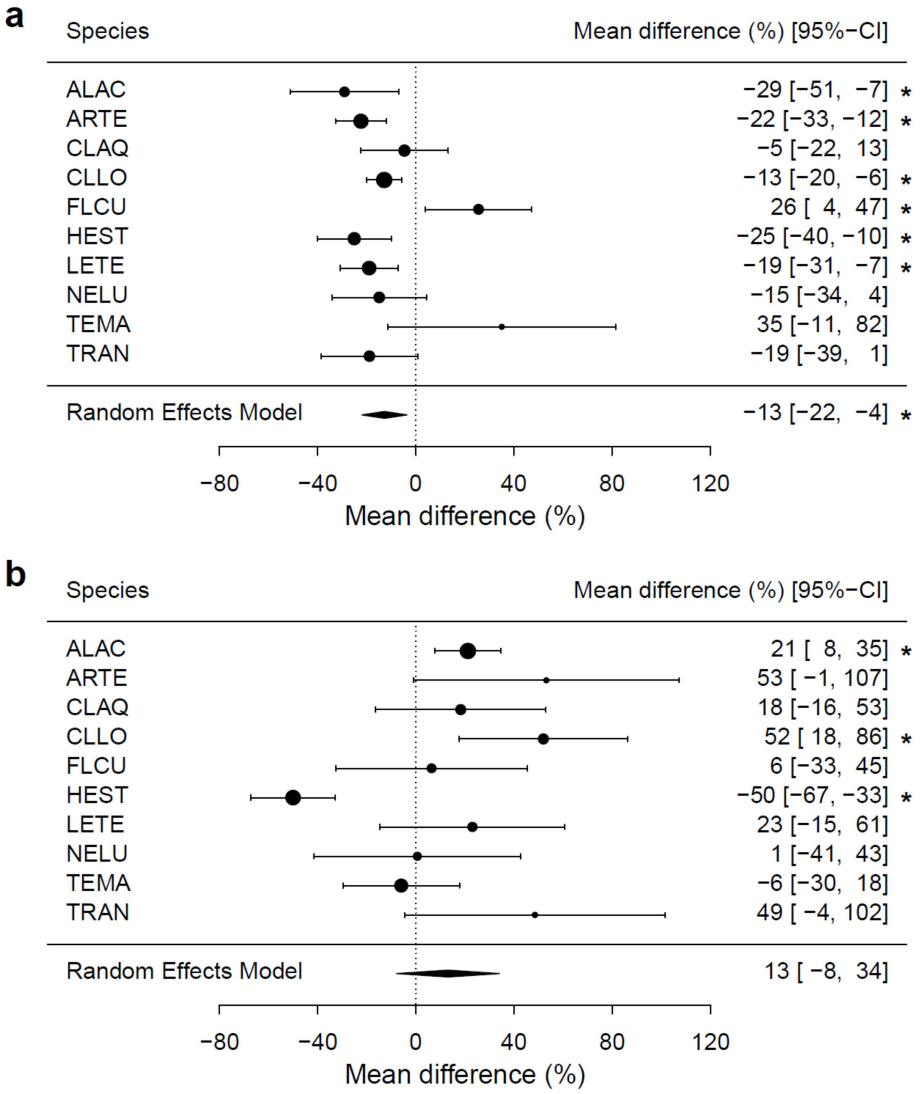



Fig. 5.2. Relative mean differences (points; ± 95%-CIs) in (a) fungal species' ergosterol and (b) DNA concentrations between tebuconazole-treated and control cultures, obtained via random-effects meta-analyses. Means on the right and the left side of the dotted line indicate higher and lower biomarker concentrations in tebuconazole-treated cultures, respectively. Point sizes indicate the weight (i.e. the inverted variance) of the respective species to the overall effect (diamonds). Fungal biomarker concentrations are significantly altered by tebuconazole-treatment, if CIs do not include zero (i.e. $p < 0.05$; ALAC: *A. acuminata*, ARTE: *A. tetracladia*, CLAQ: *C. aquatica*, CLLO: *C. longibrachiata*, FLCU: *F. curvula*, HEST: *H. stellata*, LETE: *L. terrestris*, NELU: *N. lugdunensis*, TEMA: *T. marchalianum* and TRAN: *T. angulatum*) **[Appendix A.2]**.

(26%) and *T. marchalianum* (35%; Fig. 5.2a) and reduced DNA concentrations in *H. stellata* (50%) and *T. marchalianum* (6%; Fig. 5.2b).

Table 5.4. ANOVA tables for ergosterol and DNA concentrations **[Appendix A.2]**.

| Biomarker | Source of variation | *df* | *SS* | *MS* | *F* | *p* |
|---|---|---|---|---|---|---|
| Ergosterol | Species | 9 | 165.14 | 18.349 | 40.649 | <0.001 |
| | Tebuconazole | 1 | 4.21 | 4.205 | 9.316 | 0.003 |
| | Species × tebuconazole | 9 | 18.19 | 2.021 | 4.476 | <0.001 |
| | Residuals | 76 | 34.31 | 0.451 | | |
| DNA | Species | 9 | 3632707 | 403634 | 50.524 | <0.001 |
| | Tebuconazole | 1 | 72420 | 72420 | 9.065 | 0.004 |
| | Species × tebuconazole | 9 | 199220 | 22136 | 2.771 | 0.007 |
| | Residuals | 76 | 607157 | 7989 | | |

## 5.3 CHARACTERIZATION OF FUNGAL B-EF RELATIONSHIPS

In monoculture, the four model fungi showed marked differences in leaf mass loss and fungal biomass production (based on ergosterol concentrations), with *A. acuminata* and *H. stellata* being less and *N. lugdunensis* and *T. marchalianum* being more productive (Fig. 5.3). In fungal mixed cultures, leaf mass loss and total fungal biomass did not or only marginally exceed performances of the most productive monoculture *T. marchalianum* (Fig. 5.3). The presence of bacteria resulted in lower leaf mass loss, except in the less productive species *A. acuminata* and *H. stellata* as well as their combination (Fig. 5.3a). Fungal biomass production was overall reduced by bacterial presence, while *N. lugdunensis* and

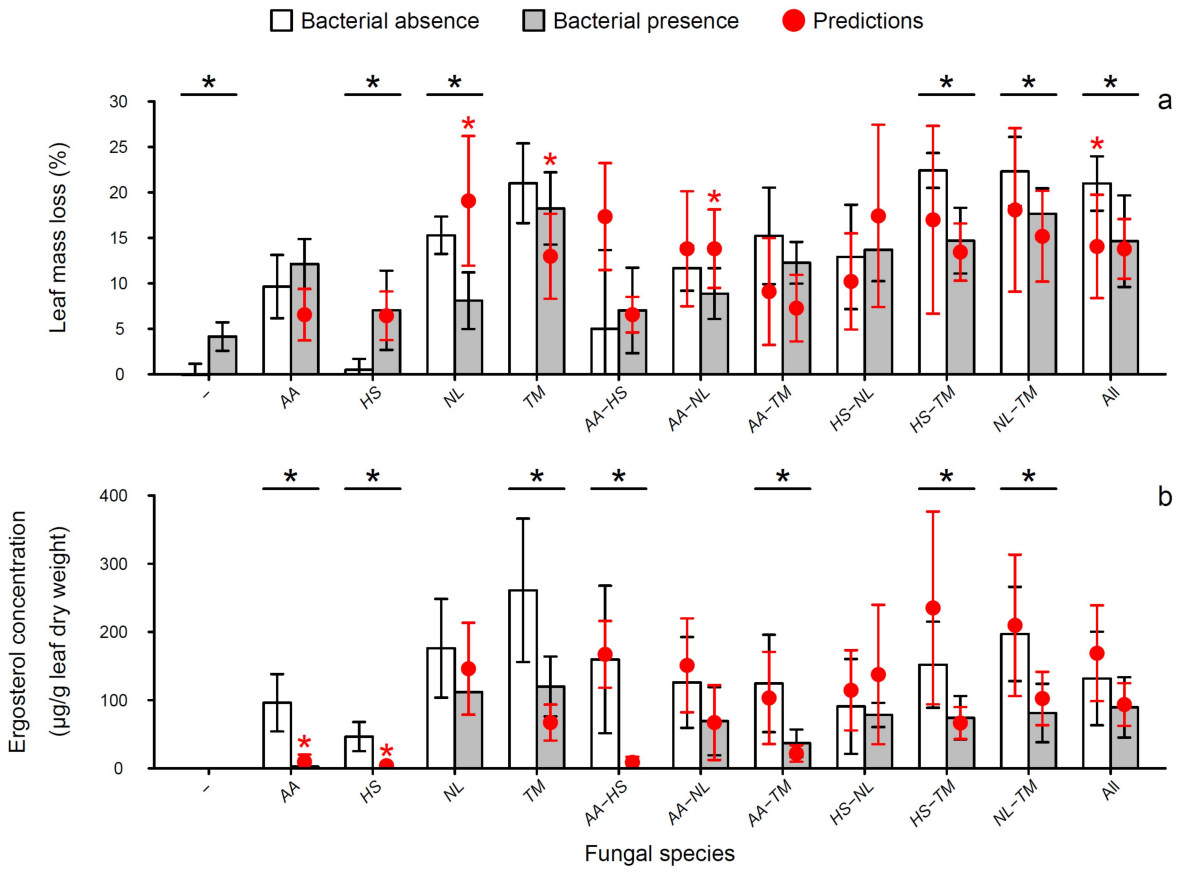



Fig. 5.3. (a) Mean leaf mass loss (in percent; relative to the initial leaf dry weight) and (b) ergosterol concentrations (in µg/g leaf dry weight; with 95%-CIs, respectively; *n*=6) in fungal cultures in absence (white bars) and presence of bacteria (grey bars). Red points indicate predictions based on fungal and bacterial biomass-specific leaf mass loss or ergosterol concentrations. Red asterisks indicate significant differences between observed and predicted leaf mass loss or ergosterol concentrations of fungal treatments containing more than one organism (i.e., presence of bacteria or further fungi). Black asterisks indicate significant differences in leaf mass loss or ergosterol concentrations between bacteria-free and bacteria-containing cultures (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*) **[Appendix A.3]**.

*T. marchalianum* were least affected (Fig. 5.3b). Generally, observed functional performances largely matched the predictions based on microbial biomass (i.e., fungal DNA and bacterial counts), while the exceptions indicate potential synergistic or antagonistic interactions between fungi and bacteria (Fig. 5.3) **[Appendix A.3]**.

When leaf mass loss is normalized to fungal biomass (i.e., ergosterol concentrations), *A. acuminata*, *N. lugdunensis*, and *T. marchalianum* showed a nearly identical leaf decomposition efficiency, which is substantially higher compared to *H. stellata* (Fig. 5.4a) **[Appendix A.3]**. While the fungi were able to produce all of the investigated enzymes (Fig. 5.5), the biomass-specific enzyme activity profiles of

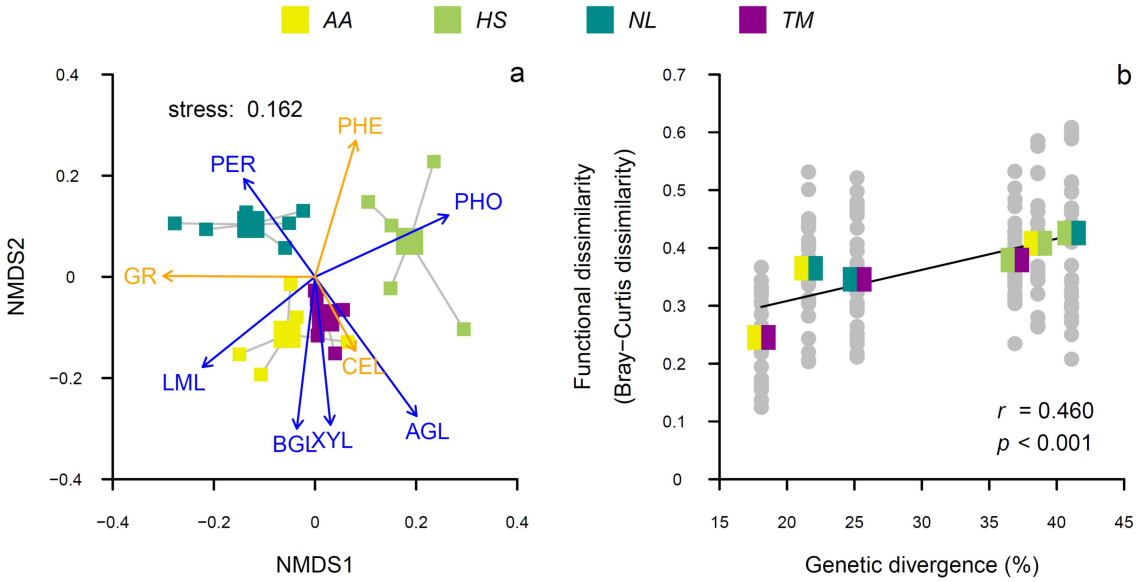



Fig. 5.4. (a) Non-metric multidimensional scaling (NMDS) plot for biomass-specific functional performances of the fungi (i.e., leaf mass loss (LML), enzyme activities (for abbreviations see below), and growth rates (GR) based on ergosterol concentrations) using Bray-Curtis dissimilarity. Group centroids and replicates are displayed as large and small squares, respectively. Blue and orange arrows display functional performances significantly and non-significantly contributing to the separation between the species, respectively (LML: $p$=0.002; GR: $p$=0.096; PHO: $p$=0.001; AGL: $p$=0.001, BGL: $p$=0.001; CEL: $p$=0.521; XYL: $p$=0.001; PER: $p$=0.011; PHE: $p$=0.122). The provided stress value indicates a reasonable fit (i.e., < 0.2; Clarke, 1993). (b) Correlation between functional dissimilarity (Bray-Curtis dissimilarity) and genetic divergence of pairs of fungi (grey circles; dissimilarities were calculated for all possible replicate combinations of two cultures). Mean functional dissimilarities between two fungi are displayed as colored squares. Pearson's correlation coefficient ($r$) and $p$-value indicate a weak but statistically significant positive correlation (Hinkle et al., 2003). The regression line was added to visualize the relationship between the variables (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, *TM*: *T. marchalianum*, PHO: phosphatase, AGL: α-1,4-glucosidase, BGL: β-1,4-glucosidase, CEL: cellobiohydrolase, XYL: β-1,4-xylosidase, PER: peroxidase, and PHE: phenol oxidase). Note the reduced sample size for *A. acuminata*, *H. stellata*, and *T. marchalianum* due to missing ergosterol or enzyme activity data for one replicate, respectively ($n$=5). Figure modified from **Appendix A.3**.

*A. acuminata* and *T. marchalianum* were characterized by hydrolases, whereas the profile of *N. lugdunensis* was characterized by oxidases (Fig. 5.4a) **[Appendix A.3]**. The profile of *H. stellata* contained only two of the investigated enzymes, namely α-1,4-glucosidase and phosphatase (Fig. 5.4a) **[Appendix A.3]**. Particularly biomass-specific activities of phosphatase, α-1,4-glucosidase, β-1,4-glucosidase, β-1,4-xylosidase, and peroxidase were driving functional separation between the species (Fig. 5.4a). Functional dissimilarity between pairs of two species – calculated from biomass-specific leaf mass loss, growth rates, and enzyme activities **[Appendix A.3]** – was positively correlated with genetic divergence (Fig. 5.4b). In fungal mixed cultures, and similarly as for leaf mass loss and fungal biomass production, enzyme activities did not exceed those observed in the most productive monocultures *N. lugdunensis* and *T. marchalianum* **[Appendix A.3]**. Bacterial presence generally induced a shift in enzyme profiles towards higher activities

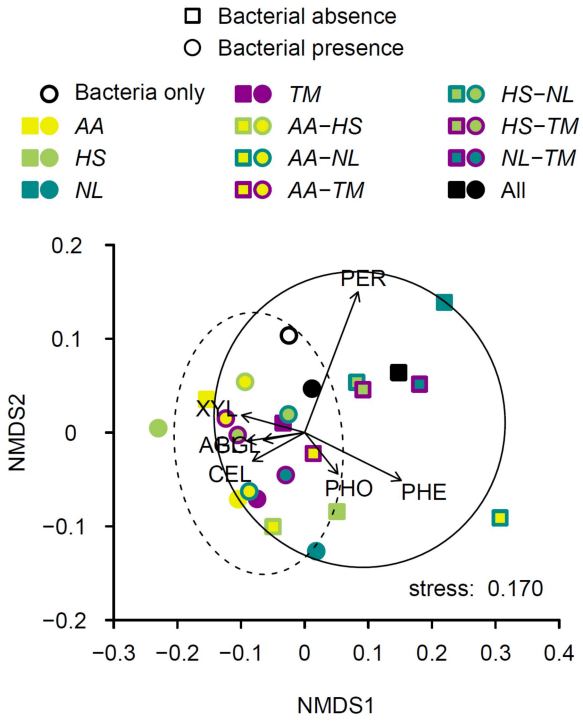



Fig. 5.5. Non-metric multidimensional scaling (NMDS) plot for enzyme activities using Bray-Curtis dissimilarity. Group centroids are displayed as colored symbols (for individual replicates see **Appendix A.3** Appendix S2: Fig. S4). Squares and circles indicate bacterial absence and presence, respectively. Solid and dashed ellipses indicate group clusters of cultures in absence and presence of bacteria, respectively. Arrows display enzyme activities significantly contributing to the separation between treatments ($p$=0.001-0.003). The provided stress value indicates a reasonable fit (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, *TM*: *T. marchalianum*, PHO: phosphatase, AGL: α-1,4-glucosidase, BGL: β-1,4-glucosidase, CEL: cellobiohydrolase, XYL: β-1,4-xylosidase, PER: peroxidase, and PHE: phenol oxidase) **[Appendix A.3]**.

of α-1,4-glucosidase, β-1,4-glucosidase, cellobiohydrolase, and β-1,4-xylosidase and lower activities of phosphatase, peroxidase, and phenol oxidase (Fig. 5.5). Moreover, particularly for peroxidase and phenol oxidase, observed activities were significantly lower than predicted based on microbial biomass (i.e., fungal DNA concentrations and bacterial counts), indicating potential interactions between fungi and bacteria **[Appendix A.3]**.

Changes in the biomass of individual fungi (based on DNA concentrations) were driven by the interplay of fungal diversity, species identity, and bacterial presence (Fig. 5.6) **[Appendix A.3]**. With few exceptions, biomass of individual fungi was lower in mixed cultures in absence and presence of bacteria compared to the respective bacteria-free and bacteria-containing fungal monoculture, respectively

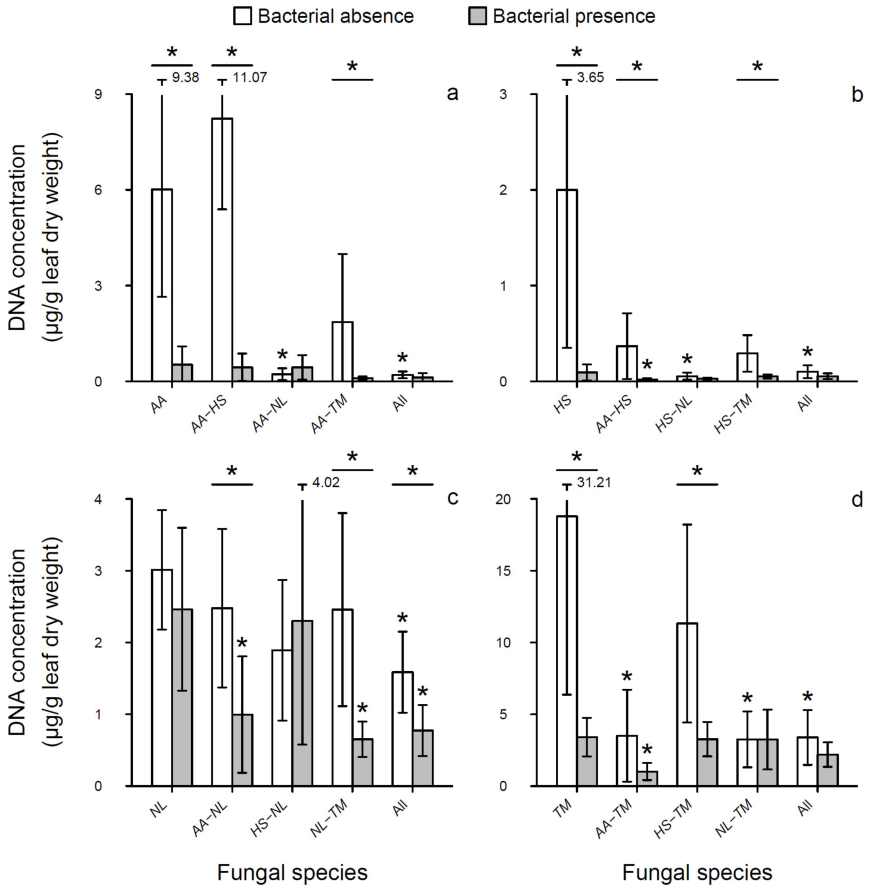



Fig. 5.6. Mean species-specific DNA concentrations (µg DNA/g leaf dry weight) of (a) *A. acuminata*, (b) *H. stellata*, (c) *N. lugdunensis*, and (d) *T. marchalianum* (with 95%-CIs) in mono, binary, and quaternary cultures in absence and presence of bacteria. Asterisks above bars indicate significant differences to the monoculture within the same bacteria treatment and asterisks above horizontal lines indicate significant differences between bacteria-free and bacteria-containing cultures within the same fungal combination (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*) **[Appendix A.3]**.

(Fig. 5.6). Only for *A. acuminata*, a (non-significantly) higher biomass was observed when co-cultured with *H. stellata* (Fig. 5.6a), indicating potential interactions between these species. Biomass of *H. stellata* was considerably reduced if any of the other fungi was present (Fig. 5.6b). Moreover, in mixed cultures, in particular the presence of *N. lugdunensis* had a substantial negative impact on biomass of the other fungi (Fig. 5.6a, b, d).

In absence of bacteria, net diversity effects on leaf colonization in fungal mixed cultures (based on fungal DNA concentrations in monocultures and mixed cultures) were on average negative (-14%; Fig. 5.7). These effects were driven by trait-independent complementarity effects (contributing 114%), while dominance and trait-dependent effects were opposed (contributing -8% and -6%, respectively; Fig. 5.7). In presence of bacteria, biodiversity effects were on average positive (43%) and driven by trait-independent complementarity, dominance, and trait-dependent complementarity effects (contributing 51%, 34%, and 17%, respectively; Fig. 5.7).

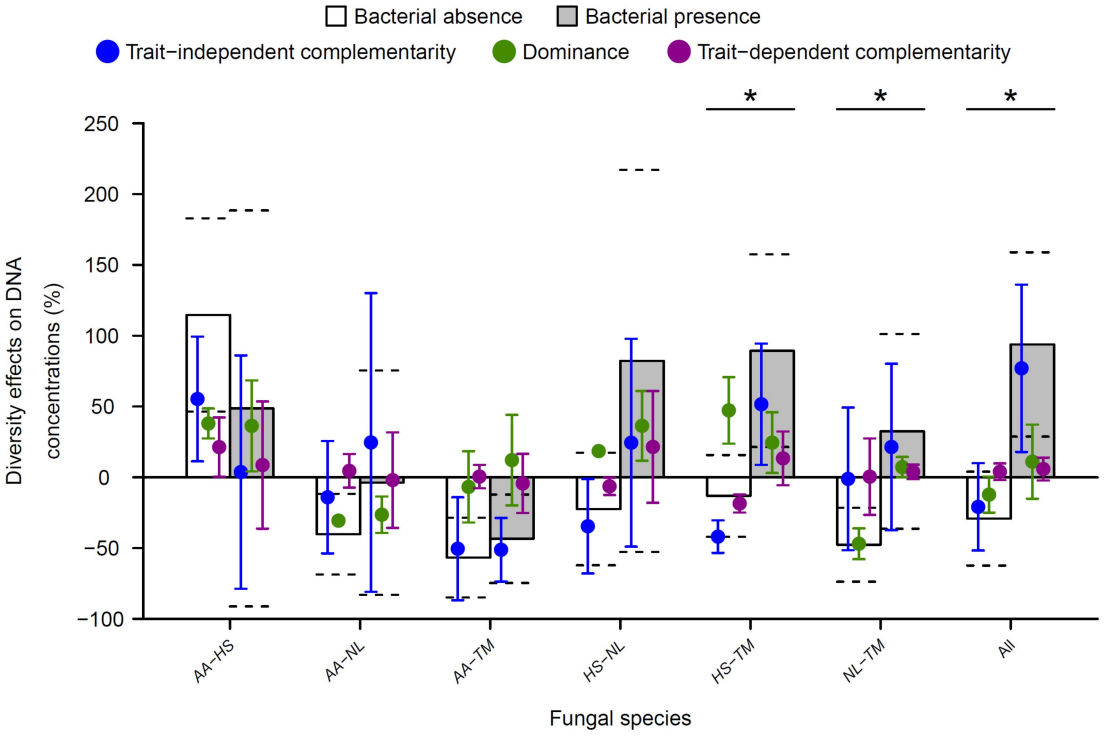



Fig. 5.7. Mean diversity effects on DNA concentrations (in percent; with 95%-CIs; *n*=6) in fungal mixed cultures based on the tripartite partitioning model. Bars indicate mean net diversity effects on fungal leaf colonization in absence (white bars) and presence of bacteria (grey bars), which are composed of the sum of trait-independent complementarity (blue points), dominance (green points), and trait-dependent complementarity effects (purple points). Asterisks indicate significant differences in net diversity effects between bacteria-free and bacteria-containing cultures (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*) **[Appendix A.3]**.

In absence of bacteria, net diversity effects were significantly negatively and positively correlated with average growth rates (Fig. 5.8a) and genetic divergence of the fungi, respectively (Fig. 5.8b). In presence of bacteria, net diversity effects were no more correlated with average growth rates (Fig. 5.8c) but still showed a significant dependence on genetic divergence (Fig. 5.8d).

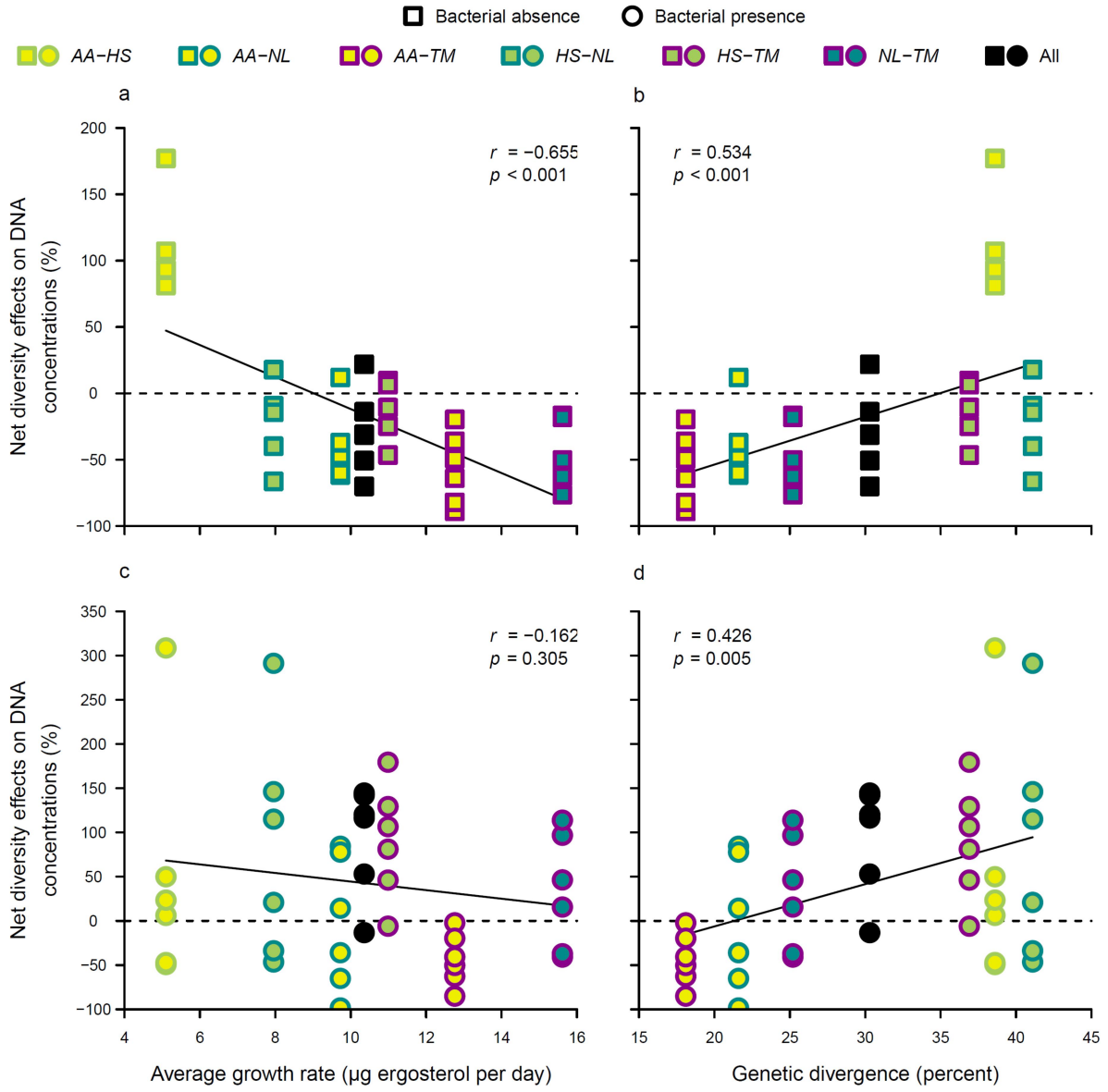



Fig. 5.8. Correlations between net diversity effects on DNA concentrations and (a, c) average growth rates or (b, d) genetic divergence of fungal mixed cultures in (a, b) bacterial absence and (c, d) presence, with Pearson's correlation coefficients (*r*) and *p*-values. The regression lines were added to visualize the relationships between the variables (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*) **[Appendix A.3]**.

## 5.4 FUNGICIDE IMPACTS ON FUNGAL B-EF RELATIONSHIPS

Overall, leaf mass loss, fungal biomass production, and enzyme activities in monocultures and mixed cultures not subjected to fungicide exposure were largely consistent with observations in bacteria-free cultures during the third phase **[Appendix A.3]**. Virtually no leaf mass loss was observed in monocultures of *A. acuminata* and *H. stellata*, which is why potential fungicide effects on this function could not be detected for these species (Fig. 5.9a). *N. lugdunensis* and

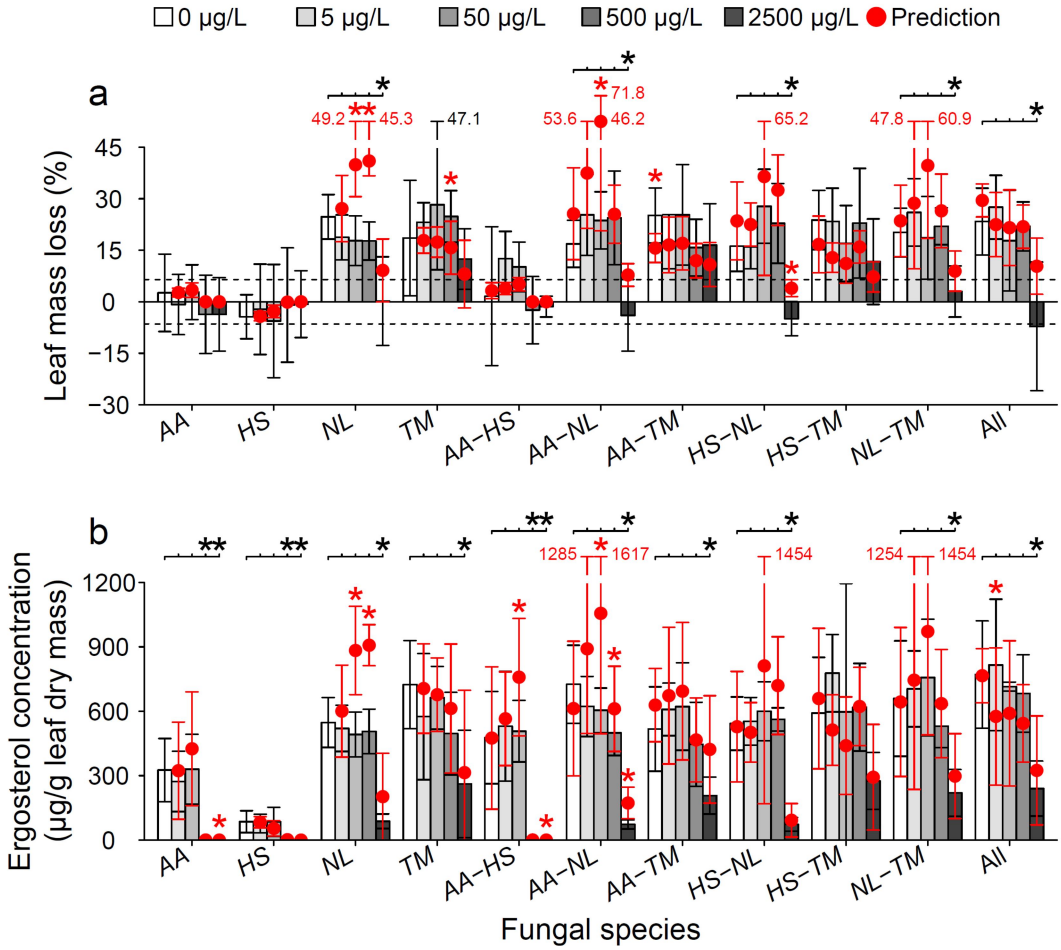



Fig. 5.9. Mean (a) leaf mass loss and (b) ergosterol concentrations (with 95%-CIs, respectively) in fungal species treatments exposed to fungicide sum concentrations of 0, 5, 50, 500, and 2500 µg/L (gray-scale bars) and mean predictions (red points with 95%-CIs) based on biomass-specific leaf mass loss or ergosterol concentrations. Solid and dashed horizontal lines indicate the mean and 95%-confidence interval, respectively, of the sterile control (a). Black asterisks indicate significant differences between fungicide treatments and fungicide-free controls within fungal species treatments (Dunnett's test; $p < 0.05$; $n = 5$). Red asterisks indicate significant differences between observed and predicted variables (paired *t*-test; $p < 0.05$; $n = 5$). Note that predictions for each species are based on biomass-specific leaf mass loss and ergosterol concentrations in fungicide-free monocultures (therefore, mean predictions equal mean observations in these treatments; *AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*) **[Appendix A.4]**.

*T. marchalianum* were capable of decomposing leaves up to a fungicide concentration of 2500 µg/L, while at this concentration only for *N. lugdunensis* this function was significantly reduced (Fig. 5.9a). Biomass of *A. acuminata* and *H. stellata* was not affected by fungicide concentrations of up to 50 µg/L but tended to be very low or zero at higher fungicide concentrations (Fig. 5.9b). *N. lugdunensis* and *T. marchalianum*, in contrast, were able to produce biomass up to the highest fungicide concentration of 2500 µg/L, although, at this concentration, significantly less compared to the controls (Fig. 5.9b). In line with observations on leaf mass loss and fungal biomass (Fig. 5.9), enzyme activity profiles were only affected at high fungicide concentrations of ≥ 500 µg/L (Fig. 5.10). In mixed cultures, leaf mass loss,

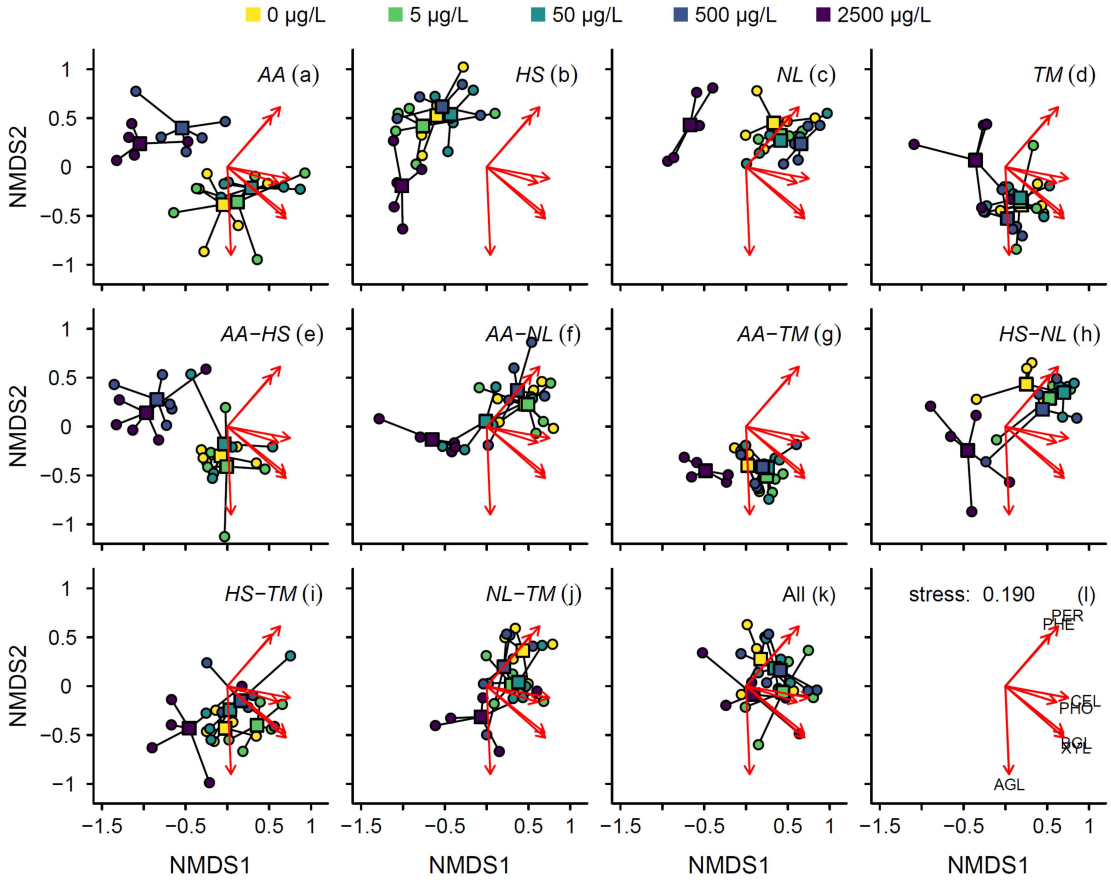



Fig. 5.10. Non-metric multidimensional scaling (NMDS) plots for enzyme activity profiles using Euclidean distance. Data shown in panels (a–l) originate from a common distance matrix. The panels show enzyme profiles for monocultures and mixed cultures exposed to fungicide concentrations of 0, 5, 50, 500, and 2500 µg/L. Group centroids and replicates are displayed as squares and circles, respectively. Red arrows display enzyme activities. The provided stress value indicates a reasonable fit (i.e., < 0.2; Clarke, 1993). For the impact of the treatment factors on enzyme activity profiles, see **Appendix A4** Table S3 (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, *TM*: *T. marchalianum*, PHO: phosphatase, AGL: α-1,4-glucosidase, BGL: β-1,4-glucosidase, CEL: cellobiohydrolase, XYL: β-1,4-xylosidase, PER: peroxidase, and PHE: phenol oxidase). Image modified from **Appendix A.4**.

fungal biomass production, and enzyme activity profiles largely reflected functional patterns observed in most productive and tolerant species in the assemblage, irrespective of the applied fungicide concentration (Fig. 5.9; Fig. 5.10). For monocultures and mixed cultures, predictions of leaf mass loss and fungal biomass production (i.e., ergosterol concentrations) based on measured fugal DNA concentrations largely met observations, while the majority of the few significant deviations concerned fungicide-exposed cultures containing *N. lugdunensis* (i.e., mostly over-predictions; Fig. 5.9). In particular, deviations between predicted and observed fungal biomass production occurring under fungicide exposure indicate discrepancies between ergosterol (Fig. 5.9b) and DNA concentrations (Fig 5.11c) as fungal biomass proxies, as investigated in the second phase **[Appendix A.2]**.

With few exceptions (see above), DNA concentrations in monocultures (Fig 5.11) were in accordance with observed ergosterol concentrations (Fig. 5.9b). In binary

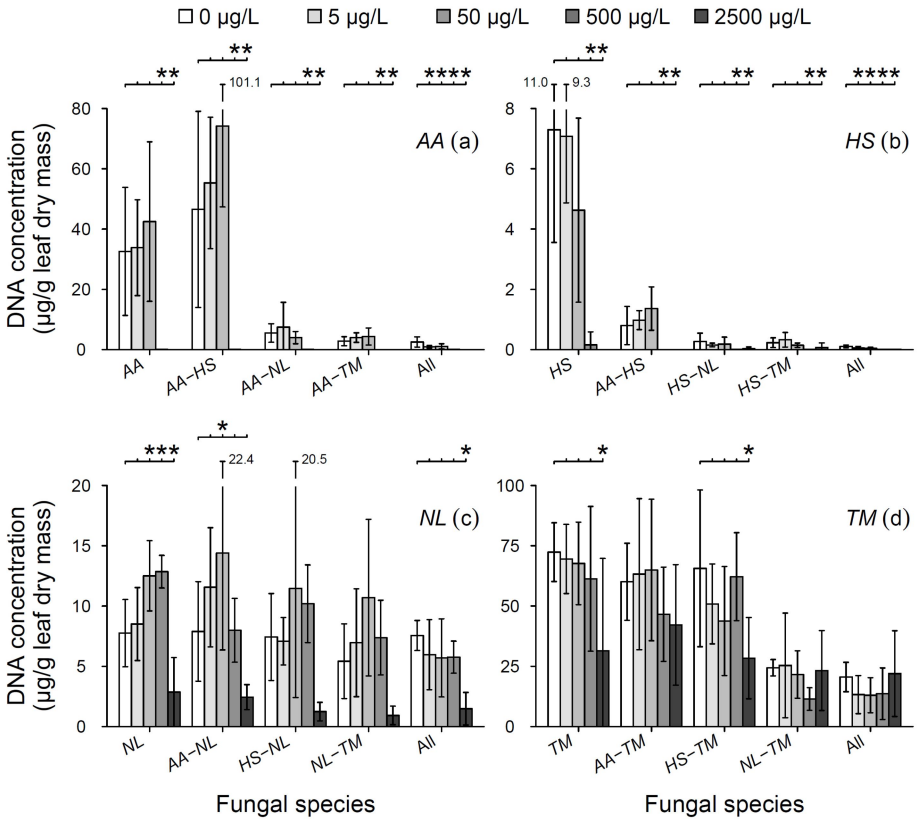



Fig 5.11. Mean DNA concentrations (with 95%-confidence intervals) of *A. acuminata*, *H. stellata*, *N. lugdunensis*, and *T. marchalianum* in fungal species treatments exposed to fungicide concentrations of 0, 5, 50, 500, and 2500 µg/L (gray-scale bars). Asterisks indicate significant differences between fungicide treatments and fungicide-free controls (Dunnett's test; $p < 0.05$; $n = 5$; *AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*). Image modified from **Appendix A.4**.

cultures, fungicide effects on DNA concentrations of the individual fungi were largely reflected by responses in monocultures (Fig 5.11). In the quaternary culture, however, DNA concentrations of *A. acuminata* and *H. stellata* were already significantly reduced at the lowest fungicide concentration of 5 µg/L (Fig 5.11a, b).

In fungicide-free mixed cultures, dependencies between net diversity effects on leaf colonization and fungal growth rates or rather genetic divergence (Fig. 5.12) were consistent with observations in the third phase (cf. Fig. 5.8a, b) **[Appendix A.3]**, demonstrating reproducibility of the experimental results. Net diversity effects remained largely unaffected by fungicide exposure in binary cultures (Fig 5.13a-f), while substantial (partially significant) reductions were observed in the quaternary culture exposed to fungicide concentrations of 5 and 50 µg/L (Fig 5.13g). This reduction was driven by alterations of trait-independent complementarity effects from positive, in the fungicide-free culture, to negative, in the fungicide-exposed cultures (Fig 5.13g). Despite the absence of fungicide effects on net diversity effects in binary cultures, dominance relationships between *A. acuminata* and *T. marchalianum* at concentrations of 5 and 50 µg/L (in favor of *A. acuminata*; Fig 5.13c), *H. stellata* and *N. lugdunensis* at 5 and 50 µg/L (in favor of *N. lugdunensis*; Fig 5.13d) as well as *N. lugdunensis* and *T. marchalianum* at 2500 µg/L (in favor of *T. marchalianum*; Fig 5.13f) were altered.

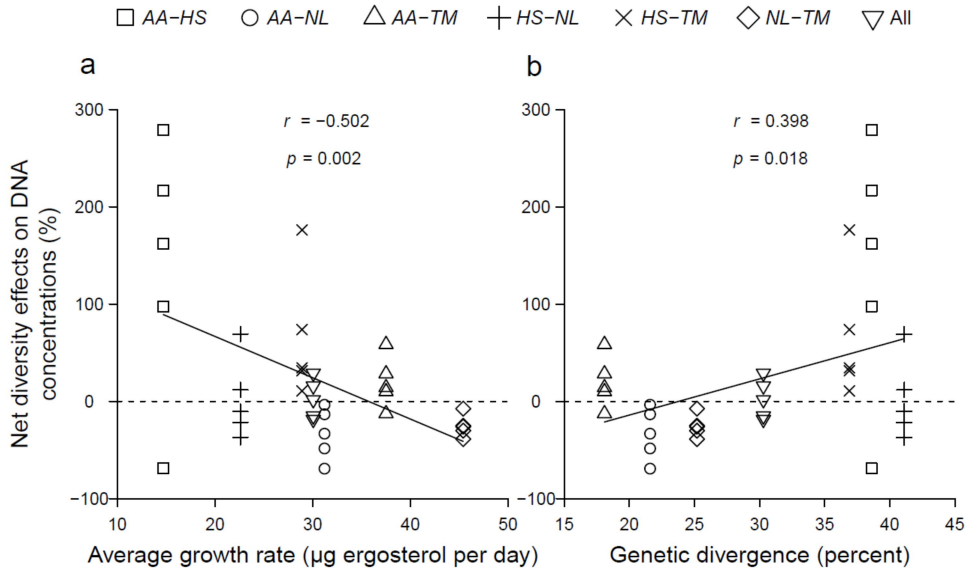



Fig. 5.12. Correlations between net diversity effects on DNA concentrations and (a) average growth rates or (b) genetic divergence of fungal mixed cultures (fungicide-free control) with Pearson's correlation coefficients (r) and p-values. The regression lines were added to visualize the relationships between the variables. (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*). **[Appendix A.4]**.

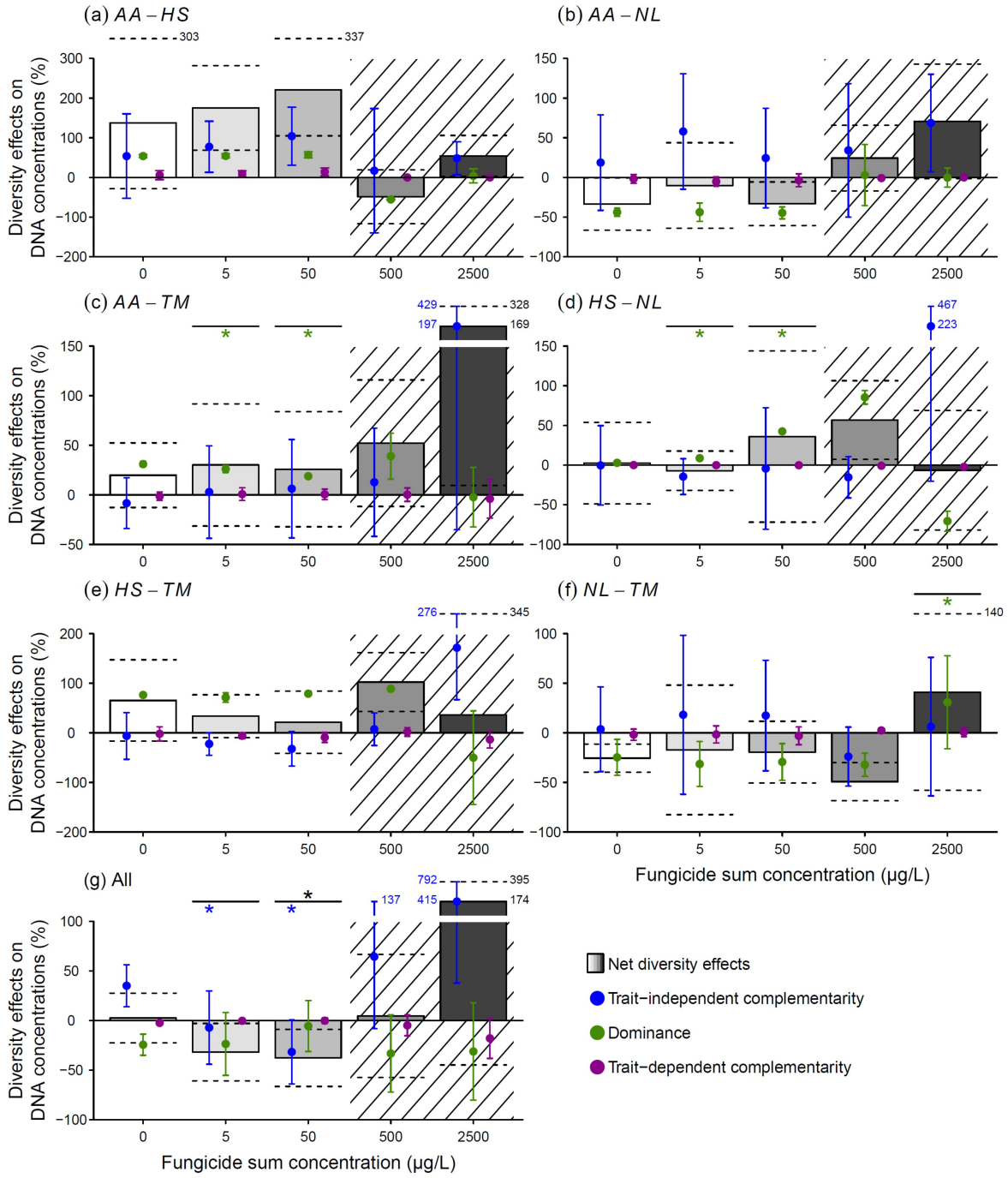



Fig 5.13. Mean diversity effects on DNA concentrations (with 95%-CIs) of mixed cultures exposed to fungicide concentrations of 0, 5, 50, 500, and 2500 µg/L. Net diversity effects (gray-scale bars) are composed of the sum of trait-independent complementarity (blue points), dominance (green points), and trait-dependent complementarity effects (purple points; for an explanation of these effects see section *Diversity effects on leaf colonization*). Black and colored asterisks indicate significant differences in net diversity effects or component interaction effects, respectively, between fungicide treatments and fungicide-free controls (Dunnett's test; $p < 0.05$; $n = 5$). Treatments plotted within shaded areas contain species, apparently incapable of surviving under the applied fungicide concentration, as no ergosterol could be detected in the respective monoculture treatment (Fig. 5.9a). Thus, these treatments likely do not reflect true interactions and are therefore excluded from statistical analysis (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*). Image modified from **Appendix A.4**.

# 6. DISCUSSION

## 6.1 RQ 1: DESIGN AND VALIDATION OF FUNGAL SPECIES-SPECIFIC BIOMASS PROXIES FOR ECO(TOXICO)LOGICAL APPLICATION

During this thesis, ten novel qPCR assays were developed and successfully validated, which made it possible to address the basic research question of this thesis (i.e., RQ 3). In the first phase of this thesis, it was demonstrated that ITS-based DNA quantification via standardized TaqMan® qPCR is a suitable method to assess the biomass of aquatic hyphomycetes at the species-level. While performances of the developed qPCR assays (Table 5.1) largely met standards for technically robust assays (Baschien and Carl, 2020), the extent of potential applications (e.g., microcosm vs. field samples) varies between assays **[Appendix A.1]**.

*In silico* validation of the assay sequences indicated the potential to reliably detect and quantify DNA of *C. aquatica*, *C. longibrachiata*, *H. stellata*, and *T. angulatum* in environmental samples bearing unknown communities (Table 5.2). For the other species, however, application of the assays in environmental samples may be associated with uncertainties due to non-species-specificity (e.g., *L. terrestris*) or strain-specificity (i.e., "too specific"; e.g., *A. acuminata;* Table 5.2), potentially leading to false-positive or false-negative results, respectively. Non-species-specificity and strain-specificity can be explained by phenomena, such as low sequence divergence within the genera of the target species (e.g., *L. terrestris* and *N. lugdunensis*) or distinct phylogenetic groups within the target species (e.g., *A. acuminata*, *N. lugdunensis*, and *T. marchalianum*), respectively (Anderson and Shearer, 2011; Baschien et al., 2006; Baschien et al., 2013; Seena et al., 2012). Deviations from the desired specificity may, on the other hand, at least to some degree, arise from fungal sequence entries deposited in publicly available databases that are incorrectly identified to the species-level (Bridge et al., 2003; Nilsson et al., 2006). Uncertainties associated with the application of some assays in field samples, may, however, be overcome by the combined use of qPCR and high-throughput sequencing techniques (HTS). Accordingly, microbial communities can be screened by HTS, whereupon particular operational taxonomic units may be specifically quantified by qPCR (cf. Pivato et al., 2007) **[Appendix A.1]**.

The experimental validation (Fig. 4.2) demonstrated that extracted DNA of each model strain can be reliably detected and quantified within microcosm samples

comprising all of the investigated fungal strains and black alder leaves as substrate. Only the qPCR assay targeting *L. terrestris* was slightly interfered by the presence of DNA of *A. tetracladia*. Nonetheless, affected reactions became clearly evident as irregular amplification curves and DNA concentrations above 10 pg DNA/PCR can be reliably quantified **[Appendix A.1]**.

Application of the qPCR assays in liquid culture extracts showed an overall higher variability of mycelial concentrations of DNA, compared to ergosterol (Table 5.3). This may be explained by a different degree of variation in the extraction yield between the extraction procedures for the two biomarkers. Furthermore, particularly the higher interspecific variability of DNA concentrations may additionally result from a distinct degree of biological variability of these biomarkers between species (Table 5.3). Since incubation was terminated once sufficient biomass was available for analyses and as the species strongly vary in their growth rate (cf. Gessner and Chauvet, 1993), cultures were potentially not sampled at the same growth stage, which may have had a decisive influence on mycelial DNA concentrations (Grimmett et al., 2013). Regardless of this uncertainty, the identified positive correlation between both biomarkers among species (Fig. 5.1) indicates that ITS-based DNA concentrations and ergosterol concentrations reflect fungal biomass in a comparable quality **[Appendix A.1]**. This assumption was further supported by the agreement between ergosterol concentrations measured and predicted (from DNA) in colonized leaf material in the third phase (Fig. 5.3b) **[Appendix A.3]**. All in all, the developed qPCR assays constitute a sensitive, precise and standardized alternative to conventional microscopic techniques (i.e., morphological identification and quantification of conidia) for assessing aquatic hyphomycete community composition **[Appendix A.1]**.

In the second phase of this thesis, it was observed that exposure to fungicides – using tebuconazole as model substance – can, however, result in an altered ratio between DNA concentrations and mycelial dry weight, on average resulting in increased DNA concentrations (Fig. 5.2b). Accordingly, this would result in an overestimation of actual fungal biomass. Considering the toxic mode of action of DMI fungicides, it is assumed that genome integrity was maintained under exposure to tebuconazole (i.e., DNA concentrations per cell were not altered by fungicide exposure) and that the observed effect on DNA concentrations was based on responses in cell or hyphal morphology. For instance, a thinning of hyphae or

reduction of hyphal length (distance between two adjacent septa), as observed for pathogenic fungi exposed to DMI fungicides (Zhang et al., 2018), may reduce the cell dry weight relative to the amount of DNA. Accordingly, DNA concentrations would increase relative to fungal biomass. For ergosterol concentrations, the opposite effect was observed in tebuconazole-exposed mycelium (Fig. 5.2a), which would lead to an underestimation of actual fungal biomass. This observation was consistent with the hypothesis based on DMI fungicides' toxic mode of action (i.e., inhibition of ERG11 reduces ergosterol biosynthesis). These fungicide-induced alterations in fungal DNA and ergosterol concentrations complicate the use of these proxies for comparisons between biological samples obtained from fungicide-exposed and unexposed systems. Additionally, as fungicide effects on concentrations of both biomarkers are species-dependent (i.e., varying effect intensity and direction; Table 5.4), species-specific responses need to be taken into account, especially when analyzing microcosm experiments comprising only few fungal species **[Appendix A.2]**.

## 6.2 RQ 2: CHARACTERIZATION OF FUNGAL B-EF RELATIONSHIPS

In the microcosm experiment in the third phase of this thesis, new insights were gained into the ecological mechanisms underlying B-EF relationships in aquatic hyphomycete communities. In addition, other microbial groups such as bacteria were found to play an essential role in the coexistence of aquatic hyphomycete species.

To facilitate an assessment of B-EF relationships, in a first step, the ecological roles of aquatic hyphomycete species involved in the experiment were investigated. While the model fungi showed nearly identical biomass-specific leaf decomposition efficiencies (with the exception of *H. stellata*), they could be functionally distinguished in terms of growth rates and enzyme activity patterns (Fig. 5.3; Fig. 5.4). This indicates differences in the quality of decomposition (i.e., species may decompose different plant structures) and niche partitioning between species, both being more pronounced with increasing genetic divergence (Fig. 5.4; cf. Andrade et al., 2016). The functional characteristics of *H. stellata* – an aquatic hyphomycete species that was so far never investigated under axenic conditions – are particularly noteworthy. This species had a ten-times lower leaf decomposition efficiency compared to the other species; in other words, it was capable of colonizing the leaf substrate ten-times more efficiently (Fig. 5.3). Accordingly, in comparison to the other species, *H. stellata* seems to pursue a different substrate colonization strategy. Colonization

by *A. acuminata*, *N. lugdunensis*, and *T. marchalianum* is based on a cellulolytic or ligninolytic utilization of the major plant polymers (Fig. 5.4a), ultimately leading to the degradation of the substrate (i.e., a common understanding of one of aquatic hyphomycetes' functional roles; Krauss et al., 2011; Fig. 5.3a). Since nearly no leaf degradation was observed for *H. stellata* (Fig. 5.3a), colonization by this species (Fig. 5.3b), seems to be rather based on the utilization of dissolved organic compounds (Fig. 5.4a), such as phosphate esters or maltose, being leached from leaves or released during progressing leaf senescence (Baldwin, 1999; Duan et al., 2014; Wildman and Parkinson, 1981) **[Appendix A.3]**.

Using the qPCR assays developed and validated in the first phase of this thesis **[Appendix A.1]**, biomass of the model fungi could be species-specifically quantified in mixed cultures. In combination with biomass-specific functional performances (based on DNA concentrations) derived from monocultures, these data could be used to identify diversity effects on functioning (predictions vs. observations) and leaf colonization (modified Price equation *sensu* Fox, 2005). Functional performances in mixed cultures did not exceed those observed in the most productive monoculture (Fig. 5.3), which is in line with earlier B-EF studies on aquatic hyphomycetes (e.g., Bärlocher and Corkum, 2003; Duarte et al., 2006). In most mixed cultures, observed functional performances could be explained by the biomass of the present species (i.e., by community composition; Fig. 5.3; Fig. 5.6), indicating the prevalence of additive interactions (zero sum game) in the fungal-mediated leaf decomposition process. Yet, in the culture bearing the highest fungal diversity (i.e., the quaternary culture), leaf mass loss was significantly higher than predicted based on community composition (Fig. 5.3a). This phenomenon may be explained by the joint action of a diverse enzymatic pool provided by all four species (Fig. 5.4a; Fig. 5.5), resulting in a more efficient decomposition, and indicates a potential for complementary functional interactions in aquatic hyphomycete communities (Gessner et al., 2010). While interactions were overall limited at the functional level (Fig. 5.4), pronounced diversity effects were identified to occur during leaf colonization (Fig. 5.6; Fig. 5.7). Contrary to expectations based on the characterization of species' traits (see previous paragraph; Fig. 5.4a), however, the fungi largely seemed to compete for similar resources in mixed cultures (i.e., niche overlap). This is indicated by mainly negative net diversity effects on colonization (i.e., antagonistic interactions; Fox, 2005), driven by negative trait-independent

complementarity (Fig. 5.7). The degree of competition increased (i.e., diversity effects decrease) with increasing average growth rate (Fig. 5.8a). This relationship may be explained by inhibitory priority effects (Fukami, 2015), which became, in this case, evident as fast-growing species (i.e., *N. lugdunensis* and *T. marchalianu*m) rapidly colonizing leaf substrate up to the carrying capacity, thereby displacing slow-growing species (i.e., *A. acuminata* and *H. stellata*; Fig. 5.3b; Fig. 5.6). In contrast, the degree of competition decreased (i.e., diversity effects increase) with increasing genetic divergence of co-occurring species (Fig. 5.8b), which might indeed be explained by a tight coupling between genetic divergence and functional dissimilarity (cf. Andrade et al., 2016; Fig. 5.4b; Fig. 5.8b) **[Appendix A.3]**.

As observed earlier, the presence of bacteria generally had an inhibitory effect on fungal activity (e.g., Romaní et al., 2006), while particularly biomass production of species with lower growth rates (i.e., *A. acuminata* and *H. stellata*) was affected (Fig. 5.3b). Therefore, the growth rate seems to be a decisive trait for fungal colonization success in the competition not only among aquatic hyphomycete species (see above) but also between these fungi and bacteria. With few exceptions, functional performances in cultures containing both organism groups could be explained by the biomass of present organisms (based on fungal DNA concentrations and bacterial cell densities; Fig. 5.3; cf. previous paragraph), which provides evidence for the prevalence of additive interactions between and within the investigated microbial groups in the leaf decomposition process. The most conspicuous exception to this were antagonistic interactions indicated by the over-predicted oxidase activities (Fig. 5.5). These interactions might be based on an inhibition of fungal oxidases by certain metabolites released by bacteria (Fernandes and Kerkar, 2017). This is in agreement with the over-predicted leaf mass loss in cultures containing bacteria and *N. lugdunensis* (Fig. 5.3a), the model fungus showing the highest biomass-specific oxidase activities (Fig. 5.4a). Surprisingly, in contrast to bacteria-free cultures, bacterial presence led to positive net diversity effects on fungal leaf colonization (Fig. 5.7), indicating synergistic interactions between fungi. This shift may be explained by a bacteria-induced alteration of fungal diversity-based feedback loops enabling coexistence. More specifically, the general inhibitory effect of bacteria on fungal growth could possibly reduce competition among fungi (i.e., alleviation of inhibitory priority effects), which allows slow-growing species (e.g., *A. acuminata* and *H. stellata*) to colonize leaf substrates in presence of

fast-growing species (e.g., *N. lugdunensis* and *T. marchalianum*). This assumption is supported by the lack of correlation between net diversity effects on leaf colonization and average growth rates in the presence of bacteria (Fig. 5.8c; cf. Fig. 5.8a). Moreover, net diversity effects on leaf colonization were still positively correlated with genetic divergence in the more environmentally realistic scenarios (cf. absence vs. presence of bacteria; Fig. 5.8b, d), which supports the hypothesis that, in aquatic fungi, genetic diversity translates into functional diversity (Andrade et al., 2016) **[Appendix A.3]**. Overall, these results indicate that there is potential for complementary interactions in aquatic hyphomycete communities, whereas it is the presence of bacteria that appears to have enabled the coexistence of aquatic hyphomycete species.

### 6.3 RQ 3: FUNGICIDE IMPACTS ON FUNGAL B-EF RELATIONSHIPS

In the microcosm experiment addressing the basic research question of this thesis, it was demonstrated that environmentally relevant fungicide concentrations can affect B-EF relationships in aquatic hyphomycete communities. As hypothesized, in monoculture, the four model fungi strongly varied in their sensitivity to the applied fungicide mixture (cf. Dijksterhuis et al., 2011). While for *A. acuminata* and *H. stellata* (nearly) no activity was observed at fungicide concentrations of 500 µg/L and higher, *N. lugdunensis* and *T. marchalianum* were able to colonize and decompose leaf substrate up to the highest fungicide concentration tested (Fig. 5.9; Fig. 5.10). The observed sensitivity pattern is in line with previous studies on near-natural aquatic hyphomycete communities, where increasing fungicide concentrations reduced the contribution of *A. acuminata* and *H. stellata* and maintained or even increased the contribution of *N. lugdunensis* and *T. marchalianum* (based on conidial counts; Bundschuh et al., 2011; Zubrod et al., 2015b). The basis for these substantial differences in sensitivity to fungicides is yet not clarified. However, since *N. lugdunensis* and *T. marchalianum* have been reported to occur in aquatic systems heavily polluted with xenobiotics and metals (Fernández et al., 2015; Solé et al., 2008; Sridhar et al., 2000; Sridhar et al., 2005), these species' remarkable tolerance can be assumed to be based on well-developed detoxification systems. Fungicide tolerance in *N. lugdunensis* may be partly based on its production of significant amounts of ligninolytic enzymes (i.e., peroxidase and phenol oxidase; Fig. 5.5a; Fig. 5.10c), which are known to degrade a variety of xenobiotic pollutants including

fungicides (Harms et al., 2011; Ikehata et al., 2004; Kang et al., 2002), thereby possibly mitigating their toxicity. However, other detoxification pathways may also play a great role, since the most tolerant species *T. marchalianum* did not show pronounced oxidase activities (Fig. 5.5a; Fig. 5.10d). In previous studies, the metal tolerance of *N. lugdunensis* and *T. marchalianum* was linked to an increased synthesis of glutathione (Braha et al., 2007; Miersch et al., 2005). Since this molecule is also involved in the elimination of reactive oxygen species formed via xenobiotic action as well as the conjugation of xenobiotics (Pócsi et al., 2004), this metabolic pathway may have also contributed to the observed fungicide tolerance in the two fungal species **[Appendix A.4]**.

Since productive species also proved to be tolerant to fungicides, leaf mass loss, biomass production, and enzyme activity profiles in mixed cultures were largely reflected by the most tolerant species in monoculture (Fig. 5.9; Fig. 5.10). Moreover, the broad agreement of predictions and observations on leaf mass loss and fungal biomass in monocultures and mixed cultures (Fig. 5.9) indicates that fungicide exposure did not affect biomass-specific performances. Accordingly, also under fungicide exposure, functional performances can be explained by community composition **[Appendix A.4]**. Cultures containing *N. lugdunensis* were, however, apparently exceptions to this conclusion, which can be attributed to fungicide-induced increases in DNA concentrations, as observed in the second phase of this thesis **[Appendix A.2]**, leading to over-predictions of leaf mass loss and ergosterol concentrations (Fig. 5.9). This is surprising, as a fungicide-induced increase in DNA concentrations was rather expected for the fungicide sensitive species *A. acuminata*, which, in the second phase, followed the identified overall pattern (i.e., reduced ergosterol and increased DNA concentrations; Fig. 5.2) **[Appendix A.2]**. The discrepancy in species-specific responses in biomarker concentrations between the two experiments might be explained by differences in test conditions (e.g., exposure conditions, test durations, and substrates applied), possibly inducing distinct response patterns **[Appendix A.2 and A.4]**.

While at the functional level, fungicide effects rather occurred at high concentrations in all fungal cultures (≥ 500 µg/L; Fig. 5.9; Fig. 5.10; cf. Pimentão et al., 2020; Zubrod et al., 2015a), community composition in the quaternary cultures was already affected by environmentally relevant fungicide concentrations (≥ 5 µg/L; Fig 5.11a, b). The observed shift in community composition, as manifested by a

displacement of *A. acuminata* and *H. stellata* (Fig 5.11a, b), could be explained by a synergistic interaction between effects of competitive pressure, exerted by *N. lugdunensis* and *T. marchalianum*, and fungicide stress (Steinberg, 2012). This shift and the simultaneous negative impact on leaf colonization (i.e., reduced biomass production; based on DNA concentrations; Fig 5.13g) indicate an impairment of the leaf conditioning function. Besides the reduction of fungal biomass production, particularly the shift in community composition indicates the potential of environmental fungicide exposures to reduce leaves' nutritional quality for detritivorous consumers. This may be hypothesized as *A. acuminata* is a species preferably consumed by invertebrate leaf consumers, whereas *N. lugdunensis* and *T. marchalianum* are species rather rejected by the same consumers (Arsuffi and Suberkropp, 1989; Rong et al., 1995). Moreover, this hypothesis is further supported by earlier studies (Bundschuh et al., 2011; Zubrod et al., 2015a), where the above-discussed preferences were demonstrated for an amphipod leaf consumer, which preferred leaves conditioned in absence of fungicides over those conditioned in presence of fungicides. This preference was accompanied by fungicide-induced shifts in aquatic hyphomycete community composition, reflecting the observations in the fourth phase of this thesis (Fig 5.11). Surprisingly, fungicide-induced alterations of interactions during leaf colonization in the quaternary culture cannot be explained by observations in binary cultures. In the latter, fungicide exposure did not affect complementary interactions but instead induced alterations in dominance relations between the species (Fig 5.13c, d, f) **[Appendix A.4]**. In absence of toxicant stress, a similar phenomenon was observed for wood decay fungi by Hiscox et al. (2017) who concluded that binary species combinations are not necessarily a predictor of the outcomes of multispecies combinations. As aquatic hyphomycete diversity in natural decomposer communities usually exceeds two species (e.g., Carl et al., 2022; Shearer and Lane, 1983) **[Appendix A.1]** – even in contaminated ecosystems (e.g., Fernández et al., 2015) – observed effects in the quaternary culture most likely more realistically reflect the situation in the field (compared to binary cultures).

## 7. CONCLUSION AND OUTLOOK

The ITS-based qPCR assays developed in this thesis constitute standardized and precise tools to investigate aquatic hyphomycete community composition under controlled conditions. Their application in an ecological or ecotoxicological context is,

however, associated with uncertainties mainly originating from the variability of DNA concentrations depending on, *inter alia*, species, mycelial age, and toxicant exposure (Table 5.3; Fig. 5.2; see also Grimmett et al., 2013; Rooney and Ward, 2005) **[Appendix A.1-A.4]**. Yet, considering these potential influencing factors in the interpretation of experimental data, the developed qPCR assays overcome drawbacks of the most widely applied methods to investigate aquatic hyphomycete community composition (i.e., quantification of total fungal biomass via ergosterol and morphological identification and quantification of conidia; see also Bermingham et al., 1995; 1996) and allow new research possibilities in fungal community and stress ecology.

Making use of these qPCR assays, this thesis provides new insights into B-EF relationships in aquatic hyphomycete communities. It was shown that functioning in terms of leaf decomposition and fungal biomass production, in essence, can be explained by community composition as B-EF relationships are predominantly based on additive interactions (Fig. 5.3; Fig. 5.9). The nature of colonization, however, is characterized by complementary interactions (Fig. 5.7; Fig 5.13g), which are favored by increasing functional and genetic divergence (Fig. 5.4; Fig. 5.8; see also Andrade et al., 2016) **[Appendix A3 and A4]**. Bacteria were found to further promote these complementary interactions during leaf colonization and appear to be a key factor enabling coexistence of aquatic hyphomycetes in the field (Fig. 5.7) **[Appendix A3]**. Fungicide exposure, in contrast, lead to a shift from complementary to competitive interactions at environmentally relevant concentrations (Fig 5.13g) and accordingly might lead to a loss of fungal diversity in the field over the long-term. The concurrent shift in community composition (Fig 5.11a, b) may, in addition, translate into diet-related effects on leaf consumers (Konschak et al., 2019; 2020; Zubrod et al., 2015a), which allows concluding that, in the field, fungicide exposures rather induce bottom-up effects in aquatic food webs than impairments in the microbial leaf decomposition process (Fig. 5.9; see also Pimentão et al., 2020) **[Appendix A.4]**. Accordingly, to assess functional integrity of detritus-based ecosystems, it seems important to consider microbially-mediated parameters associated to leaves' nutritional quality (e.g., alterations of fatty acid composition; Konschak et al., 2021) in addition to or rather than leaf mass loss, which is still widely accepted as indicator for this purpose (see e.g., Colas et al., 2019).

The universality of these findings, however, needs to be further validated, which may be pursued in future experiments using the tools developed in this thesis in combination with higher-tier test systems involving higher fungal diversity, different species combinations, and other naturally co-occurring organism groups (e.g., bacteria, green algae, and diatoms). Moreover, and although previous studies indicate a close link between aquatic hyphomycete community composition and the physiological fitness of leaf consumers (Konschak et al., 2019; 2020), the underlying mechanisms of this relationship are not clear and require further research. Shedding light on these mechanisms may foster a targeted assessment of leaves' nutritional quality, potentially serving as sensitive indicator for the evaluation of functional stream integrity.

## 8. REFERENCES


Amoatey, P., Baawain, M.S., 2019. Effects of pollution on freshwater aquatic organisms. Water Environment Research 91, 1272–1287.

Anderson, J.L., Shearer, C.A., 2011. Population genetics of the aquatic fungus *Tetracladium marchalianum* over space and time. PLoS ONE 6, e15908.

Andrade, R., Pascoal, C., Cássio, F., 2016. Effects of inter and intraspecific diversity and genetic divergence of aquatic fungal communities on leaf litter decomposition – a microcosm experiment. FEMS Microbiology Ecology 92, fiw102.

Arsuffi, T.L., Suberkropp, K., 1989. Selective feeding by shredders on leaf-colonizing stream fungi: comparison of macroinvertebrate taxa. Oecologia 79, 30–37.

Baldwin, D.S., 1999. Dissolved organic matter and phosphorus leached from fresh and 'terrestrially' aged river red gum leaves: implications for assessing river-floodplain interactions. Freshwater Biology 4, 675–685.

Bärlocher, F., 1985. The role of fungi in the nutrition of stream invertebrates. Botanical Journal of the Linnean Society 91, 83–94.

Bärlocher, F., 1991. Fungal colonization of fresh and dried leaves in the River Teign (Devon, England). Nova Hedwigia 52, 349–357.

Bärlocher, F., Corkum, M., 2003. Nutrient enrichment overwhelms diversity effects in leaf decomposition by stream fungi. Oikos 101, 247–252.

Baschien, C., Carl, S.C., 2020. Quantitative Real-Time PCR (qPCR) to Estimate Molecular Fungal Abundance, in: Bärlocher, F., Gessner, M.O., Graça, M.A.S. (Eds.), Methods to Study Litter Decomposition: A Practical Guide, 2nd ed. Springer International Publishing, pp. 327–337.

Baschien, C., Marvanová, L., Szewzyk, U., 2006. Phylogeny of selected aquatic hyphomycetes based on morphological and molecular data. Nova Hedwigia 83, 311–352.

Baschien, C., Tsui, C.K.-M., Gulis, V., Szewzyk, U., Marvanová, L., 2013. The molecular phylogeny of aquatic hyphomycetes with affinity to the Leotiomycetes. Fungal Biology 117, 660–672.

Bermingham, S., Maltby, L., Cooke, R.C., 1995. A critical assessment of the validity of ergosterol as an indicator of fungal biomass. Mycological Research 99, 479–484.

Bermingham, S., Maltby, L., Cooke, R.C., 1996. Effects of a coal mine effluent on aquatic hyphomycetes. II. Laboratory toxicity experiments. Journal of Applied Ecology 33, 1322–1328.

Bermingham, S., Maltby, L., Dewey, F.M., 1997. Use of immunoassays for the study of natural assemblages of aquatic hyphomycetes. Microbial Ecology 33, 223–229.

Braha, B., Tintemann, H., Krauss, G., Ehrman, J., Bärlocher, F., Krauss, G.-J., 2007. Stress response in two strains of the aquatic hyphomycete *Heliscus lugdunensis* after exposure to cadmium and copper ions. BioMetals 20, 93–105.

Bridge, P.D., Roberts, P.J., Spooner, B.M., Panchal, G., 2003. On the unreliability of published DNA sequences. New Phytologist 160, 43–48.

Buesing, N., 2005. Bacterial counts and biomass determination by epifluorescence microscopy., in: Graça, M.A.S., Bärlocher, F., Gessner, M.O. (Eds.), Methods to study litter decomposition: a practical guide. Springer-Verlag, Dordrecht, Netherlands, pp. 203–208.

Bundschuh, M., Zubrod, J.P., Kosol, S., Maltby, L., Stang, C., Duester, L., Schulz, R., 2011. Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. Aquatic Toxicology 104, 32–37.

Bundschuh, M., Zubrod, J.P., Wernicke, T., Konschak, M., Werner, L., Brühl, C.A., Baudy, P., Schulz, R., 2021. Bottom-up effects of fungicides on tadpoles of the European common frog (*Rana temporaria*). Ecology and Evolution 11, 4353–4365.

Carl, S., Mohr, S., Sahm, R., Baschien, C., 2022. Laboratory conditions can change the complexity and composition of the natural aquatic mycobiome on *Alnus glutinosa* leaf litter. Fungal Ecology, 101142.

Casado, J., Brigden, K., Santillo, D., Johnston, P., 2019. Screening of pesticides and veterinary drugs in small streams in the European Union by liquid chromatography high resolution mass spectrometry. Science of the Total Environment 670, 1204–1225.

Chandrashekar, K.R., Kaveriappa, K.M., 1989. Effect of pesticides on the growth of aquatic hyphomycetes. Toxicology Letters 48, 311–315.

Chandrashekar, K.R., Kaveriappa, K.M., 1994. Effect of pesticides on sporulation and germination of conidia of aquatic hyphomycetes. Journal of Environmental Biology 15, 315–324.

Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 18, 117–143.

Colas, F., Woodward, G., Burdon, F.J., Guérold, F., Chauvet, E., Cornut, J., Cébron, A., Clivot, H., Danger, M., Danner, M.C., Pagnout, C., Tiegs, S.D., 2019. Towards a simple global-standard bioassay for a key ecosystem process: organic-matter decomposition using cotton strips. Ecological Indicators 106, 105466.

Copolovici, L., Kännaste, A., Remmel, T., Niinemets, Ü., 2014. Volatile organic compound emissions from *Alnus glutinosa* under interacting drought and herbivory stresses. Environmental and Experimental Botany 100, 55–63.

Dang, C.K., Chauvet, E., Gessner, M.O., 2005. Magnitude and variability of process rates in fungal diversity-litter decomposition relationships. Ecology Letters 8, 1129–1137.

Danger, M., Cornut, J., Chauvet, E., Chavez, P., Elger, A., Lecerf, A., 2013. Benthic algae stimulate leaf litter decomposition in detritus-based headwater streams: A case of aquatic priming effect? Ecology 94, 1604–1613.

Danger, M., Gessner, M.O., Bärlocher, F., 2016. Ecological stoichiometry of aquatic fungi: current knowledge and perspectives. Fungal Ecology 19, 100–111.

Das, M., Royer, T.V., Leff, L.G., 2007. Diversity of fungi, bacteria, and actinomycetes on leaves decomposing in a stream. Applied and Environmental Microbiology 73, 756–767.

DeForest, J.L., 2009. The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and l-DOPA. Soil Biology and Biochemistry 41, 1180–1186.

Descals, E., 2005. Techniques for handling Ingoldian fungi, in: Graça, M.A.S., Bärlocher, F., Gessner, M.O. (Eds.), Methods to study litter decomposition: a practical guide. Springer-Verlag, Dordrecht, Netherlands, pp. 129–142.

Di Lorenzo, T., Hose, G.C., Galassi, D.M.P., 2020. Assessment of different contaminants in freshwater: origin, fate and ecological Impact. Water 12, 1810.

Dijksterhuis, J., van Doorn, T., Samson, R., Postma, J., 2011. Effects of seven fungicides on non-target aquatic fungi. Water, Air, and Soil Pollution 222, 421–425.

Dimitrov, M.R., Kosol, S., Smidt, H., Buijse, L., van den Brink, P.J., Van Wijngaarden, R.P.A., Brock, T.C.M., Maltby, L., 2014. Assessing effects of the fungicide tebuconazole to heterotrophic microbes in aquatic microcosms. Science of the Total Environment 490, 1002–1011.

Duan, S., Delaney-Newcomb, K., Kaushal, S.S., Findlay, S.E.G., Belt, K.T., 2014. Potential effects of leaf litter on water quality in urban watersheds. Biogeochemistry 121, 61–80.

Duarte, S., Bärlocher, F., Pascoal, C., Cássio, F., 2016. Biogeography of aquatic hyphomycetes: Current knowledge and future perspectives. Fungal Ecology 19, 169–181.

Duarte, S., Pascoal, C., Alves, A., Correia, A., Cássio, F., 2010. Assessing the dynamic of microbial communities during leaf decomposition in a low-order stream by microscopic and molecular techniques. Microbiological Research 165, 351–362.

Duarte, S., Pascoal, C., Cássio, F., Bärlocher, F., 2006. Aquatic hyphomycete diversity and identity affect leaf litter decomposition in microcosms. Oecologia 147, 658–666.

Evans, C.S., Hedger, J.N., 2001. Degradation of plant cell wall polymers, in: Gadd, G.M. (Ed.), Fungi in Bioremediation. British Mycological Society symposium series v. 23. Cambridge University Press, Cambridge, UK.

Feckler, A., Schrimpf, A., Bundschuh, M., Bärlocher, F., Baudy, P., Cornut, J., Schulz, R., 2017. Quantitative real-time PCR as a promising tool for the detection and quantification of leaf-associated fungal species – A proof-of-concept using *Alatospora pulchella*. PLoS ONE 12, e0174634.

Fernandes, I., Pereira, A., Trabulo, J., Pascoal, C., Cássio, F., Duarte, S., 2015. Microscopy- or DNA-based analyses: Which methodology gives a truer picture of stream-dwelling decomposer fungal diversity? Fungal Ecology 18, 130–134.

Fernandes, M.S., Kerkar, S., 2017. Microorganisms as a source of tyrosinase inhibitors: a review. Annals of Microbiology 67, 343–358.

Fernández, D., Voss, K., Bundschuh, M., Zubrod, J.P., Schäfer, R.B., 2015. Effects of fungicides on decomposer communities and litter decomposition in vineyard streams. Science of the Total Environment 533, 40–48.

Fox, J.W., 2005. Interpreting the 'selection effect' of biodiversity on ecosystem function. Ecology Letters 8, 846–856.

Fukami, T., 2015. Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects. Annual Review of Ecology, Evolution, and Systematics 46, 1–23.

Fungicide Resistance Action Committee, 2020. FRAC code list 2020: Fungal control agents sorted by cross resistance pattern and mode of action (including FRAC Code numbering).

Garnett, H., Bärlocher, F., Giberson, D., 2000. Aquatic hyphomycetes in Catamaran Brook: colonization dynamics, seasonal patterns, and logging effects. Mycologia 92, 29–41.

Gessner, M.O., 2005. Ergosterol as a measure of fungal biomass, in: Graça, M.A.S., Bärlocher, F., Gessner, M.O. (Eds.), Methods to study litter decomposition: a practical guide. Springer-Verlag, Dordrecht, Netherlands, pp. 189–195.

Gessner, M.O., 2020. Ergosterol as a Measure of Fungal Biomass, in: Bärlocher, F., Gessner, M.O., Graça, M.A.S. (Eds.), Methods to Study Litter Decomposition: A Practical Guide, 2nd edition ed. Springer International Publishing, pp. 247–255.

Gessner, M.O., Chauvet, E., 1993. Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. Applied and Environmental Microbiology 59, 502–507.

Gessner, M.O., Gulis, V., Kuehn, K.A., Chauvet, E., Suberkropp, K., 2007. Fungal decomposers of plant litter in aquatic ecosystems, in: Kubicek, C.P., Druzhinina, I.S. (Eds.), Microbial and Environmental Relationships, 2nd ed. Springer, Berlin, Germany, pp. 301–324.

Gessner, M.O., Swan, C.M., Dang, C.K., McKie, B.G., Bardgett, R.D., Wall, D.H., Hättenschwiler, S., 2010. Diversity meets decomposition. Trends in Ecology and Evolution 25, 372–380.

Grimmett, I.J., Shipp, K.N., Macneil, A., Bärlocher, F., 2013. Does the growth rate hypothesis apply to aquatic hyphomycetes? Fungal Ecology 6, 493–500.

Grossart, H.-P., Rojas-Jimenez, K., 2016. Aquatic fungi: targeting the forgotten in microbial ecology. Current Opinion in Microbiology 31, 140–145.

Gulis, V., Marvanová, L., Descals, E., 2020. An Illustrated Key to the Common Temperate Species of Aquatic Hyphomycetes, in: Bärlocher, F., Gessner, M.O., Graça, M.A.S. (Eds.), Methods to Study Litter Decomposition: A Practical Guide, 2nd edition ed. Springer International Publishing, pp. 223–239.

Gulis, V., Suberkropp, K., 2003. Leaf litter decomposition and microbial activity in nutrient-enriched and unaltered reaches of a headwater stream. Freshwater Biology 48, 123–134.

Halbach, K., Möder, M., Schrader, S., Liebmann, L., Schäfer, R.B., Schneeweiss, A., Schreiner, V.C., Vormeier, P., Weisner, O., Liess, M., Reemtsma, T., 2021. Small streams-large concentrations? Pesticide monitoring in small agricultural streams in Germany during dry weather and rainfall. Water Research 203, 117535.

Harms, H., Schlosser, D., Wick, L.Y., 2011. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nature Reviews Microbiology 9, 177–192.

Hieber, M., Gessner, M.O., 2002. Contribution of stream detrivores, fungi, and bacteria to leaf breakdown based on biomass estimates. Ecology 83, 1026–1038.

Hinkle, D.E., Wiersma, W., Jurs, S.G., 2003. Applied Statistics for the Behavioral Sciences, 5th edition ed. Houghton Mifflin, Boston, USA.

Hiscox, J., Savoury, M., Toledo, S., Kingscott-Edmunds, J., Bettridge, A., Waili, N.A., Boddy, L., 2017. Threesomes destabilise certain relationships: multispecies interactions between wood decay fungi in natural resources. FEMS Microbiology Ecology 93, fix014.

Ikehata, K., Buchanan, I.D., Smith, D.W., 2004. Recent developments in the production of extracellular fungal peroxidases and laccases for waste treatment. Journal of Environmental Engineering and Science 3, 1–19.

Kang, K.-H., Dec, J., Park, H., Bollag, J.-M., 2002. Transformation of the fungicide cyprodinil by a laccase of *Trametes villosa* in the presence of phenolic mediators and humic acid. Water Research 36, 4907–4915.

Konschak, M., Zubrod, J.P., Baudy, P., Englert, D., Herrmann, B., Schulz, R., Bundschuh, M., 2019. Waterborne and diet-related effects of inorganic and

organic fungicides on the insect leaf shredder *Chaetopteryx villosa* (Trichoptera). Aquatic Toxicology 206, 33–42.

Konschak, M., Zubrod, J.P., Baudy, P., Fink, P., Kenngott, K., Lüderwald, S., Englert, K., Jusi, C., Schulz, R., Bundschuh, M., 2020. The importance of diet-related effects of the antibiotic ciprofloxacin on the leaf-shredding invertebrate *Gammarus fossarum* (Crustacea; Amphipoda). Aquatic Toxicology 222, 105461.

Konschak, M., Zubrod, J.P., Baudy, P., Fink, P., Kenngott, K.G.J., Englert, D., Röder, N., Ogbeide, C., Schulz, R., Bundschuh, M., 2021. Chronic effects of the strobilurin fungicide azoxystrobin in the leaf shredder *Gammarus fossarum* (Crustacea; Amphipoda) via two effect pathways. Ecotoxicology and Environmental Safety 209, 111848.

Krauss, G.-J., Solé, M., Krauss, G., Schlosser, D., Wesenberg, D., Bärlocher, F., 2011. Fungi in freshwaters: ecology, physiology and biochemical potential. FEMS Microbiology Reviews 35, 620–651.

Maltby, L., Brock, T.C.M., van den Brink, P.J., 2009. Fungicide risk assessment for aquatic ecosystems: importance of interspecific variation, toxic mode of action, and exposure regime. Environmental Science & Technology 43, 7556–7563.

Manoli, K., Morrison, L.M., Sumarah, M.W., Nakhla, G., Ray, A.K., Sharma, V.K., 2019. Pharmaceuticals and pesticides in secondary effluent wastewater: Identification and enhanced removal by acid-activated ferrate(VI). Water Research 148, 272–280.

Miersch, J., Neumann, D., Menge, S., Bärlocher, F., Baumbach, R., Lichtenberger, O., 2005. Heavy metals and thiol pool in three strains of *Tetracladium marchalianum*. Mycological Progress 4, 185–194.

Millennium Ecosystem Assessment, 2005a. Ecosystems and Human Well-being: Synthesis, Washington, DC.

Millennium Ecosystem Assessment, 2005b. Ecosystems and Human Well-being: Wetlands and Water Synthesis, Washington, DC.

Newman, M.M., Liles, M.R., Feminella, J.W., 2015. Litter Breakdown and Microbial Succession on Two Submerged Leaf Species in a Small Forested Stream. PLoS ONE 10, e0130801.

Nilsson, R.H., Ryberg, M., Kristiansson, E., Abarenkov, K., Larsson, K.-H., Kõljalg, U., 2006. Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. PLoS ONE 1, e59.

Pascoal, C., Cássio, F., Marvanová, L., 2005. Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. Archiv für Hydrobiologie 162, 481–496.

Petersen, R.C., Cummins, K.W., 1974. Leaf processing in a woodland stream. Freshwater Biology 4, 343–368.

Pimentão, A.R., Pascoal, C., Castro, B.B., Cássio, F., 2020. Fungistatic effect of agrochemical and pharmaceutical fungicides on non-target aquatic decomposers does not translate into decreased fungi- or invertebrate-mediated decomposition. Science of the Total Environment 712, 135676.

Pivato, B., Mazurier, S., Lemanceau, P., Siblot, S., Berta, G., Mougel, C., van Tuinen, D., 2007. *Medicago* species affect the community composition of arbuscular mycorrhizal fungi associated with roots. The New phytologist 176, 197–210.

Pócsi, I., Prade, R.A., Penninckx, M.J., 2004. Glutathione, Altruistic Metabolite in Fungi, in: Poole, R.K. (Ed.), Advances in microbial physiology, vol. 49. Advances in Microbial Physiology. Elsevier/Academic Press, Amsterdam, pp. 1–76.

Raidl, S., Bonfigli, R., Agerer, R., 2005. Calibration of quantitative real-time TaqMan PCR by correlation with hyphal biomass and ITS copies in mycelia of *Piloderma croceum*. Plant Biology 7, 713–717.

Romaní, A.M., Fischer, H., Mille-Lindblom, C., Tranvik, L.J., 2006. Interactions of bacteria and fungi on decomposing litter: differential extracellular enzyme activities. Ecology 87, 2559–2569.

Rong, Q., Sridhar, K.R., Bärlocher, F., 1995. Food selection in three leaf-shredding stream invertebrates. Hydrobiologia 316, 173–181.

Rooney, A.P., Ward, T.J., 2005. Evolution of a large ribosomal RNA multigene family in filamentous fungi: birth and death of a concerted evolution paradigm. Proceedings of the National Academy of Sciences of the United States of America 102, 5084–5089.

Schäfer, R.B., Kühn, B., Malaj, E., König, A., Gergs, R., 2016. Contribution of organic toxicants to multiple stress in river ecosystems. Freshwater Biology 61, 2116–2128.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proceedings of the National Academy of Sciences of the United States of America 109, 6241–6246.

Schreiner, V.C., Feckler, A., Fernández, D., Frisch, K., Muñoz, K., Szöcs, E., Zubrod, J.P., Bundschuh, M., Rasmussen, J.J., Kefford, B.J., Axelsen, J., Cedergreen, N., Schäfer, R.B., 2018. Similar recovery time of microbial functions from fungicide stress across biogeographical regions. Scientific Reports 8, 17021.

Seena, S., Duarte, S., Pascoal, C., Cássio, F., 2012. Intraspecific variation of the aquatic fungus *Articulospora tetracladia*: an ubiquitous perspective. PLoS ONE 7, e35884.

Shearer, C.A., Descals, E., Kohlmeyer, B., Kohlmeyer, J., Marvanová, L., Padgett, D., Porter, D., Raja, H.A., Schmit, J.P., Thorton, H.A., Voglymayr, H., 2007. Fungal biodiversity in aquatic habitats. Biodiversity and Conservation 16, 49–67.

Shearer, C.A., Lane, L.C., 1983. Comparison of three techniques for the study of aquatic hyphomycete communities. Mycologia 75, 498–508.

Solé, M., Fetzer, I., Wennrich, R., Sridhar, K.R., Harms, H., Krauss, G., 2008. Aquatic hyphomycete communities as potential bioindicators for assessing anthropogenic stress. Science of the Total Environment 389, 557–565.

Sridhar, K.R., Bärlocher, F., Krauss, G.-J., Krauss, G., 2005. Response of aquatic hyphomycete communities to changes in heavy metal exposure. International Review of Hydrobiology 90, 21–32.

Sridhar, K.R., Kaveriappa, K.M., 1986. Effect of pesticides on sporulation and spore germination of water-borne hyphomycetes. Environmental Biology - Coastal Ecosystem, 195–204.

Sridhar, K.R., Krauss, G., Bärlocher, F., Wennrich, R., Krauss, G.J., 2000. Fungal diversity in heavy metal polluted waters in central Germany. Fungal Diversity 5, e129.

Steinberg, C.E.W., 2012. Stress Ecology: Environmental Stress as Ecological Driving Force and Key Player in Evolution. Springer, Dordrecht, Netherlands.

Stenersen, J., 2004. Chemical pesticides mode of action and toxicology. CRC Press, Brussels.

Vannote, R.L., Minshall, G.W., Cummins, K.W., Sedell, J.R., Cushing, C.E., 1980. The River Continuum Concept. Canadian Journal of Fisheries and Aquatic Sciences 37, 130–137.

Vörösmarty, C.J., McIntyre, P.B., Gessner, M.O., Dudgeon, D., Prusevich, A., Green, P., Glidden, S., Bunn, S.E., Sullivan, C.A., Liermann, C.R., Davies, P.M., 2010. Global threats to human water security and river biodiversity. Nature 467, 555–561.

Wallace, J.B., 1997. Multiple trophic levels of a forest stream linked to terrestrial litter inputs. Science 277, 102–104.

Webster, J., 1959. Experiments with spores of aquatic hyphomycetes: I. Sedimentation, and impaction on smooth surfaces. Annals of Botany 23, 595–611.

Webster, J., Descals, E., 1981. Morphology, distribution, and ecology of conidial fungi in freshwater habitats, in: Cole, G.T., Kendrick, B. (Eds.), Biology of conidial fungi. Volume 1. Academic Press, New York, USA, pp. 295–355.

Wildman, H.G., Parkinson, D., 1981. Seasonal changes in water-soluble carbohydrates of *Populus tremuloides* leaves. Canadian Journal of Botany 59, 862–869.

Wright, A.J., Wardle, D.A., Callaway, R., Gaxiola, A., 2017. The overlooked role of facilitation in biodiversity experiments. Trends in Ecology and Evolution 32, 383–390.

Zhang, J., Hu, S., Xu, Q., You, H., Zhu, F., 2018. Baseline sensitivity and control efficacy of propiconazole against *Sclerotinia sclerotiorum*. Crop Protection 114, 208–214.

Zubrod, J.P., Bundschuh, M., Arts, G., Brühl, C.A., Imfeld, G., Knäbel, A., Payraudeau, S., Rasmussen, J.J., Rohr, J., Scharmüller, A., Smalling, K., Stehle, S., Schulz, R., Schäfer, R.B., 2019. Fungicides: an overlooked pesticide class? Environmental Science & Technology 53, 3347–3365.

Zubrod, J.P., Englert, D., Feckler, A., Koksharova, N., Konschak, M., Bundschuh, R., Schnetzer, N., Englert, K., Schulz, R., Bundschuh, M., 2015a. Does the current fungicide risk assessment provide sufficient protection for key drivers in aquatic ecosystem functioning? Environmental Science & Technology 49, 1173–1181.

Zubrod, J.P., Englert, D., Wolfram, J., Wallace, D., Schnetzer, N., Baudy, P., Konschak, M., Schulz, R., Bundschuh, M., 2015b. Waterborne toxicity and diet-related effects of fungicides in the key leaf shredder *Gammarus fossarum* (Crustacea: Amphipoda). Aquatic Toxicology 169, 105–112.

## APPENDIX

Manuscripts **[A.1-A.4]** represent the latest versions submitted to the respective journal.

**A.1: A glance into the black box: Novel species-specific quantitative real-time PCR assays to disentangle aquatic hyphomycete community composition**
Baudy, Zubrod, Röder, Baschien, Feckler, Schulz, Bundschuh **56**

**A.2: The fungicide tebuconazole confounds concentrations of molecular biomarkers estimating fungal biomass**
Baudy, Konschak, Sakpal, Baschien, Schulz, Bundschuh, Zubrod **88**

**A.3: Fungal-fungal and fungal-bacterial interactions in aquatic decomposer communities: bacteria promote fungal diversity**
Baudy, Zubrod, Konschak, Kolbenschlag, Pollitt, Baschien, Schulz, Bundschuh **101**

**A.4: Environmentally relevant fungicide levels modify fungal community composition and interactions but not functioning**
Baudy, Zubrod, Konschak, Röder, Nguyen, Schreiner, Baschien, Schulz, Bundschuh **150**

**A.5: Curriculum vitae** **182**

**APPENDIX A.1**

# A glance into the black box: Novel species-specific quantitative real-time PCR assays to disentangle aquatic hyphomycete community composition

Patrick Baudy, Jochen P. Zubrod, Nina Röder, Christiane Baschien, Alexander Feckler, Ralf Schulz, Mirco Bundschuh

Fungal Ecology 42, 100858
Impact Factor (2020): 3.404

**ABSTRACT**

Aquatic hyphomycetes (AH) are ubiquitous fungi playing a key role in the decomposition of leaf litter in streams. Though their functional performance is modulated by their community composition, this ecological relationship remains poorly investigated due to a lack of suitable methods to identify the biomass-contribution of individual species to AH communities. We, therefore, designed and validated TaqMan® probe-based qPCR assays targeting ten AH species common in temperate regions, allowing detection and quantification of these species within complex communities. In a further step, we compared qPCR-obtained DNA levels to concentrations of the traditional fungal biomass proxy ergosterol. We demonstrate that the qPCR assays are valid for use and that DNA and ergosterol concentrations were significantly positively correlated, suggesting DNA levels as a suitable species-specific biomass proxy. Accordingly, the use of these assays may facilitate multi-species experiments to address major research issues in stress and community ecology including biodiversity-ecosystem functioning relationships.

**KEYWORDS**

*Alatospora acuminata* – *Articulospora tetracladia* – *Clavariopsis aquatica* – *Clavatospora longibrachiata* – *Flagellospora curvula* – *Heliscella stellata* – *Lemonniera terrestris* – *Neonectria lugdunensis* – *Tetracladium marchalianum* – *Tricladium angulatum*

**INTRODUCTION**

Aquatic hyphomycetes (AH) are a ubiquitously occurring polyphyletic group of asexual fungi (Baschien et al., 2013; Duarte et al., 2016) that colonize submerged plant material in surface waters and disperse via the release of conspicuous conidia (Webster and Descals, 1981; Gulis, 2001). Their activity contributes to ecologically important key functions in forested stream ecosystems, which heavily rely on allochthonous plant material (particularly leaf litter) as the primary source of energy (Petersen and Cummins, 1974; Wallace, 1997). These functions include the direct decomposition of leaf litter and the concurrent release of energy and nutrients (Hieber and Gessner, 2002), as well as the chemical and physical modification of such leaf litter (i.e., conditioning by softening), by which the resource accessibility of leaves is increased for detritivorous stream invertebrates (Cummins and Klug, 1979).

The efficiency of these functions through AH communities is modulated by their structure (i.e., community composition) and contributing species' functional traits such as enzyme production and nutrient stoichiometry (Gessner et al., 2010; Bärlocher, 2016; Danger et al., 2016). Community structure, in turn, heavily depends on the environmental context (Pascoal et al., 2005; Baudoin et al., 2008; Duarte et al., 2016). Mechanisms behind the interplay of environmental variables, community composition, and functioning (e.g., niche adaptation, biodiversity-ecosystem functioning relationships, or taxa-area relationships), however, remain poorly understood (Bell et al., 2009).

To investigate the mechanisms behind these ecological relationships, it is essential to acquire knowledge of the biomass-contribution of individual AH species, featuring specific functional traits, to a community (Bärlocher, 2016). Conventionally applied methods, namely quantifying fungal biomass using the fungal membrane molecule ergosterol as proxy and the morphological identification and counting of spores, do not or only partially fulfill this demand. While the former method measures ergosterol as the sum parameter of total fungal biomass (i.e., not species-specific; Gessner et al., 2003), the latter method is rather indicative of AHs' reproductive activity and can neither provide proof of species' absence nor does it necessarily correlate with species' biomass (Bermingham et al., 1996a, 1997; Fernandes et al., 2015). Although high-throughput sequencing (HTS) techniques promise a more comprehensive picture of AH diversity (Fernandes et al., 2015), methodological limitations prevent a precise quantification of single species (Elbrecht and Leese, 2015; Nilsson et al., 2019). This may be realized using enzyme-linked immunosorbent assays (ELISA; Bermingham et al., 1996b), fluorescence in situ hybridization (FISH; Baschien et al., 2008), or quantitative real-time PCR (qPCR; e.g., Feckler et al., 2017). Among these methods, qPCR offers the highest level of sensitivity and standardization for routine application (Haffar and Gilbride, 2010; Krauss et al., 2011; Bärlocher, 2016). Accordingly, qPCR constitutes a suitable tool to disentangle AH community composition and in turn may provide a deeper understanding of mechanisms linking environmental context and biodiversity-ecosystem functioning relationships in AH communities.

Currently only four AH species are covered by available qPCR assays (Fernandes et al., 2011; Feckler et al., 2017), preventing the assessment of more complex, natural or artificial (i.e., laboratory) AH communities. Although, compared to

HTS, qPCR does not allow for an all-encompassing picture of community composition, its use for species-specific biomass quantification may help to fill major research gaps. Hence, our aim was to establish a set of species/ taxon-specific qPCR assays for AH species common in fungal communities in streams of the temperate latitudes. We generated DNA sequence data from various AH strains isolated from streams in Germany as a basis to design TaqMan® probe-based qPCR assays. Assay sequences (primer pairs and probes) were designed to target sequence signatures in the internal transcribed spacer region of the ribosomal DNA operon – used as the primary barcoding region for fungi (Schoch et al., 2012). The assays were validated experimentally and in silico to demonstrate their suitability for a species-specific assessment of AH biomass in complex leaf-associated microbial communities. The suitability of DNA quantity (e.g., obtained by qPCR) as fungal biomass proxy is, however, still under debate (e.g., Marstorp et al., 2000; Baldrian et al., 2013). Therefore, in a final step, we tested whether concentrations of DNA and ergosterol, a widely accepted reference biomarker for fungal biomass estimations (Gessner, 2005), are positively correlated among the AH species targeted by our qPCR assays.

## MATERIAL AND METHODS

### FUNGAL STRAINS

The 81 AH strains used to design and validate qPCR assays were prepared from single spores (as described in Descals, 2005) collected from streams located in Germany (Table S1). Isolated strains were maintained on Petri dishes containing 2% (w/v) malt extract agar (MEA; 20 g/L malt extract, 20 g/L agar) at 16°C in complete darkness for the duration of the experimental procedures. Subcultures of the isolated strains were deposited in the open collection of the Leibniz Institute DSMZ (German Collection for Microorganisms and Cell Cultures, Germany) for sequencing of the internal transcribed spacer (ITS) region (covering the subregions ITS1, 5.8S, and ITS2) and the D1/D2 domain of the 28S large subunit (LSU; for a full description of methods, see Document S1), identification, and preservation in liquid nitrogen. The ten AH species e belonging to the ergosterol-containing ascomycetes (Newell, 1992) e presented in Table 1 were selected as target species for qPCR assay development. This selection covers species frequently detected in AH communities of the temperate latitudes, which exhibit varying sensitivities to anthropogenic stress (e.g.,

Schlief and Mutz, 2011; Zubrod et al., 2015; Fernandez et al., 2016). Hence, they can be considered as promising model species to study adaptation processes or indicator species for aquatic biomonitoring. One model strain per targeted species (also referred to as a target strain; Table 1) was used for experimental analyses. The nine model strains related to species not targeted by an assay, may in this context also be referred to as non-target strain.

Mycelium of the model strains for qPCR and ergosterol analyses was obtained from liquid cultures prepared under sterile conditions. Therefore, for every strain, eight agar plugs with 5 mm diameter were punched from the growing edge of a colony maintained on 2% MEA. The plugs were homogenized in 20 mL of a 2% (w/v) malt extract solution (MES; 20 g/L malt extract) using an Ultra-turrax® blender (IKA®-Werke GmbH & Co. KG, Germany) at 3500 rpm. Subsequently, 0.5 mL of the homogenate was used to inoculate a liquid culture consisting of 30 mL of MES in a 50-mL Erlenmeyer flask. Liquid cultures were replicated five times ($n$ = 5) for each of the ten model strains ($N$ = 50). The flasks were stoppered with cellulose plugs and kept at 16°C on a rotary shaker (model KS 15, Edmund Bühler GmbH, Germany) at 120 rpm in complete darkness, while growth of the cultures was visually inspected on a daily basis. Due to strongly varying growth rates among strains (cf. Gessner and Chauvet, 1993), sampling sufficient biomasses for further analyses (i.e., > 5mg dry weight per culture for each biomarker) was prioritized over sampling a particular growth phase. Accordingly, incubation times ranged from 7 to 24 d. After incubation, the cultures were transferred to 50-mL Falcon tubes and washed three times with approximately 45 mL of sterile deionized water to remove MES residues and afterwards stored at -20°C until further processing.

GENETIC MATERIAL

Fungal genomic DNA extracts used for qPCR analysis were prepared from lyophilized mycelia of liquid cultures stored at 20°C (see above). Therefore, 6-17 mg dry weight per culture e depending on the availability of biomass e was weighed to the nearest 0.01 mg (only for samples in which DNA was put in relation to biomass). Afterwards, DNA was extracted using the FastDNA® Spin Kit for Soil in conjunction with the FastPrep™-24 5G Instrument (MP Biomedicals, Germany) according to the manufacturer's instructions. Since our final aim was to detect and quantify the target species associated with plant detritus, DNA of black alder (*Alnus glutinosa*) leaves

was extracted as described above and used as additional non-target DNA for experimental assay validation. For this purpose, leaf material was collected shortly before abscission in 2016 near Landau, Germany (49.20116N, 8.09331E) and stored at -20°C until further use (for ~2 y).

DNA standards were prepared from target AH strains and leaf extracts. To prepare the initial standards with a DNA concentration of approximately 10 (±2) ng/mL, genomic DNA concentration in pure extracts was quantified using a NanoDrop 1000 Spectrophotometer (NanoDrop products, USA), before diluting extracts with sterile DNase-free water ($ddH_2O$). Afterwards, standards were serially diluted ten-fold down to $10^{-9}$ ng/mL (i.e., ten standards for each AH strain and black alder). For use in the experimental validation, a non-target mixture was prepared for each qPCR assay, containing 1 ng/mL of DNA from black alder leaves and each of the non-target strains, yielding a sum DNA concentration of 10 ng/mL.

## ASSAY DESIGN AND PROCEDURE

The ITS region and the D1/D2 domain of the adjacent LSU were used as a basis for the design of the qPCR assays for each of the ten AH strains. The procedure generally followed the method described in Feckler et al. (2017). Criteria for the design of suitable assay sequences were species-specificity and appropriate primer and probe hybridization properties (e.g., oligonucleotide length, melting temperature, guanine-cytosine content, and amplicon length) as defined by the default settings of the software Primer Express (version 2.0, Applied Biosystems, USA). Using the bioinformatics software Geneious (version 10.0.9, Biomatters, New Zealand), ITS sequences of the model strains were ClustalW-aligned against sequences of target and closely related non-target species. This procedure comprised sequences identified via GenBank's “basic local alignment search tool” algorithm (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) and all sequences produced in this study (Table S1). After a suitable region was visually identified in the alignment, primers and probe of each assay were designed based on a template ITS sequence (Table 1) using the manual design function of the Primer Express software. Assays were validated in silico (see section 2.5 *In silico* assay validation) and synthesized by Applied Biosystems, Germany. TaqMan® probes contained the fluorescent reporter dye 6-carboxyfluorescein (6-FAM) on the 5’-end and the non-fluorescent quencher (MGBNFQ) on the 3’-end. Each assay was obtained as a mix

Table 1. Information on qPCR assay designations, targeted species, including the used model strain and template sequences as well as technical properties including melting temperature, guanine-cytosine content, binding region, amplicon length, efficiency parameters, inter- as well as intra-assay coefficients of variation (CV), limits of detection as well as quantification (expressed as cycle of quantification (Cq) and DNA concentration per PCR), and quantitative ranges covered by DNA standards.

| qPCR assay designation | Target species | Target strain (DSM number) | Template ITS sequence (GenBank accession number) | Primer/ probe | Sequence (5'-3') | Primer/ probe length (bp) | Melting temperature (°C) | Guanine-cytosine content (%) | Binding region | Amplicon length (bp) | Slope | $R^2$ | Amplification efficiency (%) | Overall inter-assay variation and range (CV in %; *n*=7) | Overall intra-assay variation and range (CV in %; *n*=5) | Limit of detection | | Limit of quantification | | Quantitative standards covered by the assay (ng/µL) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | | | $C_q$ value | fg/ PCR | $C_q$ value | fg/ PCR | |
| ALAC-tqmn | *Alatospora acuminata* | DSM 104360 | **MH930815** | Forward | CGTAGTAATTTTCTCGCTTTGGAGA | 21 | 59.0 | 52 | ITS2 | 82 | -3.599 | 1.000 | 90 | 0.9 (0.6-1.1) | 0.4 (0.2-1.0) | 39.04 | 3.57 | 34.50 | 65.22 | $10\text{-}10^{-5}$ |
| | | | | Reverse | CCTGATCCGAGGTCAACCTTT | 21 | 59.0 | 52 | ITS2/LSU | | | | | | | | | | | |
| | | | | Probe | 6 FAM-TTTGCCAACAACCC-MGBNFQ | 14 | 68.0 | 50 | ITS2 | | | | | | | | | | | |
| ARTE-tqmn | *Articulospora tetracladia* | DSM 104345 | **MH930816** | Forward | GCCCTGTGGTATTCCGCA | 18 | 59.0 | 61 | 5.8S | 77 | -4.267 | 0.999 | 72 | 0.8 (0.2-1.6) | 0.7 (0.4-1.0) | 44.44 | 38.54 | 41.15 | 227.47 | $10\text{-}10^{-4}$ |
| | | | | Reverse | AGGCCCCAATACCAAGCG | 18 | 59.5 | 61 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-TCATTTCAACCCATCAAG-MGBNFQ | 18 | 68.0 | 39 | 5.8S/ITS2 | | | | | | | | | | | |
| CLAQ-tqmn | *Clavariopsis aquatica* | DSM 104362 | **MH930817** | Forward | AATTCATTGGCAGCCGGTAA | 20 | 59.0 | 45 | ITS2 | 82 | -3.435 | 1.000 | 95 | 0.6 (0.4-1.1) | 0.6 (0.2-0.9) | 40.08 | 0.65 | 35.79 | 11.56 | $10\text{-}10^{-5}$ |
| | | | | Reverse | AAGAGGCTTGATGGAAGGAGGTA | 23 | 59.2 | 48 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-TGTGTCGCGATCCAGT-MBGNFQ | 16 | 70.0 | 56 | ITS2 | | | | | | | | | | | |
| CLLO-tqmn | *Clavatospora longibrachiata* | DSM 104365 | **MH930818** | Forward | GTTTGACCAAGACACAGCAAAATG | 24 | 59.6 | 42 | ITS2 | 89 | -3.450 | 1.000 | 95 | 0.4 (0.2-0.6) | 0.9 (0.3-2.0) | 41.55 | 3.26 | 37.09 | 63.99 | $10\text{-}10^{-5}$ |
| | | | | Reverse | CTACGTTTCTCTTACGAAAAACTGTTTGT | 29 | 59.6 | 34 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-CTGGAAATTTGAACTATTTG-MGBNFQ | 20 | 69.0 | 30 | ITS2 | | | | | | | | | | | |
| FLCU-tqmn | *Flagellospora curvula* | DSM 104334 | **MH930819** | Forward | CTCAAGCTCTGCTTGGTATTGG | 22 | 57.8 | 50 | ITS2 | 108 | -3.339 | 1.000 | 99 | 0.7 (0.6-0.9) | 1.0 (0.4-2.2) | 39.16 | 0.72 | 35.03 | 12.33 | $10\text{-}10^{-5}$ |
| | | | | Reverse | GGTCCTCCAGAGCGAGAAGA | 20 | 58.1 | 60 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-CGCCTTAAAGACAGTGGC-MGBNFQ | 18 | 70.0 | 56 | ITS2 | | | | | | | | | | | |
| HEST-tqmn | *Heliscella stellata* | DSM 104386 | **MH930820** | Forward | GCGTCGTAGTTTGACCAAGACA | 22 | 58.9 | 50 | ITS2 | 79 | -3.445 | 0.999 | 95 | 0.8 (0.5-1.2) | 0.5 (0.1-1.2) | 41.41 | 7.71 | 37.05 | 142.13 | $10\text{-}10^{-4}$ |
| | | | | Reverse | AAACAGATGATTCAAGGCTAATCGT | 25 | 58.3 | 36 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-CAATGTGAAACTAAGTCAGATAA-MGBNFQ | 23 | 70.0 | 30 | ITS2 | | | | | | | | | | | |
| LETE-tqmn | *Lemonniera terrestris* | DSM 104344 | **MH930821** | Forward | AGCGTCATTACAACCCCTCAAG | 22 | 59.1 | 50 | 5.8S/ITS2 | 81 | -3.498 | 1.000 | 93 | 0.6 (0.4-1.2) | 0.6 (0.1-1.6) | 40.58 | 2.01 | 36.13 | 37.55 | $10\text{-}10^{-5}$ |
| | | | | Reverse | ATGGCACCGCCACTGAGT | 18 | 58.6 | 61 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-TGGTATTGGAGCATGCG-MGBNFQ | 17 | 70.0 | 53 | ITS2 | | | | | | | | | | | |
| NELU-tqmn | *Neonectria lugdunensis* | DSM 104361 | **MH930822** | Forward | TGTAGCTTCCTCTGCGTAGTAGCA | 24 | 59.0 | 50 | ITS2 | 90 | -3.431 | 1.000 | 96 | 0.9 (0.5-1.8) | 0.5 (0.1-1.3) | 38.02 | 3.52 | 33.73 | 62.56 | $10\text{-}10^{-5}$ |
| | | | | Reverse | CCGAGGTCAACCTTTCAGAAGT | 22 | 58.0 | 50 | ITS2/LSU | | | | | | | | | | | |
| | | | | Probe | 6 FAM-TCGCACTGGAAAGC-MGBNFQ | 14 | 69.0 | 57 | ITS2 | | | | | | | | | | | |
| TEMA-tqmn | *Tetracladium marchalianum* | DSM 104373 | **MH930823** | Forward | GCTGTCAGGCTCTAAGCGTAGTAA | 24 | 58.0 | 50 | ITS2 | 64 | -3.484 | 1.000 | 94 | 0.6 (0.3-1.2) | 0.5 (0.1-0.9) | 39.11 | 2.03 | 34.75 | 36.16 | $10\text{-}10^{-5}$ |
| | | | | Reverse | GTTCTGGCGAGTGTCCATCA | 20 | 58.0 | 55 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-CTCTCTCGCTACAGACAC-MGBNFQ | 18 | 69.0 | 56 | ITS2 | | | | | | | | | | | |
| TRAN-tqmn | *Tricladium angulatum* | DSM 104374 | **MH930824** | Forward | CCTGTTCGAGCGTCATCAAA | 20 | 58.5 | 50 | 5.8S/ITS2 | 129 | -3.523 | 1.000 | 92 | 0.6 (0.3-1.1) | 0.5 (0.2-1.8) | 42.16 | 0.72 | 37.68 | 13.36 | $10\text{-}10^{-5}$ |
| | | | | Reverse | CCGACGTCTATAGCGAGAAGAATT | 24 | 59.0 | 46 | ITS2 | | | | | | | | | | | |
| | | | | Probe | 6 FAM-CCTGGCAGCCCTTA-MGBNFQ | 14 | 68.0 | 64 | ITS2 | | | | | | | | | | | |

containing 18 µM of each primer and 5 µM probe. In the following, particular qPCR assays will be referred to by a combination of the first two letters of the target species' genus and epithet and the ending “-tqmn” (e.g., *Tricladium angulatum*: TRAN-tqmn; Table 1).

All qPCR reactions were performed in a Mastercycler® ep gradient S (Eppendorf, Germany) using clear 0.2-mL 8-tube strips covered with clear flat optical 8-cap strips (Sarstedt AG & Co., Germany). The total reaction volume of 10 mL consisted of 2 mL sample, standard, or $ddH_2O$ and 8 mL of master mix containing 4 mL of 2x Environmental Mastermix 2.0 (Applied Biosystems, Germany), 3.48 mL of $ddH_2O$, and 0.52 mL of primer and probe mix (i.e., final reaction concentrations of 940 nM of each primer and 260 nM probe). Reactions were performed in duplicate. Each qPCR run comprised six to seven DNA standards of the target strain and no-template controls in addition to the samples to be analyzed. The qPCR program (as recommended by the assay manufacturer) covered an initial uracil-N-glycosylase incubation step of 2 min at 50°C, a DNA polymerase activation step of 10 min at 95°C, 50 cycles of denaturation for 15 s at 95°C and hybridization and extension for 1 min at 60°C. Fluorescence intensity was measured at the end of each cycle. Finally, the cycle of quantification ($C_q$) was determined for each reaction as a measure of DNA quantity (Heid et al., 1996; for $C_q$ value calculation see section 2.8 Data analysis).

ASSAY PERFORMANCE EVALUATION

Each assay was calibrated in seven independent qPCR runs using ten DNA standards ranging from $10^{-9}$-10 ng/mL, with one replicate each. DNA standards showing a $C_q$ standard deviation of ≤ 0.5 were considered as quantitative standards and were used to calculate linearity, amplification efficiency, repeatability, and precision. Theoretical limits of detection (LOD) and limits of quantification (LOQ) were calculated as described in Feckler et al. (2017) and references therein. Briefly, PCR forming units (PFU) representing the truly amplified copies of the standards were determined following the SIMQUANT assay (Berdal et al., 2008). The LOD was defined as the $C_q$ value on the standard curve corresponding to 3 PFUs, while the LOQ was calculated from the quantitative standard with the lowest DNA concentration. If it was not possible to determine these limits according to the method described above, the LOD was defined by the standard with the lowest DNA

concentration showing a detection frequency of 95% (Forootan et al., 2017), while the LOQ was defined by the lowest calibration level (i.e., the quantitative standard with the lowest DNA concentration). Finally, for each assay an additional qPCR run was conducted with five replicates of each quantitative standard to examine intra-assay variations.

*IN SILICO* ASSAY VALIDATION

Using BLAST, assay sequences were in silico cross-checked against the International Nucleotide Sequence Database (INSD; http://www.insdc.org/). For forward and reverse primer sequences, we used the primer-BLAST feature (https://www.ncbi.nlm.nih.gov/ tools/primer-blast/). For probe sequences, we used the nucleotide-BLAST feature with adjustment for short input sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Parameters for both BLAST searches were adjusted to exclude uncultured and environmental samples and to return a maximum of 1000 highly similar database entries. BLAST results were checked for species showing 100% similarity with assay sequences in both searches. Identified species were used to investigate assays' species-specificity more closely. Therefore, all deposited sequences of these identified species covering the complete assay target region (i.e., ITS2 and partially the flanking regions) and having no unidentified nucleotides in this region were downloaded from the INSD and species-wise ClustalW-aligned using the Geneious software. Subsequently, a primer test was performed on each alignment to check for the number of sequences compatible with the respective assay. Sequences showing 100% similarity with both primers and the probe were considered compatible with the respective assay (Rodríguez et al., 2015). Assays were considered species-specific if only sequences related to the target species were compatible.

EXPERIMENTAL ASSAY VALIDATION

Each assay was experimentally cross-validated for species-specificity and PCR inhibition. Species-specificity was assessed using positive/negative reactions containing only 20 ng of target, non-target, or non-target mixture DNA (Fig. 1 A). Reactions were rated as positive if both replicates showed a $C_q$ and if the average $C_q$ was below the LOD of the respective assay, otherwise it was rated as negative. To assess PCR inhibition, reactions containing quantitative standards of the target

species were spiked by substituting 2 mL of dd$H_2O$ of the master mix with 2 mL of non-target DNA. Standards were spiked with similar DNA concentrations of each non-target species individually (Fig. 1 B, C). Additionally, to simulate a worst-case situation with low amounts of target DNA in the presence of high amounts of non-target DNA originating from various species, a fixed amount of 20 ng of the non-target DNA mixture was used (Fig. 1 D).

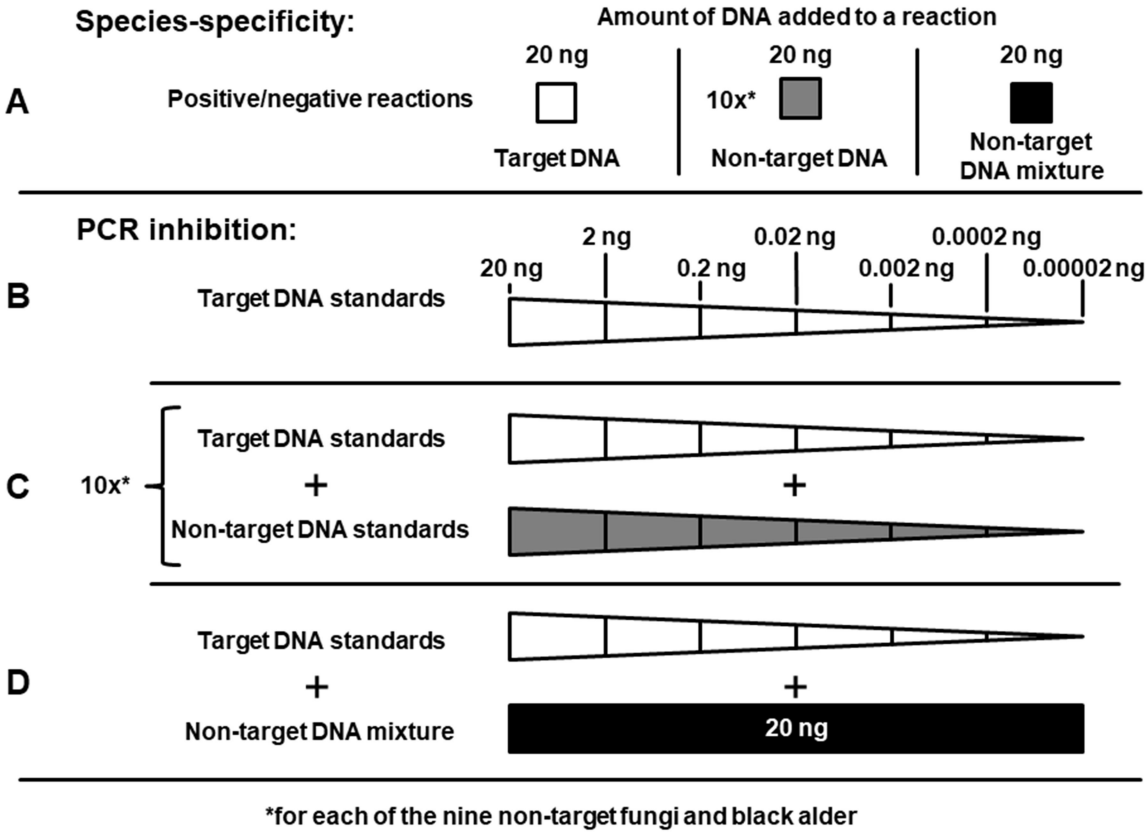



Fig. 1. Schematic layout of the experimental validation. (A) The assessment of species-specificity comprised single reactions containing target, non-target, or non-target mixture DNA. (B-D) The assessment of PCR inhibition included the analysis of target DNA standards (B) without the addition of non-target DNA, (C) with addition of similar concentrations of non-target DNA, and (D) with addition of a fixed concentration of a non-target DNA mixture. Each reaction was replicated twice.

## ERGOSTEROL ANALYSIS

Ergosterol was analyzed in all liquid culture samples using lyophilized mycelium following the procedure described in Gessner and Chauvet (1993). Briefly, ergosterol was extracted from 7-20 mg dry weight per culture – depending on the availability of biomass – weighed to the nearest 0.01 mg, in 10 mL of alkaline methanol. The extract was purified by solid-phase extraction (Sep-Pak® Vac RC tC18 500mg sorbent, Waters, USA) and ergosterol was quantified using a high-

performance liquid chromatography (HPLC) system (1200 Series, Agilent Technologies, USA). The detailed procedure is described in Zubrod et al. (2011).

DATA ANALYSIS

All qPCR runs were analyzed with the software Realplex 2.2 (Eppendorf, Germany). Determined $C_q$ values are inversely proportional to the log10-transformed initial target DNA quantity in a reaction and were automatically calculated from individual fluorescence curves via the software-implemented CalQPlex algorithm. To assess amplification quality, each fluorescence curve was visually inspected for irregularities by comparison with standard curves via the amplification plot of each qPCR run. For each assay, two types of calibration curves were prepared from the seven calibration runs modeling $C_q$ values as a linear function of either log10transformed PFUs or log10-transformed DNA amounts of the quantitative standards. The PFU calibration curve was used to calculate the LOD and LOQ as described above (see section 2.4 Assay performance evaluation). The DNA calibration curve was used to calculate amounts of target DNA from $C_q$ values of the reactions (i.e., ng DNA/PCR). For liquid culture samples, the amount of DNA/PCR was extrapolated to the total amount of target DNA in the initial extraction volume (i.e., 100 mL) and divided by the dry weight of the sample used for extraction (i.e., ng DNA/mg dry weight). Ergosterol concentrations obtained via HPLC were analogously extrapolated (i.e., mg ergosterol/mg dry weight). Intra-assay and inter-assay variations as well as variations in ergosterol and DNA concentrations among and within fungal species were expressed as coefficient of variation (CV; in percent), which was calculated as for instance described in Everitt and Skrondal (2002).

Prior to statistical analyses, ergosterol and DNA concentration data were checked for normality using Shapiro-Wilk's test. If the assumption for parametric testing were met, associations between ergosterol and DNA concentrations among and within the species were evaluated by Pearson's correlation. Otherwise, Spearman's correlation was conducted. To validate target DNA quantification in the presence of non-target DNA, linear models of standards (Fig. 1 B) and spiked standards (Fig. 1 C, D) were compared against each other. Using analysis of covariance (ANCOVA), we assessed whether target DNA concentration, non-target DNA treatment, or their interaction affect $C_q$ values. All figures and statistics were prepared using R version 3.5.2 (R Core Team, 2018). The term “significant(ly)” is

exclusively used with regard to statistical significance (i.e., $p < 0.05$) throughout this paper.

## RESULTS AND DISCUSSION

### ASSAY PERFORMANCE

All qPCR assays (for assay designations, sequences, properties, and performances see Table 1) were designed to target the ITS2 region, which proved more suitable than ITS1 to create primer and probe sequences with adequate hybridization properties, yet maximizing species-specificity. Accordingly, assay performances in terms of efficiency, sensitivity, repeatability, and precision are of comparable quality to well-established assays in the literature (e.g., Pfaffl, 2001; Ginzinger, 2002; Tomlinson et al., 2005; Bhuiyan et al., 2018). The only exception is the ARTE-tqmn assay, which we refrained from optimizing – e.g., by testing other cycling conditions than suggested by the manufacturer – to maintain standardization. The ARTE-tqmn assay had a low amplification efficiency of 72%, which may be explained by substantial intragenomic ITS2 variability in the targeted species Articulospora tetracladia (Seena et al., 2012). Due to this low amplification efficiency, the calculation of the LOD and LOQ was not possible via the SIMQUANT approach and had to be realized via the alternative method (as described in section 2.4 Assay performance evaluation). Accordingly, LOD and LOQ were 38.54 and 227.47 fg DNA/PCR, respectively. In contrast, amplification efficiencies of the other assays ranged from 90 to 99% with LODs and LOQs ranging from 0.65 to 7.71 fg/PCR and 11.56 to 142.13 fg DNA/PCR, respectively. Quantitative standards for all assays (including the ARTE-tqmn assay) covered four to five log units, ranging from 10 ng DNA/mL down to 10 or 100 fg DNA/mL. Moreover, repeatability and precision were high in all assays as indicated by overall inter-assay and intra-assay variations of 0.4-0.9% and 0.4-1.0%, respectively.

### *IN SILICO* ASSAY VALIDATION

qPCR assay design, based on the AHs' ITS barcoding region, frequently required compromises between species-specificity and suitable assay hybridization properties, partially resulting in suboptimal specificities. Nonetheless, the in silico analysis based on INSD queries (accessed on 09/06/2018) revealed that the majority of assays, namely ALAC-tqmn, ARTE-tqmn, CLAQ-tqmn, CLLO-tqmn, HEST-tqmn,

and TRAN-tqmn, can be considered species-specific (Table 2; Table S2). Non-specificity of the LETE-tqmn and NELU-tqmn assays appears evident, due to a low sequence divergence in the subunits of the ribosomal DNA operon within the genera of the target species *Lemonniera terrestris* and *Neonectria lugdunensis*, respectively (e.g., Baschien et al., 2006, 2013). However, due to a comparatively lower number of publicly available reference sequences (e.g., INSD), and the fact that ITS is the most widely used locus for the barcoding of fungi, we refrained from using other loci (e.g., the translation elongation factor 1-α or β-tubulin genes). Also, non-species-specificity of the FLCU-tqmn and TEMA-tqmn assays has been observed. In both cases only one sequence each, allocated to strains identified as genetically and morphologically closely related to the target species (i.e., *Flagellospora fusarioides* and *Tetracladium breve*, respectively), matched the respective assay (Table 2; Table S2) demonstrating the difficulty of using ITS as the only marker (Johnston et al., 2019).

The assays matched approximately 90-100% of target species sequences (i.e., coverage) deposited in the INSD (including sequences produced in this study), except for the ALAC-tqmn, NELU-tqmn, and TEMA-tqmn assays (Table 2; Table S2). The non-coverage of target strains may similarly, at least to some degree, be attributed to single strains being misidentified (Bridge et al., 2003) or common intraspecific genetic variations at the assay binding sites (Seena et al., 2012). However, the particularly low coverage of the ALAC-tqmn, NELU-tqmn, and TEMA-tqmn assays may furthermore be related to distinct phylogenetic groups within the targeted species *Alatospora acuminata*, *N. lugdunensis*, and *Tetracladium marchalianum*, respectively. In fact, this has already been suggested based on analyses with other molecular approaches (Baschien et al., 2006, 2013; Anderson and Shearer, 2011). However, it is noteworthy that the results of *in silico* analyses generally need to be interpreted with caution since a substantial percentage of fungal sequence entries in publicly available databases is suspected to be incorrectly identified to the species-level (e.g., INSD ~20%; Nilsson et al., 2006). Moreover, assay sequences should be regularly re-evaluated for species-specificity against newly deposited sequences of continuously growing nucleotide databases (Duarte et al., 2014) and may be adjusted if necessary (cf. Baschien et al., 2008).

Table 2. List of assay compatible species identified during the in silico validation as well as numbers of related INSD entries covering the full assay target region and the number entries being 100% compatible with the respective assay. Species targeted by the respective assay are printed in bold. Values in parentheses indicate the proportion of sequences produced in this study, included in the number of INSD sequences outside the parentheses. For a complete list of INSD entries used for the *in silico* validation see Table S2.

| | | INSD entries | | |
|---|---|---|---|---|
| qPCR assay designation | Species | Number of entries covering the full assay target region | Number of entries showing 100% similarity with all assay sequences | Entry coverage (%) |
| ALAC-tqmn | ***Alatospora acuminata*** | 18 (8) | 5 (2) | 28 |
| ARTE-tqmn | ***Articulospora tetracladia*** | 172 (6) | 161 (5) | 94 |
| CLAQ-tqmn | ***Clavariopsis aquatica*** | 13 (3) | 13 (3) | 100 |
| CLLO-tqmn | ***Clavatospora longibrachiata*** | 6 (4) | 6 (4) | 100 |
| FLCU-tqmn | ***Flagellospora curvula*** | 8 (3) | 7 (3) | 88 |
| | *F. fusarioides* | 1 | 1 | 100 |
| HEST-tqmn | ***Heliscella stellata*** | 2 (2) | 2 (2) | 100 |
| LETE-tqmn | *Lemonniera aquatica* | 9 (3) | 8 (3) | 89 |
| | *L. centrosphaera* | 3 (1) | 3 (1) | 100 |
| | *L. cornuta* | 2 | 2 | 100 |
| | ***L. terrestris*** | 7 (3) | 7 (3) | 100 |
| | *Margaritispora aquatica* | 2 (2) | 2 (2) | 100 |
| NELU-tqmn | *Cylindrocarpon obtusisporum* | 12 | 6 | 50 |
| | ***Neonectria lugdunensis*** | 36 (14) | 27 (6) | 75 |
| | *Phaeoacremonium vibratile* | 1 | 1 | 100 |
| TEMA-tqmn | *Tetracladium breve* | 7 (1) | 1 (0) | 14 |
| | ***T. marchalianum*** | 77 (6) | 57 (5) | 74 |
| TRAN-tqmn | ***Tricladium angulatum*** | 11 (3) | 11 (3) | 100 |

## EXPERIMENTAL ASSAY VALIDATION

Experimental validation indicates a reliable species-specific DNA detection and quantification by all assays within their technical limits. Reactions containing only non-target DNA as template (i.e., 20 ng of DNA of the respective nine non-target fungi or black alder and the non-target mixture; Fig. 1 A) were consistently negative (Table S3). Moreover, target DNA quantification in each of the assays was not affected (e.g., by PCR inhibition) in the presence of non-target DNA (Table S4; ANCOVA; all *p*-values ≥ 0.72). The only exception to this is the LETE-tqmn assay in combination with DNA of A. tetracladia. Reactions containing high DNA concentrations of this non-target species (i.e., individually and within the non-target mixture) yielded positive signals with Cq values of 27.72 and higher (corresponding to ~10 pg of target DNA/PCR and less). Accordingly, quantification of target DNA was affected in the presence of the non-target mixture above $C_q$ values of

approximately 30 (i.e., reactions containing less than ~2 pg of target DNA/PCR). However, affected reactions could be clearly identified by visual inspection of the amplification plots as shapes of amplification curves notably deviated from non-affected reactions (Fig. S1). Consequently, the LETE-tqmn assay may still be considered species-specific for analysis of target DNA in complex samples, provided that only results of target DNA concentrations above 10 pg/PCR are considered and amplification plots are carefully checked.

APPLICATION

Observed ergosterol and DNA concentrations (Table 3) varied among the species by factors of approximately two and five, respectively, and were well in the range of concentrations reported for AH cultured under similar conditions (ergosterol: 2.3-11.5 mg/mg; Gessner and Chauvet, 1993;DNA:40-3960 ng/mg; Grimmett et al., 2013). Moreover, inter- and average intraspecific variability in DNA concentrations (CV = 59% and 26%, respectively) was approximately twice as high as in ergosterol concentrations (CV = 31% and 14%, respectively). The generally higher interspecific variability may be due to species-specific differences in biomarker concentrations. In addition, also the different incubation times (Table 3) and the consequent sampling of cultures during different growth phases, which can affect concentrations of both biomarkers (Gessner and Chauvet, 1993; Grimmett et al., 2013), might have contributed to the higher interspecific variability. The higher intraspecific and interspecific variability of DNA concentrations compared to ergosterol might be explained by a higher variation in extraction yields during sample preparation for qPCR analysis. A further explanation may be the substantial differences in the distribution of mycelia of varying ages among the cultures. As ergosterol can even persist in dead mycelia (Mille-Lindblom et al., 2004) it may be more homogeneously distributed in the entire mycelia of a culture. DNA quantity, in contrast, declines with mycelial age (Grimmett et al., 2013) and might accordingly be more indicative of biomass involved in leaf decomposition. Nevertheless, our results indicate that DNA is a promising proxy for the biomass of the investigated AH species (for other fungi also see Raidl et al., 2005; Lopez-Mondéjar et al., 2010; Tellenbach et al., 2010). This is also indicated by a positive relationship between biomass estimates derived from DNA and ergosterol concentrations. Although the correlation between

Table 3. Incubation time and mean ergosterol as well as DNA concentrations (with 95% confidence intervals and coefficients of variation (CV)) in freeze-dried liquid cultures of the target species.

| Species | Liquid culture incubation time (d) | Ergosterol | | DNA | |
|---|---|---|---|---|---|
| | | Concentration (µg/mg) | CV (%) | Concentration (ng/mg) | CV (%) |
| *Alatospora acuminata* | 18 | 5.75 (4.88 - 6.63) | 12 | 548.93 (475.62 - 622.25) | 11 |
| *Articulospora tetracladia* | 17 | 3.28 (3.05 - 3.52) | 5 | 112.70 (41.70 - 183.72) | 40 |
| *Clavariopsis aquatica* | 24 | 6.10 (5.48 - 6.71) | 8 | 227.20 (169.23 - 285.18) | 21 |
| *Clavatospora longibrachiata* | 17 | 5.69 (5.25 - 6.14) | 6 | 527.14 (418.78 - 635.50) | 17 |
| *Flagellospora curvula* | 17 | 5.86 (4.35 - 7.37) | 21 | 481.28 (239.20 - 723.37) | 41 |
| *Heliscella stellata* | 18 | 4.41 (3.60 - 5.22) | 15 | 164.71 (129.46 - 199.95) | 17 |
| *Lemonniera terrestris* | 20 | 3.18 (2.89 - 3.47) | 7 | 114.73 (61.56 - 167.90) | 37 |
| *Neonectria lugdunensis* | 7 | 2.95 (2.45 - 3.45) | 14 | 381.60 (232.04 - 531.16) | 32 |
| *Tetracladium marchalianum* | 14 | 2.67 (0.80 - 4.55) | 44 | 212.39 (133.95 - 290.84) | 23 |
| *Tricladium angulatum* | 17 | 3.83 (3.38 - 4.27) | 9 | 171.24 (128.95 - 213.53) | 20 |

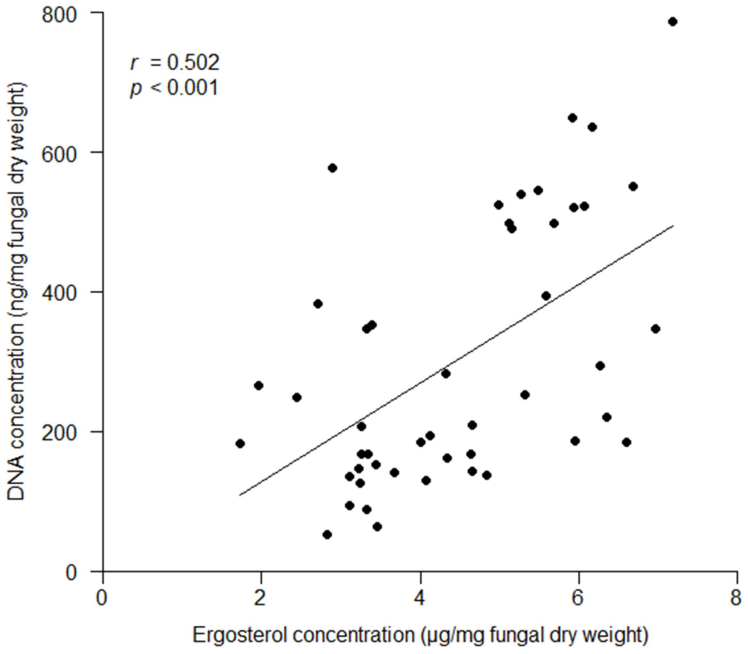



Fig. 2. Correlation between ergosterol (mg/mg of fungal dry weight) and DNA concentrations (ng/mg of fungal dry weight) in the tested strains with Spearman's correlation coefficient (*r*) and *p*-value. The regression line was added to visualize the positive relationship between the variables. Note that the initial number of replicates was reduced to *N* = 48 due to the loss of two replicates through contamination.

concentrations of these two biomarkers was not evident at the species-level (Table S5), which is likely related to the low number of replicates available per species (i.e., $n = 4/5$), it was significantly positive among all AH cultures (Fig. 2; Spearman's correlation; $r = 0.502$; $p < 0.001$; $N = 48$).

The qPCR assays designed during this study hence constitute novel tools to estimate the biomass of individual AH species in complex communities, which is a prerequisite to an understanding of their contribution to ecological functions (Bärlocher, 2016). Accordingly, individual AH species' traits in terms of roles in the enzymatic decomposition of leaf litter and in the nutrition of detritivorous stream invertebrates may be unraveled, which facilitates inter alia the assessment of biodiversity-ecosystem functioning relationships in AH communities. Moreover, it may be resolved how AH species' occurrence and functional performance are linked to the environmental context (e.g., physico-chemical water quality). Prospectively, these qPCR assays may thus provide new avenues for addressing open questions in stress and community ecology. Due to AHs' small size and short asexual (reproduction) cycle times, they constitute ideal model organisms to develop microcosm experiments to study selection and adaptation processes (cf. Zubrod et al., 2019). An application in more complex systems bearing unknown communities (e.g., in the field), however, may for some of the developed qPCR assays be subject to substantial uncertainties, due to non-species-specificity or non-coverage of target strains (Table 2). But these uncertainties may be reduced via the combined application of qPCR and HTS techniques (cf. Murray et al., 2011). Accordingly, HTS based on ITS allows for preliminary screening of microbial communities to identify organisms that may be included in subsequent qPCR analyses. In the future, this problem may be circumvented, by the design and evaluation of qPCR assays targeting alternative species-specific loci such as protein coding genes preferably in multiplex assays. Additionally this would overcome the quantification bias introduced by the genomic multi-copy occurrence of ribosomal genes.

Generally, extrapolations from single species' DNA amount to the total AH species' biomass should be interpreted carefully, since studies on the suitability of DNA as fungal biomass indicator in environmental samples are scarce and concluding recommendations for its use are more or less supportive (e.g., Marstorp et al., 2000; Baldrian et al., 2013). A major issue which needs to be considered is that the mycelium of AH species can cover several colonization stages, while DNA can be

gradually translocated from older to younger cells (Grimmett et al., 2013). Thus, DNA-based biomass estimates may be confounded by species' colonization stage. Moreover, it needs to be taken into account that comparing DNA-based biomass estimates among AH species may entail uncertainties, given that the number of qPCR assay-targeted ITS copies and the total amount of DNA per cell can substantially vary among fungal species (Rooney and Ward, 2005). Regardless of these aspects, the qPCR assays presented in this study constitute standardized and precise tools overcoming drawbacks of the most widely applied methods used to investigate AH community composition (e.g., ergosterol analysis or spore counts) and may open new research avenues in the fields of community and stress ecology.

## ACKNOWLEDGEMENTS

This study was funded by the German Research Foundation (DFG): Project AQUA-REG (DFG; SCHU 2271/14-1) and BIO2FUN (DFG; BU 2439/5e1). We would like to thank Andreas Bruder for constructive discussions and Flavius Popa for his valuable help in the sampling of aquatic hyphomycete conidia in the Schwarzwald national park. Furthermore, we thank Therese Bürgi, Dominic Englert, Marco Konschak, Sebastian Pietz, Harshada Sakpal, Britta Wahl-Ermel, and Sebastian Wendland for assistance in the laboratory.

## REFERENCES

Anderson, J. L., Shearer, C. A., 2011. Population genetics of the aquatic fungus *Tetracladium marchalianum* over space and time. PLoS One 6, e15908.

Baldrian, P., Vetrovský, T., Cajthaml, T., Dobiasova, P., Petrankova, M., Snajdr, J., Eichlerova, I., 2013. Estimation of fungal biomass in forest litter and soil. Fungal Ecology 6, 1-11.

Bärlocher, F., 2016. Aquatic hyphomycetes in a changing environment. Fungal Ecology 19, 14-27.

Baschien, C., Manz, W., Neu, T. R., Marvanova, L., Szewzyk, U., 2008. *In situ* detection of freshwater fungi in an alpine stream by new taxon-specific fluorescence *in situ* hybridization probes. Applied and Environmental Microbiology 74, 6427-6436.

Baschien, C., Marvanova, L., Szewzyk, U., 2006. Phylogeny of selected aquatic hyphomycetes based on morphological and molecular data. Nova Hedwigia 83, 311-352.

Baschien, C., Tsui, C. K.-M., Gulis, V., Szewzyk, U., Marvanova, L., 2013. The molecular phylogeny of aquatic hyphomycetes with affinity to the Leotiomycetes. Fungal Biology 117, 660-672.

Baudoin, J. M., Guerold, F., Felten, V., Chauvet, E., Wagner, P., Rousselle, P., 2008. Elevated aluminium concentration in acidified headwater streams lowers aquatic hyphomycete diversity and impairs leaf-litter breakdown. Microbial Ecology 56, 260-269.

Bell, T., Gessner, M. O., Griffiths, R. I., McLaren, J. R., Morin, P. J., van der Heijden, M., van der Putten, W. H., 2009. Microbial biodiversity and ecosystem functioning under controlled conditions and in the wild. In: Naeem, S., Bunker, D. E., Hector, A., Loreau, M., Perrings, C. (Eds.), Biodiversity, Ecosystem Functioning, and Human Wellbeing: an Ecological and Economic Perspective. Oxford University Press, Oxford, UK, pp. 121-133.

Berdal, K. G., Bøydler, C., Tengs, T., Holst-Jensen, A., 2008. A statistical approach for evaluation of PCR results to improve the practical limit of quantification (LOQ) of GMO analyses (SIMQUANT). European Food Research and Technology 227, 1149-1157.

Bermingham, S., Maltby, L., Cooke, R. C., 1996a. Effects of a coal mine effluent on aquatic hyphomycetes. II. Laboratory toxicity experiments. Journal of Applied Ecology 33, 1322-1328.

Bermingham, S., Maltby, L., Dewey, F. M., 1996b. Monoclonal antibodies as tools to quantify mycelium of aquatic hyphomycetes. New Phytologist 132, 593-601.

Bermingham, S., Maltby, L., Dewey, F. M., 1997. Use of immunoassays for the study of natural assemblages of aquatic hyphomycetes. Microbial Ecology 33, 223-229.

Bhuiyan, M. A. H. B., Groom, T., Nicolas, M. E., Taylor, P. W. J., 2018. TaqMan PCR assay for detection and quantification of *Stagonosporopsis tanaceti* in pyrethrum seed and seedlings. European Journal of Plant Pathology 150, 1041-1048.

Bridge, P. D., Roberts, P. J., Spooner, B. M., Panchal, G., 2003. On the unreliability of published DNA sequences. New Phytologist 160, 43-48.

Cummins, K. W., Klug, M. J., 1979. Feeding ecology of stream invertebrates. Annual Review of Ecology and Systematics 10, 147-172.

Danger, M., Gessner, M. O., Bärlocher, F., 2016. Ecological stoichiometry of aquatic fungi: current knowledge and perspectives. Fungal Ecology 19, 100-111.

Descals, E., 2005. Techniques for handling Ingoldian fungi. In: Graça, M. A. S., Bärlocher, F., Gessner, M. O. (Eds.), Methods to Study Litter Decomposition: a Practical Guide. Springer-Verlag, Dordrecht, Netherlands, pp. 129-142.

Duarte, S., Bärlocher, F., Cassio, F., Pascoal, C., 2014. Current status of DNA barcoding of aquatic hyphomycetes. Sydowia 66, 191-202.

Duarte, S., Bärlocher, F., Pascoal, C., Cassio, F., 2016. Biogeography of aquatic hyphomycetes: current knowledge and future perspectives. Fungal Ecology 19, 169-181.

Elbrecht, V., Leese, F., 2015. Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass-sequence relationships with an innovative metabarcoding protocol. PLoS One 10, e0130324.

Everitt, B., Skrondal, A., 2002. The Cambridge Dictionary of Statistics. Cambridge University Press, Cambridge, UK.

Feckler, A., Schrimpf, A., Bundschuh, M., Bärlocher, F., Baudy, P., Cornut, J., Schulz, R., 2017. Quantitative real-time PCR as a promising tool for the detection and quantification of leaf-associated fungal species – a proof-of-concept using *Alatospora pulchella*. PLoS One 12, e0174634.

Fernandes, I., Pascoal, C., Cassio, F., 2011. Intraspecific traits change biodiversity effects on ecosystem functioning under metal stress. Oecologia 166, 1019-1028.

Fernandes, I., Pereira, A., Trabulo, J., Pascoal, C., Cassio, F., Duarte, S., 2015. Microscopy- or DNA-based analyses: which methodology gives a truer picture of stream-dwelling decomposer fungal diversity? Fungal Ecology 18, 130-134.

Fernandez, D., Tummala, M., Schreiner, V. C., Duarte, S., Pascoal, C., Winkelmann, C., Mewes, D., Munoz, K., Schäfer, R. B., 2016. Does nutrient enrichment compensate fungicide effects on litter decomposition and decomposer communities in streams? Aquatic Toxicology 174, 169-178.

Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., Kubista, M., 2017. Methods to determine limit of detection and limit of quantification in

quantitative real-time PCR (qPCR). Biomolecular Detection and Quantification 12, 1-6.

Gessner, M. O., 2005. Ergosterol as a measure of fungal biomass. In: Graça, M. A. S., Bärlocher, F., Gessner, M. O. (Eds.), Methods to Study Litter Decomposition: a Practical Guide. Springer-Verlag, Dordrecht, Netherlands, pp. 189-195.

Gessner, M. O., Bärlocher, F., Chauvet, E., 2003. Qualitative and quantitative analyses of aquatic hyphomycetes in streams. Fungal Diversity Research Series 10, 127-157.

Gessner, M.O., Chauvet, E., 1993. Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. Applied and Environmental Microbiology 59, 502-507.

Gessner, M. O., Swan, C. M., Dang, C. K., McKie, B. G., Bardgett, R. D., Wall, D. H., Hättenschwiler, S., 2010. Diversity meets decomposition. Trends in Ecology and Evolution 25, 372-380.

Ginzinger, D. G., 2002. Gene quantification using real-time quantitative PCR. Experimental Hematology 30, 503-512.

Grimmett, I. J., Shipp, K. N., Macneil, A., Bärlocher, F., 2013. Does the growth rate hypothesis apply to aquatic hyphomycetes? Fungal Ecology 6, 493-500.

Gulis, V., 2001. Are there any substrate preferences in aquatic hyphomycetes? Mycological Research 105, 1088-1093.

Haffar, M., Gilbride, K. A., 2010. The utility and application of real-time PCR and FISH in the detection of single-copy gene targets in *Escherichia coli* O157:H7 and *Salmonella* Typhimurium. Canadian Journal of Microbiology 56, 254-262.

Heid, C. A., Stevens, J., Livak, K. J., Williams, P. M., 1996. Real time quantitative PCR. Genome Research 6, 986-994.

Hieber, M., Gessner, M. O., 2002. Contribution of stream detrivores, fungi, and bacteria to leaf breakdown based on biomass estimates. Ecology 83, 1026-1038.

Johnston, P. R., Quijada, L., Smith, C. A., Baral, H. O., Hosoya, T., Baschien, C., Pärtel, K., Zhuang, W. Y., Haelewaters, D., Park, D., Carl, S., Lopez-Giraldez, F., Wang, Z., Townsend, J. P., 2019. A multigene phylogeny toward a new phylogenetic classification of Leotiomycetes. IMA Fungus 10. https://doi.org/10.1186/s43008-019-0002-x.

Krauss, G.-J., Sole, M., Krauss, G., Schlosser, D., Wesenberg, D., Bärlocher, F., 2011. Fungi in freshwaters: ecology, physiology and biochemical potential. FEMS Microbiology Reviews 35, 620-651.

Lopez-Mondéjar, R., Anton, A., Raidl, S., Ros, M., Pascual, J. A., 2010. Quantification of the biocontrol agent *Trichoderma harzianum* with real-time TaqMan PCR and its potential extrapolation to the hyphal biomass. Bioresource Technology 101, 2888-2891.

Marstorp, H., Guan, X., Gong, P., 2000. Relationship between dsDNA, chloroform labile C and ergosterol in soils of different organic matter contents and pH. Soil Biology and Biochemistry 32, 879-882.

Mille-Lindblom, C., von Wachenfeldt, E., Tranvik, L. J., 2004. Ergosterol as a measure of living fungal biomass: persistence in environmental samples after fungal death. Journal of Microbiological Methods 59, 253-262.

Murray, D. C., Bunce, M., Cannell, B. L., Oliver, R., Houston, J., White, N. E., Barrero, R. A., Bellgard, M. I., Haile, J., 2011. DNA-based faecal dietary analysis: a comparison of qPCR and high throughput sequencing Approaches. PLoS One 6, e25776.

Newell, S. Y., 1992. Estimating fungal biomass and productivity in decomposing litter. In: Carroll, G. C., Wicklow, D. T. (Eds.), The Fungal Community, second ed. Marcel-Dekker, New York, USA, pp. 101-107.

Nilsson, R. H., Anslan, S., Bahram, M., Wurzbacher, C., Baldrian, P., Tedersoo, L., 2019. Mycobiome diversity: high-throughput sequencing and identification of fungi. Nature Reviews Microbiology 17, 95-109.

Nilsson, R. H., Ryberg, M., Kristiansson, E., Abarenkov, K., Larsson, K.-H., Köljalg, U., 2006. Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. PLoS One 1, e59.

Pascoal, C., Cassio, F., Marvanova, L., 2005. Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. Archiv für Hydrobiologie 162, 481-496.

Petersen, R. C., Cummins, K. W., 1974. Leaf processing in a woodland stream. Freshwater Biology 4, 343-368.

Pfaffl, M. W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29, 2002-2007.

R Core Team, 2018. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Raidl, S., Bonfigli, R., Agerer, R., 2005. Calibration of quantitative real-time TaqMan PCR by correlation with hyphal biomass and ITS copies in mycelia of *Piloderma croceum*. Plant Biology 7, 713-717.

Rodríguez, A., Rodríguez, M., Córdoba, J. J., Andrade, M. J., 2015. Design of primers and probes for quantitative real-time PCR methods. In: Basu, C. (Ed.), PCR Primer Design. Springer, New York, NY, pp. 31-56.

Rooney, A. P., Ward, T. J., 2005. Evolution of a large ribosomal RNA multigene family in filamentous fungi: birth and death of a concerted evolution paradigm. Proceedings of the National Academy of Sciences of the United States of America 102, 5084-5089.

Schlief, J., Mutz, M., 2011. Leaf decay processes during and after a supra-seasonal hydrological drought in a temperate lowland stream. International Review of Hydrobiology 96, 633-655.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*. Proceedings of the National Academy of Sciences of the United States of America 109, 6241-6246.

Seena, S., Duarte, S., Pascoal, C., Cassio, F., 2012. Intraspecific variation of the aquatic fungus *Articulospora tetracladia*: an ubiquitous perspective. PLoS One 7, e35884.

Tellenbach, C., Grünig, C. R., Sieber, T. N., 2010. Suitability of quantitative real-time PCR to estimate the biomass of fungal root endophytes. Applied and Environmental Microbiology 76, 5764-5772.

Tomlinson, J. A., Boonham, N., Hughes, K. J. D., Griffin, R. L., Barker, I., 2005. On-site DNA extraction and real-time PCR for detection of *Phytophthora ramorum* in the field. Applied and Environmental Microbiology 71, 6702-6710.

Wallace, J. B., 1997. Multiple trophic levels of a forest stream linked to terrestrial litter inputs. Science 277, 102-104.

Webster, J., Descals, E., 1981. Morphology, distribution, and ecology of conidial fungi in freshwater habitats. In: Cole, G. T., Kendrick, B. (Eds.), Biology of Conidial Fungi, vol. 1. Academic Press, New York, USA, pp. 295-355.

Zubrod, J. P., Bundschuh, M., Arts, G., Brühl, C. A., Imfeld, G., Knäbel, A., Payraudeau, S., Rasmussen, J. J., Rohr, J., Scharmüller, A., Smalling, K., Stehle, S., Schulz, R., Schäfer, R. B., 2019. Fungicides: an overlooked pesticide class? Environmental Science & Technology 53, 3347-3365.

Zubrod, J. P., Bundschuh, M., Feckler, A., Englert, D., Schulz, R., 2011. Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. Environmental Toxicology and Chemistry 30, 2718-2724.

Zubrod, J. P., Feckler, A., Englert, D., Koksharova, N., Rosenfeldt, R. R., Seitz, F., Schulz, R., Bundschuh, M., 2015. Inorganic fungicides as routinely applied in organic and conventional agriculture can increase palatability but reduce microbial decomposition of leaf litter. Journal of Applied Ecology 52, 310-322.

**SUPPLEMANTARY DATA**

DOCUMENT S1: EXTRACTION, PCR, AND SEQUENCING

All procedures described below were performed at the Leibniz Institute DSMZ (German Collection for Microorganisms and Cell Cultures, Germany). Fungal DNA extracts for the purpose of PCR product sequencing were prepared using mycelia directly harvested from cultures grown on 2% malt extract agar plates. DNA was extracted using the MasterPure™ Yeast DNA Purification Kit (Epicentre, USA) in conjunction with the FastPrep™-24 Instrument (MP Biomedicals, Germany) according to the manufacturer's instructions.

Fungal isolates were PCR amplified and sequenced targeting the internal transcribed spacers and *5.8S* gene and the D1/D2 domain of the *28S* large subunit of ribosomal DNA. PCR mixtures contained *Taq* polymerase and PCR buffer (Takara Bio Inc., Japan), 5 pM of each primer (Eurofins, Germany), 200 µM dNTPs (Roche, Germany), 40-200 ng of extracted genomic DNA, and nuclease-free water. The PCR program included an initial denaturation step for 2 min at 94°C, followed by 25-35 cycles of denaturation for 1 min at 94°C, primer annealing for 45 s at 50°C, elongation for 1 min at 72°C, and a final elongation step for 5 min at 72°C. Amplicon quality was checked on 1.2% agarose gels stained with GelStar™ (Lonza, Switzerland) under UV light using a 1 kb DNA ladder (GeneRuler, Thermo Scientific, Germany). The amplicons were purified with the QIAquick® PCR Purification Kit (Quiagen, Germany). Sequences were generated with an ABI 3500xL Analyzer using

BigDye chemistry and analyzed with the sequence analysis software version 3.3 (Applied Biosystems, USA). All sequences generated were used as queries in DSMZ databases, Mycobank, and GenBank's "basic local alignment search tool" algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with default stringency and restriction to type strain references when appropriate. All sequences belonging to AH strains used for assay design as well as validation procedures were deposited in the GenBank nucleotide database (https://www.ncbi.nlm.nih.gov/genbank/; see also Table S1).

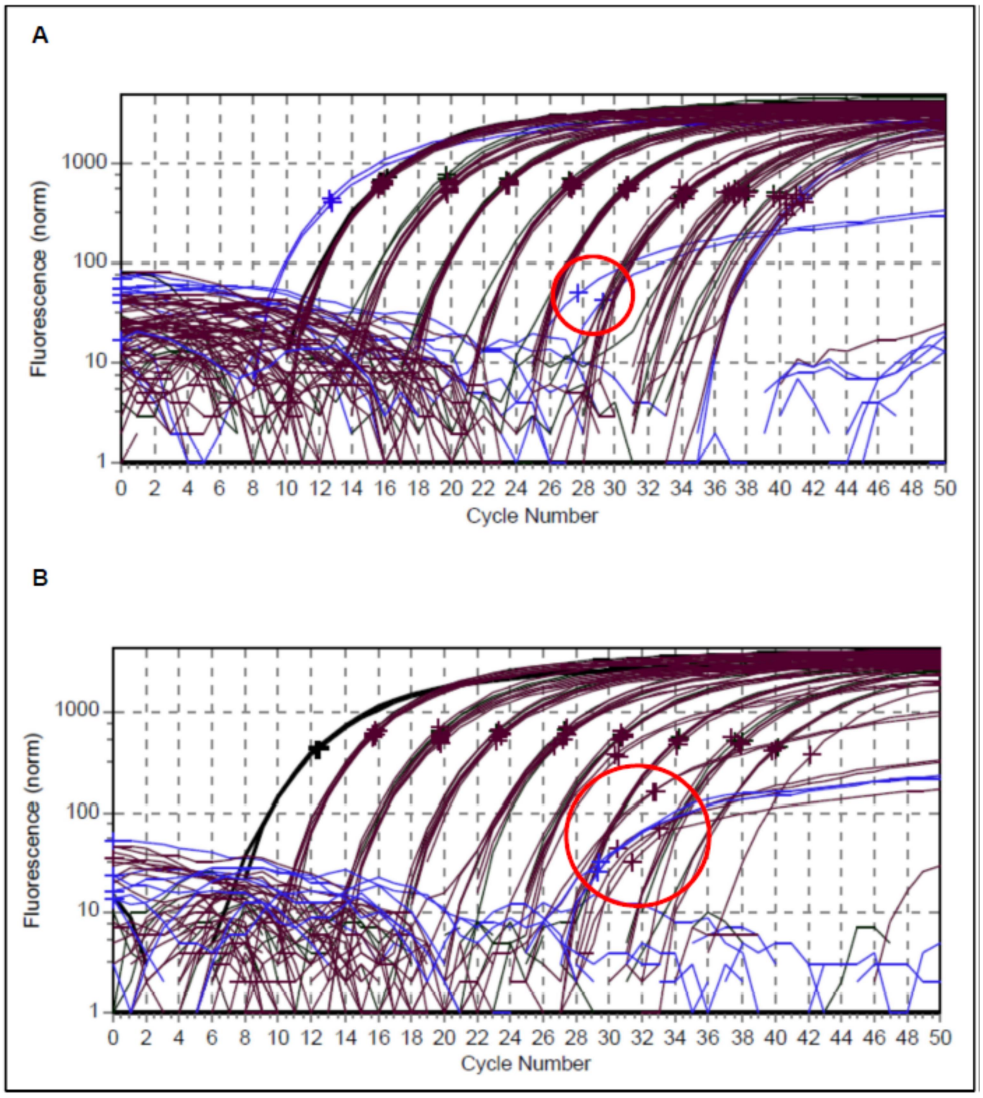



Fig. S1. Amplification plots (log10-transformed fluorescence intensity as a function of PCR cycle number) of two LETE-tqmn assay runs showing deviating amplification curves (crosses within red circles) in reactions containing (A) only 20 ng of *Articulospora tetracladia* DNA (blue curves) and (B) 20 ng of DNA of the non-target mixture (blue curves) as well as additionally 2-200 fg of target DNA (i.e., *Lemonniera terrestris*; black curves). Crosses indicate the calculated $C_q$ values.

Table S1. List of isolated species with information on sampling location and date as well as strain designation and associated GenBank accession numbers. Model strains and related sequences used for experimental validation and as template for qPCR assay design are printed in bold. The asterisk indicates a potentially new species.

| | Sampling data | | | | | |
|---|---|---|---|---|---|---|
| Species | Stream | Coordinates (decimal degrees; latitude, longitude) | Near the city of | Date of isolation (yyyy/mm/dd) | Strain (DSM number) | *ITS* sequence (GenBank accession number) |
| *Alatospora acuminata* | Hainbach | 49.24202, 8.04586 | Landau | 2015/10/01 | **DSM 104360** | **MH930815** |
| | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/09/01 | DSM 104377 | MK353091 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 104353 | MK353090 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2017/05/28 | DSM 105546 | MK353088 |
| | Schönmünz | 48.56943, 8.26608 | Offenburg | 2017/06/11 | DSM 105578 | MK353089 |
| | Schönmünz | 48.56943, 8.26608 | Offenburg | 2017/06/11 | DSM 105582 | MK371720 |
| | Schönmünz | 48.57950, 8.31872 | Offenburg | 2017/06/11 | DSM 105590 | MK353092 |
| | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/09/01 | DSM 105539 | MK353087 |
| *Amniculicola longissima* | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/09/01 | DSM 104380 | MK371721 |
| | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/09/01 | DSM 104384 | MK353142 |
| | Hockgraben | 47.67227, 9.19998 | Konstanz | 2017/06/11 | DSM 105553 | MK353143 |
| *Anguillospora crassa* | Hainbach | 49.24202, 8.04586 | Landau | 2016/07/22 | DSM 104363 | MK353094 |
| | Hainbach | 49.24202, 8.04586 | Landau | 2016/08/04 | DSM 104359 | MK353093 |
| | Hainbach | 49.24202, 8.04586 | Landau | 2016/08/04 | DSM 104370 | MK371722 |
| *Articulospora proliferata* | Hockgraben | 47.67227, 9.19998 | Konstanz | 2017/06/11 | DSM 105551 | MK353095 |
| *A. tetracladia* | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 105543 | MK353100 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | **DSM 104345** | **MH930816** |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 104352 | MK353096 |
| | Schönmünz | 48.56943, 8.26608 | Offenburg | 2017/06/11 | DSM 105580 | MK353097 |
| | Schönmünz | 48.57950, 8.31872 | Offenburg | 2017/06/11 | DSM 105596 | MK353098 |
| | Schönmünz | 48.57950, 8.31872 | Offenburg | 2017/06/11 | DSM 105598 | MK353099 |
| *Clavariopsis aquatica* | Hainbach | 49.24202, 8.04586 | Landau | 2016/07/22 | **DSM 104362** | **MH930817** |
| | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/09/01 | DSM 104382 | MK353101 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2017/05/28 | DSM 105547 | MK353102 |
| *Clavatospora longibrachiata* | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/07/18 | DSM 104364 | MK353103 |
| | Hainbach | 49.24202, 8.04586 | Landau | 2016/08/04 | **DSM 104365** | **MH930818** |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 105078 | MK353105 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 105079 | MK353104 |
| *Filosporella annelidica* | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/10/11 | DSM 104337 | MK353106 |
| | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/10/11 | DSM 104341 | MK353107 |
| | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/09/01 | DSM 104383 | MK353109 |
| | Dobrabach | 51.28908, 13.61765 | Dresden | 2017/01/01 | DSM 105084 | MK353110 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 104358 | MK353108 |
| *Flagellospora curvula* | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/10/11 | **DSM 104334** | **MH930819** |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 104349 | MK353111 |
| | Dobrabach | 51.28908, 13.61765 | Dresden | 2017/01/01 | DSM 105082 | MK353112 |
| *Heliscella stellata* | Hainbach | 49.24202, 8.04586 | Landau | 2016/07/22 | **DSM 104386** | **MH930820** |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 104357 | MK353113 |

Table S1 continued.

| Species | Sampling data | | | | Strain (DSM number) | *ITS* sequence (GenBank accession number) |
|---|---|---|---|---|---|---|
| | Stream | Coordinates (decimal degrees; latitude, longitude) | Near the city of | Date of isolation (yyyy/mm/dd) | | |
| *Lemonniera aquatica* | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/10/11 | DSM 104336 | MK353144 |
| | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/10/11 | DSM 105542 | MK353145; MK371724 |
| *L. centrosphaera* | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/10/11 | DSM 104342 | MK353146 |
| *L. terrestris* | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 104343 | MK353147 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | **DSM 104344** | **MH930821** |
| | Schönmünz | 48.57950, 8.31872 | Offenburg | 2017/06/11 | DSM 105593 | MK353114 |
| *Margaritispora aquatica* | Dobrabach | 51.28908, 13.61765 | Dresden | 2017/01/01 | DSM 105080 | MK353138 |
| | Dobrabach | 51.28908, 13.61765 | Dresden | 2017/01/01 | DSM 105081 | MK353139 |
| *M. monticola* | Hainbach | 49.24202, 8.04586 | Landau | 2016/08/04 | DSM 104367 | MK353141 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 104356 | MK353140 |
| *Neonectria lugdunensis* | Hainbach | 49.24202, 8.04586 | Landau | 2015/10/01 | **DSM 104361** | **MH930822**; MK371726 |
| | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/09/01 | DSM 104372 | MK371727 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 104354 | MK371725 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2017/05/28 | DSM 105549 | MK371729 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2017/05/28 | DSM 105550 | MK371728 |
| | Hockgraben | 47.67227, 9.19998 | Konstanz | 2017/06/11 | DSM 105558 | MK353115 |
| | Gänsbach | 47.84683, 8.44736 | Titisee-Neustadt | 2017/06/11 | DSM 105566 | MK371723 |
| | Legerbächle | 48.57286, 8.24130 | Offenburg | 2017/06/11 | DSM 105573 | MK353117 |
| | Schönmünz | 48.57950, 8.31872 | Offenburg | 2017/06/11 | DSM 105592 | MK353116 |
| *Pseudoanguillospora stricta* | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/10/11 | DSM 104335 | MK353118 |
| *Tetracladium breve* | Hockgraben | 47.67227, 9.19998 | Konstanz | 2017/06/11 | DSM 105555 | MK371730 |
| *T. furcatum* | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/10/11 | DSM 104333 | MK353119 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 104355 | MK353120 |
| | Gänsbach | 47.84683, 8.44736 | Titisee-Neustadt | 2017/06/11 | DSM 105565 | MK353121 |
| | Schönmünz | 48.57950, 8.31872 | Offenburg | 2017/06/11 | DSM 105594 | MK353122 |
| *T. marchalianum* | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/09/01 | **DSM 104373** | **MH930823** |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 104346 | MK353123 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 104347 | MK353124 |
| | Gänsbach | 47.84683, 8.44736 | Titisee-Neustadt | 2017/06/11 | DSM 105561 | MK353125 |
| | Gänsbach | 47.84683, 8.44736 | Titisee-Neustadt | 2017/06/11 | DSM 105562 | MK353126 |
| | Gänsbach | 47.84683, 8.44736 | Titisee-Neustadt | 2017/06/11 | DSM 105563 | MK353127 |
| *T. maxilliforme* | Schönmünz | 48.56943, 8.26608 | Offenburg | 2017/06/11 | DSM 105583 | MK353128 |
| *Tricladium angulatum* | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/09/01 | **DSM 104374** | **MH930824** |
| | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/09/01 | DSM 104375 | MK353129 |
| | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/09/01 | DSM 104376 | MK371731 |

Table S1 continued.

| Species | Sampling data: Stream | Coordinates (decimal degrees; latitude, longitude) | Near the city of | Date of isolation (yyyy/mm/dd) | Strain (DSM number) | *ITS* sequence (GenBank accession number) |
|---|---|---|---|---|---|---|
| *T. biappendiculatum* | Legerbächle | 48.57286, 8.24130 | Offenburg | 2017/06/11 | DSM 105574 | MK353130 |
| | Legerbächle | 48.57286, 8.24130 | Offenburg | 2017/06/11 | DSM 105575 | MK353131 |
| *Tricladium sp.** | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/10/11 | DSM 104338 | MK353132 |
| *T. splendens* | Hainbach | 49.24202, 8.04586 | Landau | 2016/08/04 | DSM 104369 | MK353134 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 104348 | MK353133 |
| | Lützelnbach | 50.72498, 8.12911 | Siegen | 2016/10/23 | DSM 105544 | MK353135 |
| | Schönmünz | 48.57950, 8.31872 | Offenburg | 2017/06/11 | DSM 105595 | MK353136 |
| *Tumularia aquatica* | Hainbach | 49.24202, 8.04586 | Landau | 2016/08/04 | DSM 104371 | MK371732 |
| | Rodenbach | 49.56932, 8.03489 | Grünstadt | 2016/09/01 | DSM 104379 | MK371733 |
| | Gänsbach | 47.84683, 8.44736 | Titisee-Neustadt | 2017/06/11 | DSM 105568 | MK353137 |
| *T. tuberculata* | Hainbach | 49.24202, 8.04586 | Landau | 2016/08/04 | DSM 104368 | MK371734 |

Table S2. INSD sequence accession numbers associated to species, which were identified as compatible with a qPCR assay designed during this study. Sequences being 100% compatible with the respective assay are underlined. Sequences generated in this study are printed in bold.

| qPCR assay designation | Species identified as assay compatible | GenBank sequence accession numbers covering the complete assay binding region |
|---|---|---|
| ALAC-tqmn | *Alatospora acuminata* | **MH930815; MK353089;** AY204587; AY204589; KF730798; **MK353087; MK353088; MK353090; MK353091; MK353092; MK371720;** AY204588; AY204590; AY204591; KF730795; KF730796; KF730797; KU519109 |
| ARTE-tqmn | *Articulospora tetracladia* | **MH930816; MK353096; MK353098; MK353099; MK353100;** EU998914; EU998915; EU998916; EU998917; EU998919; EU998920; EU998921; EU998922; EU998923; EU998924; EU998925; EU998926; EU998927; EU998928; EU998929; FJ000377; FJ000378; FJ000379; FJ000380; FJ000381; FJ000382; FJ000384; FJ000385; FJ000386; FJ000387; FJ000388; FJ000389; FJ000390; FJ000391; FJ000392; FJ000393; GQ152144; GQ152145; GQ411284; GQ411285; GQ411286; GQ411287; GQ411288; GQ411289; GQ411290; GQ411291; GQ411292; GQ411293; GU938614; GU938615; JF895397; JF895398; JF895399; JF895400; JF895401; JF895402; JF895403; JF895404; JF895405; JF895406; JF895407; JF895408; JF895409; JF895410; JF895411; JF895412; JF895413; JF895414; JF895415; JF895416; JF895417; JF895418; JF895419; JF895420; JF895421; JF895422; JF895423; JF895424; JF895425; JF895426; JF895427; JF895428; JF895429; JF895430; JF895431; JF895432; JF895433; JF895434; JF895435; JF895436; JF895437; KF952606; KF952607; KF952608; KF952609; KF952610; KF952611; KF952612; KF952613; KF952614; KF952615; KF952616; KF952617; KF952618; KF952619; KF952620; KF952621; KF952622; KF952623; KF952624; KF952625; KF952626; KF952627; KF952628; KF952629; KF952630; KF952631; KF952632; KF952633; KF952634; KF952635; KF952636; KF952637; KF952638; KF952639; KF952640; KF952641; KF952642; KF952643; KF952644; KF952645; KF952646; KF952647; KF952648; KF952649; KF952650; KF952651; KF952652; KF952653; KF952654; KF952655; KF952656; KF952657; KF952658; KF952659; KF952660; KF952661; KF952662; KF952663; KF952664; KP234358; KP234359; KP234360; KP234368; KP234369; KP234370; KP234371; KU892281; KX858606; KX858607; MH078059; **MK353097;** JN569103; KP234356; KP234366; KP234384; LC130987; LC130997; LC131000; LC131004; LC131005; LC131023 |
| CLAQ-tqmn | *Clavariopsis aquatica* | ***MH930817; MK353101; MK353102;*** *GQ152143; GQ411316; GQ411317; GQ411318; GQ411319; KX858608; KX858609; KX858610; KX858611; MH047194* |
| CLLO-tqmn | *Clavatosopora longibrachiata* | **MH930818; MK353103; MK353104; MK353105;** KF730808; KF730809 |
| FLCU-tqmn | *Flagellospora curvula* | **MH930819; MK353111; MK353112;** KC834045; KC834050; KX858613; KX858614; AY729939 |
| | *F. fusarioides* | KC834048 |

Table S2 continued.

| qPCR assay designation | Species identified as assay compatible | GenBank sequence accession numbers covering the complete assay binding region |
|---|---|---|
| HEST-tqmn | *Heliscella stellata* | **MH930820; MK353113** |
| LETE-tqmn | *Lemonniera aquatica* | **MK353144; MK353145; MK371724;** KF730819; KF730820; KF730821; KF730822; KF730823; KX858621 |
| | *L. centrosphaera* | **MK353146; KC834063;** NR155313 |
| | *L. cornuta* | KU519115; KX858620 |
| | *L. terrestris* | **MH930821; MK353114; MK353147;** KU519116; KX858615; KX858622; KX858623 |
| | *Margaritispora aquatica* | **MK353138; MK353139** |
| NELU-tqmn | *Cylindrocarpon obtusisporum* | AM419061; AM419064; GU726752; HQ713775; JX077072; KM248556; AM419060; DQ888723; KP411555; KP411558; KU214526; LC206668 |
| | *Neonectria lugdunensis* | **MH930822; MK353115; MK353117; MK371723; MK371725; MK371726;** DQ247775; DQ247777; DQ247778; DQ247779; DQ247780; DQ247781; FJ000394; GQ411329; GQ411330; GQ411331; GQ411332; GQ411333; GQ411334; GQ411335; GQ411336; GQ411337; GQ411338; GQ411339; GU726754; KU214521; MF110613; **MF110615; MF110617; MF110618; MF110619; MK353116; MK371727; MK371728; MK371729;** AY148103 |
| | *Phaeoacremonium vibratile* | KF764573 |
| TEMA-tqmn | *Tetracladium marchalianum* | **MH930823; MK353123; MK353125; MK353126; MK353127;** AF411023; AF411024; AY204620; AY204621; AY204622; AY204623; AY204624; AY204625; EU883413; EU883414; EU883415; EU883416; EU883417; EU883423; FJ000357; FJ000358; FJ000359; FJ000360; FJ000361; FJ000370; FJ205456; FJ205463; GQ411294; GQ411295; GQ411296; GQ411298; GQ411299; KF952702; KF952705; KF952707; KF952708; KF952710; KF952712; KF952713; KF952714; KF952717; KF952718; KF952719; KF952720; KF952722; KF952723; KF952724; KF952725; KF952727; KX858635; KX858638; KX858639; KX858640; KX858641; KX858642; KX858643; KX858644; **MK353124;** AF411022; KF952703; KF952704; KF952706; KF952709; KF952711; KF952715; KF952716; KF952721; KF952726; KX858626; KX858627; KX858628; KX858636; KX858637; KX858645; KX858646; KX858647; KX858648 |
| | *T. breve* | EU883418; **MK371730**; EU883431; FJ000364; FJ000405; GQ411301; KU519117 |
| TRAN-tqmn | *Tricladium angulatum* | **MH930824; MK353129; MK371731;** AY204609; AY204610; KF730830; KF730831; KF730832; KF730833; KU519121; KX858631 |

Table S3. Experimental species-specificity cross-validation showing positive (+) and negative (-) qPCR reactions. Each assay was tested for DNA of all target strains and black alder leaves as well as the respective non-target mixture. Each reaction was replicated twice.

| | 20 ng of DNA | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| qPCR assay designation | *Alatospora acuminata* | *Articulospora tetracladia* | *Clavariopsis aquatica* | *Clavatospora longibrachiata* | *Flagellospora curvula* | *Heliscella stellata* | *Lemonniera terrestris* | *Neonectria lugdunensis* | *Tetracladium marchalianum* | *Tricladium angulatum* | Black alder leaves | Non-target mixture | No-template control |
| ALAC-tqmn | + | - | - | - | - | - | - | - | - | - | - | - | - |
| ARTE-tqmn | - | + | - | - | - | - | - | - | - | - | - | - | - |
| CLAQ-tqmn | - | - | + | - | - | - | - | - | - | - | - | - | - |
| CLLO-tqmn | - | - | - | + | - | - | - | - | - | - | - | - | - |
| FLCU-tqmn | - | - | - | - | + | - | - | - | - | - | - | - | - |
| HEST-tqmn | - | - | - | - | - | + | - | - | - | - | - | - | - |
| LETE-tqmn | - | + | - | - | - | - | + | - | - | - | - | + | - |
| NELU-tqmn | - | - | - | - | - | - | - | + | - | - | - | - | - |
| TEMA-tqmn | - | - | - | - | - | - | - | - | + | - | - | - | - |
| TRAN-tqmn | - | - | - | - | - | - | - | - | - | + | - | - | - |

Table S4. *p*-values of ANCOVAs assessing the effects of target DNA concentration (A), presence of non-target DNA (B), as well as their interaction (A × B) on $C_q$ values. All *p*-values < 0.05 are printed in bold.

| | | Presence of non-target DNA | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| qPCR assay designation | Source of variation | *Alatospora acuminata* | *Articulospora tetracladia* | *Clavariopsis aquatica* | *Clavatospora longibrachiata* | *Flagellospora curvula* | *Heliscella stellata* | *Lemonniera terrestris* | *Neonectria lugdunensis* | *Tetracladium marchalianum* | *Tricladium angulatum* | Black alder leaves | Non-target mixture |
| ALAC-tqmn | A | - | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| | B | - | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 0.990 | 1.000 | 1.000 |
| | A × B | - | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 0.998 | 1.000 | 1.000 |
| ARTE-tqmn | A | **<0.001** | - | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| | B | 0.999 | - | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 | 0.994 | 0.999 | 0.999 |
| | A × B | 1.000 | - | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| CLAQ-tqmn | A | **<0.001** | **<0.001** | - | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| | B | 1.000 | 1.000 | - | 1.000 | 1.000 | 0.717 | 0.717 | 0.717 | 0.998 | 0.998 | 0.868 | 0.868 |
| | A × B | 1.000 | 1.000 | - | 1.000 | 1.000 | 0.978 | 0.978 | 0.978 | 1.000 | 1.000 | 0.995 | 0.995 |
| CLLO-tqmn | A | **<0.001** | **<0.001** | **<0.001** | - | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| | B | 0.998 | 0.998 | 0.998 | - | 0.998 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| | A × B | 1.000 | 1.000 | 1.000 | - | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| FLCU-tqmn | A | **<0.001** | **<0.001** | **<0.001** | **<0.001** | - | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| | B | 1.000 | 1.000 | 1.000 | 1.000 | - | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| | A × B | 1.000 | 1.000 | 1.000 | 1.000 | - | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| HEST-tqmn | A | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | - | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| | B | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| | A × B | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| LETE-tqmn | A | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | - | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| | B | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - | 1.000 | 1.000 | 0.894 | 1.000 | 1.000 |
| | A × B | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - | 1.000 | 1.000 | 0.986 | 1.000 | 1.000 |
| NELU-tqmn | A | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | - | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| | B | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - | 1.000 | 1.000 | 1.000 | 1.000 |
| | A × B | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - | 1.000 | 1.000 | 1.000 | 1.000 |
| TEMA-tqmn | A | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | - | **<0.001** | **<0.001** | **<0.001** |
| | B | 1.000 | 1.000 | 1.000 | 1.000 | 0.997 | 0.997 | 0.997 | 0.997 | - | 1.000 | 0.997 | 0.997 |
| | A × B | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - | 1.000 | 1.000 | 1.000 |
| TRAN-tqmn | A | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | - | **<0.001** | **<0.001** |
| | B | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 0.999 | - | 1.000 | 1.000 |
| | A × B | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - | 1.000 | 1.000 |

Table S5. Correlation between ergosterol and DNA concentrations in the tested aquatic hyphomycete species with Pearson's correlation coefficient (*r*) and *p*-value.

| Species | *r* | *p*-value |
|---|---|---|
| *Alatospora acuminata* | 0.373 | 0.537 |
| *Articulospora tetracladia* | -0.180 | 0.820 |
| *Clavariopsis aquatica* | -0.282 | 0.646 |
| *Clavatospora longibrachiata* | 0.438 | 0.461 |
| *Flagellospora curvula* | 0.588 | 0.297 |
| *Heliscella stellata* | -0.133 | 0.831 |
| *Lemonniera terrestris* | 0.686 | 0.201 |
| *Neonectria lugdunensis* | 0.842 | 0.801 |
| *Tetracladium marchalianum* | -0.549 | 0.451 |
| *Tricladium angulatum* | -0.357 | 0.556 |

**APPENDIX A.2**

## The fungicide tebuconazole confounds concentrations of molecular biomarkers estimating fungal biomass

Patrick Baudy, Marco Konschak, Harshada Sakpal, Christiane Baschien, Ralf Schulz, Mirco Bundschuh, Jochen P. Zubrod

Bulletin of Environmental Contamination and Toxicology 105, 620-625
Impact Factor (2020): 2.151

## ABSTRACT

Due to their ecological importance, fungi are suitable indicator organisms for anthropogenic stress. To estimate fungal biomass, the fungal membrane molecule ergosterol is often quantified as a proxy. Estimates based on ergosterol may, however, be distorted by exposure to demethylase inhibiting (DMI) fungicides, interfering with sterol synthesis. To test this hypothesis, we exposed ten fungal species to the DMI fungicide tebuconazole and measured concentrations of ergosterol and DNA per unit dry mass of the fungal hyphae. The latter served as alternative biomass proxy that is not specifically targeted by tebuconazole. Effects of tebuconazole on ergosterol concentrations were species-specific, while concentrations were on average reduced by 13%. In contrast, DNA concentrations were on average increased by 13%. We demonstrate that DMI fungicides – at close to field relevant levels – can distort fungal biomass estimation, complicating the use of this endpoint for environmental management.

## KEYWORDS

Ergosterol – Azole fungicides – qPCR – Freshwater fungi – Ascomycetes – Community composition

## INTRODUCTION

Fungi are, due to their ecological importance (Dighton and White 2017), frequently utilized as indicator organisms in laboratory or field studies assessing impacts of anthropogenic stress on the integrity of aquatic and terrestrial systems (e.g. Rajapaksha et al. 2004; Solé et al. 2008; Murugan et al. 2014; Pesce et al. 2016). Along with their functional performance, fungal biomass is widely quantified by molecular biomarkers. These include, inter alia, the fungal membrane molecule ergosterol and taxon-specific nucleic acid sequence motifs (Gessner 2005; Manerkar et al. 2008; Baudy et al. 2019). Particularly fungicides harbour substances designed to interfere with the metabolism and synthesis of the above mentioned molecular biomarkers in fungi (Fungicide Resistance Action Committee 2018). Consequently, the presence of certain fungicides may confound biomass estimates, meaning that differences in biomarker concentrations may not reflect true differences in biomass.

To test this hypothesis, we targeted the fungal biomass proxy ergosterol as molecular biomarker using the demethylase inhibitor (DMI) tebuconazole. This

fungicide affects cytochrome P450 lanosterol 14α-demethylase (ERG11), an enzyme involved in the sterol biosynthesis pathway (Fungicide Resistance Action Committee 2018). Additionally, we analyzed fungal desoxyribonucleic acid (DNA) as an alternative biomass proxy via quantitative real-time PCR (qPCR; Baudy et al. 2019). DNA is not specifically targeted by DMI fungicides. To assess fungicide responses, biomarker quantities were normalized to actual fungal biomass (i.e., mycelial dry weight). We used ten fungal species, belonging to a polyphyletic group of true fungi (i.e., aquatic hyphomycetes; Baschien et al. 2013), which were incubated in monoxenic liquid cultures. Incubation was performed in absence and presence of tebuconazole at a concentration of 35 µg/L, being one order of magnitude above mean fungicide concentrations detected in surface waters (Zubrod et al. 2019). Irrespective of potential growth inhibiting effects, that were not the subject of this investigation, we expected tebuconazole exposure to reduce ergosterol concentrations in the model fungi (cf. Joseph-Horne et al. 1995a, b), while DNA concentrations were assumed to remain unaffected.

## MATERIALS AND METHODS

Monoxenic liquid cultures of ten model fungi (for acronyms see Table 1) covering three ascomycete classes (Table 1) were exposed to tebuconazole, which was applied as the commercially available formulation Folicur® (Bayer Crop-Science, Germany). After a preliminary experiment, a tebuconazole concentration of 35 µg/L was chosen as concentration potentially triggering effects on fungal ergosterol levels and at the same time not completely inhibiting growth of the two species considered most sensitive (i.e. ALAC and HEST; Bundschuh et al. 2011; Fig. S1). Accordingly, all species were exposed to 35 µg/L tebuconazole and a fungicide-free control. Each treatment comprised five replicates (n = 5) consisting of a 50-mL Erlenmeyer flask filled with 30 mL of 2% (w/v) malt extract solution (MES; 20 g malt extract/L of deionized water) containing the respective tebuconazole concentration and stoppered with cellulose plugs. The preparation was performed under sterile conditions (for details see Baudy et al. 2019).

Before test initiation, eight agar plugs with 5 mm in diameter were punched from the growing edge of a single species colony cultured on malt extract agar (20 g malt extract and 20 g agar/L of deionized water). These agar plugs were homogenized in MES at 6500 rpm for 10 s using an Ultra-turrax blender (IKA®-Werke

GmbH & Co. KG, Germany). From this homogenate 0.5 mL served as inoculum for each replicate of both treatments. The replicates were incubated on a rotary shaker (model KS 15, Edmund Bühler GmbH, Germany) at 120 rpm at 16°C in complete darkness. The cultures were visually checked daily and were sampled as soon as the amount of biomass was considered sufficient for further analyses (i.e. > 5 mg dry weight per culture for each biomarker). As growth rates vary strongly among aquatic hyphomycete species (cf. Gessner and Chauvet 1993), the incubation times ranged from 7 to 24 days (Table 1). Therefore, cultures may have been sampled during different growth phases. After incubation, mycelium in liquid cultures was transferred to 50-mL falcon tubes and rinsed three times with 45 mL of sterile deionized water to remove any MES residues. Decanted falcon tubes containing the mycelium were stored frozen at − 20°C until further processing.

Table 1 List of species, associated classes, acronyms used in text, used strains with DSM accession numbers (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures), and incubation times.

| Species | Class | Acronym | Strain | Incubation time (d) |
|---|---|---|---|---|
| *Alatospora acuminata* Ingold | Leotiomycetes | ALAC | DSM 104360 | 18 |
| *Articulospora tetracladia* Ingold | Leotiomycetes | ARTE | DSM 104345 | 17 |
| *Clavariopsis aquatica* De Wild. | Dothideomycetes | CLAQ | DSM 104362 | 24 |
| *Clavatospora longibrachiata* (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson | Sordariomycetes | CLLO | DSM 104365 | 17 |
| *Flagellospora curvula* Ingold | Leotiomycetes | FLCU | DSM 104334 | 17 |
| *Heliscella stellata* (Ingold & V.J. Cox) Marvanová | Dothideomycetes incertae sedis | HEST | DSM 104386 | 18 |
| *Lemonniera terrestris* Tubaki | Leotiomycetes | LETE | DSM 104344 | 20 |
| *Neonectria lugdunensis* (Saccardo & Therry) L. Lombard & Crous | Sordariomycetes | NELU | DSM 104361 | 7 |
| *Tetracladium marchalianum* De Wild. | Leotiomycetes | TEMA | DSM 104373 | 14 |
| *Tricladium angulatum* Ingold | Leotiomycetes | TRAN | DSM 104374 | 17 |

Prior to ergosterol and DNA extraction, mycelium samples were freeze dried. Afterwards, the full amount of mycelium was transferred to pre-weighed extraction vials and dry weight was determined to the nearest 0.01 mg. Extraction and analysis of ergosterol generally followed the method described by Gessner (2005). Briefly,

ergosterol was extracted in 20 mL glass vials using 10 mL of alkaline methanol and subsequently purified via solid-phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent, Waters, USA). Quantification of ergosterol in the extract was performed via high-performance liquid chromatography (HPLC, 1200 Agilent Technologies, USA; Zubrod et al. 2011). Extraction and quantification of fungal DNA followed Baudy et al. (2019). Briefly, DNA was extracted using the FastDNA® Spin Kit for Soil in conjunction with the FastPrep-24 5G Instrument (MP Biomedicals, Germany) as per manufacturer's instructions. The extracts were 20-fold diluted before analysis. DNA amounts were estimated via base signatures in the internal transcribed spacer (ITS) region, which is situated between rRNA genes of the ribosomal DNA. The ITS region is the most frequently used barcoding region for fungi (Schoch et al. 2012) and allows for fungal taxon-specific biomass quantification (Baudy et al. 2019). In the qPCR reactions, extracted ITS fragments were amplified and fluorometrically quantified via primers and TaqMan® probes, containing a fluorescent reporter dye (Applied Biosystems, Germany). The reactions were performed in a Mastercycler® ep gradient S (Eppendorf, Germany).

To verify initial tebuconazole concentrations, 10-mL samples of fungicide-free medium and tebuconazole stock solutions were taken and stored at − 20°C. After thawing, tebuconazole was measured via an ultra-high-performance liquid chromatography system (Thermo Fisher Scientific, Germany) following Zubrod et al. (2015; Table S1).

Ergosterol and DNA levels were normalized to the mycelial dry weight of each replicate. Ergosterol and DNA concentrations were expressed as µg ergosterol/mg dry weight and ng DNA/mg dry weight, respectively. Due to the relatively low sample size (i.e. n = 4/5) data had to be assumed as normally distributed. Factorial analysis of variance (ANOVA) was performed to assess the impact of species, tebuconazole, and their interaction on ergosterol and DNA concentrations. To test for consistent and species-specific impacts of tebuconazole on concentrations of each biomarker, separate random-effects meta-analyses were conducted, using the R extension package "metafor" (Viechtbauer 2010). Statistics and figures were prepared using R version 3.5.2 (R Core Team 2019). For all analyses the alpha level was set at 0.05.

## RESULTS

As hypothesized, tebuconazole exposure reduced ergosterol concentrations among the species, as indicated by a significant overall mean difference of −13% (n = 10; p = 0.007; Fig. 1a). However, responses were highly species-specific (Table 2): while reductions in ergosterol concentrations ranged from 13% to 29%, concentration in FLCU was even increased by 35% (Fig. 1a). Against our expectation and in contrast to ergosterol, DNA concentrations tended to be increased among the

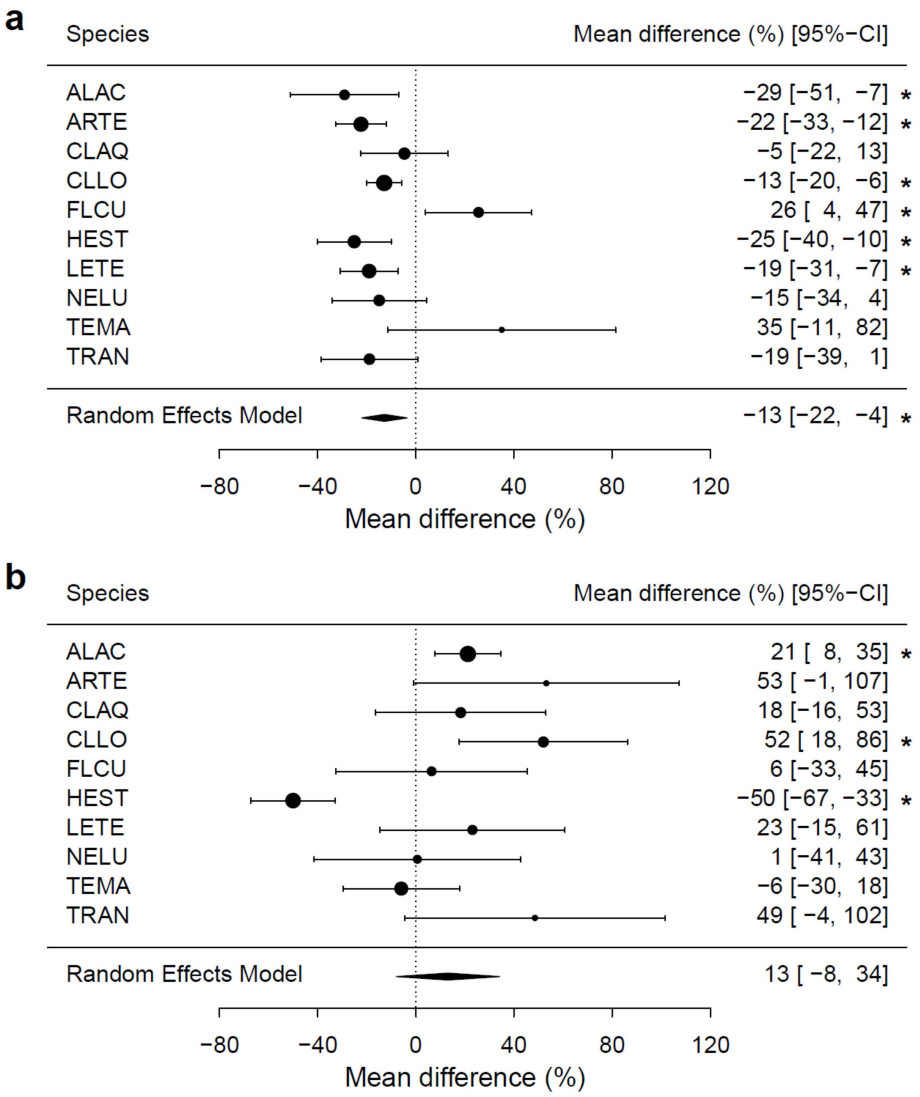



Fig. 1 Relative mean differences (points; ± 95%-CIs) in fungal species' ergosterol (a) and DNA concentrations (b) between tebuconazole-treated and control cultures, obtained via random-effects meta-analyses. Means on the right and the left side of the dotted line indicate higher and lower biomarker concentrations in tebuconazole-treated cultures, respectively. Point sizes indicate the weight (i.e. the inverted variance) of the respective species to the overall effect (diamonds). Fungal biomarker concentrations are significantly altered by tebuconazole-treatment, if CIs do not include zero (i.e. $p < 0.05$)

species by exposure to tebuconazole, as indicated by an overall mean difference of 13% (n = 10; p = 0.222; Fig. 1b), which was, however, not significant, potentially due to relatively high variability in DNA concentrations. Responses were again highly species-specific (Table 2): increments in DNA concentrations ranged among species from 21% to 53%, while concentration in HEST was reduced by 50% (Fig. 1b).

Table 2 ANOVA tables for ergosterol and DNA concentrations

| Biomarker | Source of variation | *df* | *SS* | *MS* | *F* | *p* |
|---|---|---|---|---|---|---|
| Ergosterol | Species | 9 | 165.14 | 18.349 | 40.649 | <0.001 |
| | Tebuconazole | 1 | 4.21 | 4.205 | 9.316 | 0.003 |
| | Species × tebuconazole | 9 | 18.19 | 2.021 | 4.476 | <0.001 |
| | Residuals | 76 | 34.31 | 0.451 | | |
| DNA | Species | 9 | 3632707 | 403634 | 50.524 | <0.001 |
| | Tebuconazole | 1 | 72420 | 72420 | 9.065 | 0.004 |
| | Species × tebuconazole | 9 | 199220 | 22136 | 2.771 | 0.007 |
| | Residuals | 76 | 607157 | 7989 | | |

## DISCUSSION

As hypothesized, exposure to tebuconazole reduced average fungal ergosterol concentrations, with species-specific variations in effect size and direction (Fig. 1a), pointing to variable physiological responses among species (Table 2). Reductions in ergosterol concentrations likely result from the inhibition of cytochrome P450 lanosterol 14α-demethylase activity (Joseph-Horne et al. 1995b). Moreover, the magnitude of this effect appears concentration-dependent (Fig. S1) and indicates reductions in ergosterol concentrations at environmentally relevant DMI fungicide levels (Rabiet et al. 2010; Hvězdová et al. 2018; Zubrod et al. 2019). Such reduced ergosterol concentrations in tebuconazole-exposed fungi, which do not reflect mycelial biomass, result in an underestimation of biomass. In contrast, increases in ergosterol concentrations, as observed for FLCU and TEMA (Fig. 1a), may be explained by an upregulation of ERG11 and other enzymes involved in the ergosterol biosynthesis pathway, which is a known DMI fungicide resistance mechanism in fungi (as reviewed by Zavrel et al. 2014). This is in line with FLCU and TEMA being considered fungicide-tolerant as these species are frequently dominating fungal

communities exposed to tebuconazole in the laboratory and field (e.g., Fernández et al. 2015; Zubrod et al. 2015; Feckler et al. 2017). The observed increases in ergosterol concentrations, which do not reflect true biomass increases, would result in a biomass overestimation.

In contrast to ergosterol and against our expectations, tebuconazole tended to increase average fungal DNA concentrations, while responses at the species level have again been highly variable (Fig. 1b, Table 2). Both, increments and reductions in DNA concentrations may be explained by species-specific responses in hyphal morphology, as reported for plant pathogenic fungi exposed to DMI fungicides: for instance a thinning of hyphae (Zhang et al. 2018) might increase the amount of DNA relative to the total biomass, while a progressing necrosis of hyphal cytoplasm or irregular thickening of cell walls (Kang et al. 2001) may cause the opposite effect. Analogous to ergosterol concentrations, such increases and decreases in this alternative biomass proxy may result in an underestimation and overestimation of fungal biomass, respectively.

Although drawing final conclusions on the universality of our findings requires further experimentation (involving e.g. the use of other taxa, such as basidiomycetes, fungicides, and test systems), our study provides evidence that ergosterol and DNA-based biomass estimates in fungi can be confounded by environmentally relevant DMI fungicide levels (Hvězdová et al. 2018; Zubrod et al. 2019). This, in turn, may question the use of these biomarkers in laboratory or field studies where the presence of fungicides is given or cannot be ruled out. Yet, for assessing systems with potential exposures to DMI fungicides, we recommend to complement ergosterol analyses with additional investigations, especially if effect sizes for ergosterol are close to the overall effect observed in the present study (i.e. − 13%; Fig. 1a). A suitable complementary approach may be the combined use of community composition analyses and rapidly emerging fungal trait databases comprising inter alia the (pollution) stress tolerance of individual fungi (e.g., Fun$^{Fun}$; Zanne et al. 2020). In contrast, DNA concentrations in the tolerant model fungi (i.e., FLCU, TEMA, and NELU), which generally dominate in communities shaped by chronic disturbance (Solé et al. 2008; Duarte et al. 2009; Feckler et al. 2017), remained unaltered (Fig. 1b). Accordingly, using DNA, the biomass of such insensitive species may be accurately estimated. Since the investigated species belong to a cosmopolitan and ecologically meaningful group of freshwater fungi (Bärlocher 1992), ITS copy

numbers of tolerant relative to all fungal species (as obtained via taxon-specific qPCR; e.g. Manerkar et al. 2008; Baudy et al. 2019) might serve as a globally applicable index of anthropogenic stress (cf. Mateu-Vicens et al. 2014). Moreover, since qPCR allows for a species-specific quantification, the sensitivities of individual species – potentially provided by emerging trait databases in the near future (Zanne et al. 2020) – can be taken into account when assessing fungal communities exposed to DMI fungicides, preventing biased biomass estimation via DNA.

When using ergosterol or DNA as fungal biomass proxies in field studies, we recommend checking for potential exposure to DMI fungicides. If contamination is intended (e.g., in laboratory experiments) or to be expected (e.g., in agricultural landscapes; Rabiet et al. 2010; Hvězdová et al. 2018) ergosterol or DNA-based fungal biomass data should be carefully interpreted. If the exposure situation is uncertain, an accompanying chemical analysis tailored to local fungicide use patterns, screening for specific DMI fungicides, should be considered. Moreover, global change is predicted to trigger an intensified agricultural and medical use of DMI fungicides (Anderson et al. 2004; Jones et al. 2006; Hakala et al. 2011), which may further exacerbate environmental contamination. Accordingly, it will become increasingly important to consider the potential of this fungicide group to bias fungal monitoring, ultimately misguiding environmental management.

## ACKNOWLEDGEMENTS

This study was funded by the German Research Foundation, Project AQUA-REG (DFG; SCHU227/14-1). We thank Therese Bürgi, Sebastian Pietz, and Britta Wahl-Ermel for their assistance in the laboratory.

## REFERENCES

Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR, Daszak P (2004) Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. Trends Ecol Evol 19:535–544

Bärlocher F (ed) (1992) The ecology of aquatic hyphomycetes. Springer, Berlin, New York

Baschien C, Tsui CK-M, Gulis V, Szewzyk U, Marvanová L (2013) The molecular phylogeny of aquatic hyphomycetes with affinity to the Leotiomycetes. Fungal Biol 117:660–672

Baudy P, Zubrod JP, Röder N, Baschien C, Feckler A, Schulz R, Bundschuh M (2019) A glance into the black box: novel species-specific quantitative real-time PCR assays to disentangle aquatic hyphomycete community composition. Fungal Ecol 42:100858

Bundschuh M, Zubrod JP, Kosol S, Maltby L, Stang C, Duester L, Schulz R (2011) Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. Aquat Toxicol 104:32–37

Dighton J, White JF (eds) (2017) The fungal community: its organization and role in the ecosystem. CRC Press, Boca Raton

Duarte S, Pascoal C, Garabétian F, Cássio F, Charcosset J (2009) Microbial decomposer communities are mainly structured by trophic status in circumneutral and alkaline streams. Appl Environ Microbiol 75:6211–6221

Feckler A, Goedkoop W, Konschak M, Bundschuh R, Kenngott KGJ, Schulz R, Zubrod JP, Bundschuh M (2017) History matters: heterotrophic microbial community structure and function adapt to multiple stressors. Glob Change Biol. https ://doi.org/10.1111/gcb.13859

Fernández D, Voss K, Bundschuh M, Zubrod JP, Schäfer RB (2015) Effects of fungicides on decomposer communities and litter decomposition in vineyard streams. Sci Total Environ 533:40–48

Fungicide Resistance Action Committee (2018) FRAC code list 2018: fungicides sorted by mode of action (including FRAC code numbering)

Gessner MO (2005) Ergosterol as a measure of fungal biomass. In: Graça MAS, Bärlocher F, Gessner MO (eds) Methods to study litter decomposition: a practical guide. Springer, Dordrecht, pp 189–195

Gessner MO, Chauvet E (1993) Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. Appl Environ Microbiol 59:502–507

Hakala K, Hannukkala AO, Huusela-Veistola E (2011) Pests and diseases in a changing climate a major challenge for Finnish crop production. Agric Food Sci 20:3–14

Hvězdová M, Kosubová P, Košíková M, Scherr KE, Šimek Z, Brodský L, Šudoma M, Škulcová L, Sáňka M, Svobodová M, Krkošková L, Vašíčková J, Neuwirthová N, Bielská L, Hofman J (2018) Currently and recently used pesticides in Central European arable soils. Sci Total Environ 613–614:361–370

Jones OAH, Voulvoulis N, Lester JN (2006) Partitioning behavior of five pharmaceutical compounds to activated sludge and river sediment. Arch Environ Contam Toxicol 50:297–305

Joseph-Horne T, Hollomon D, Loeffler RST, Kelly SL (1995a) Altered P450 activity associated with direct selection for fungal azole resistance. FEBS Lett 374:174–178

Joseph-Horne T, Carelli A, Hollomon D, Kelly SL (1995b) Investigation of azole resistance in the *Ustilago maydis* sterol demethylase mutant erg 40. Pestic Sci 44:33–38

Kang Z, Huang L, Krieg U, Mauler-Machnik A, Buchenauer H (2001) Effects of tebuconazole on morphology, structure, cell wall components and trichothecene production of *Fusarium culmorum* in vitro. Pest Manag Sci 57:491–500

Manerkar MA, Seena S, Bärlocher F (2008) Q-RT-PCR for assessing archaea, bacteria, and fungi during leaf decomposition in a stream. Microb Ecol 56:467–473

Mateu-Vicens G, Khokhlova A, Sebastian-Pastor T (2014) Epiphytic foraminiferal indices as bioindicators in mediterranean seagrass meadows. J Foramin Res 44:325–339

Murugan R, Loges R, Taube F, Sradnick A, Joergensen RG (2014) Changes in soil microbial biomass and residual indices as ecological indicators of land use change in temperate permanent grassland. Microb Ecol 67:907–918

Pesce S, Zoghlami O, Margoum C, Artigas J, Chaumot A, Foul-quier A (2016) Combined effects of drought and the fungicide tebuconazole on aquatic leaf litter decomposition. Aquat Toxicol 173:120–131

R Core Team (2019) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Rabiet M, Margoum C, Gouy V, Carluer N, Coquery M (2010) Assessing pesticide concentrations and fluxes in the stream of a small vineyard catchment Effect of sampling frequency. Environ Pollut 158:737–748

Rajapaksha RMCP, Tobor-Kapłon MA, Bååth E (2004) Metal toxicity affects fungal and bacterial activities in soil differently. Appl Environ Microbiol 70:2966–2973

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a

universal DNA barcode marker for Fungi. Proc Natl Acad Sci USA 109:6241–6246

Solé M, Fetzer I, Wennrich R, Sridhar KR, Harms H, Krauss G (2008) Aquatic hyphomycete communities as potential bioindicators for assessing anthropogenic stress. Sci Total Environ 389:557–565

Viechtbauer W (2010) Conducting meta-analyses in R with the metafor package. J Stat Softw 36:1–48

Zanne AE, Abarenkov K, Afkhami ME, Aguilar-Trigueros CA, Bates S, Bhatnagar JM, Busby PE, Christian N, Cornwell WK, Crowther TW, Flores-Moreno H, Floudas D, Gazis R, Hibbett D, Kennedy P, Lindner DL, Maynard DS, Milo AM, Nilsson RH, Powell J, Schildhauer M, Schilling J, Treseder KK (2020) Fungal functional ecology: bringing a trait-based approach to plant-associated fungi. Biol Rev 95:409–433

Zavrel M, Esquivel BD, White TC (2014) The ins and outs of azole antifungal drug resistance: molecular mechanisms of transport. In: Gotte M, Berghuis A, Matlashewski G, Wainberg M, Shep-pard D (eds) Handbook of antimicrobial resistance. Springer, New York, pp 1–27

Zhang J, Hu S, Xu Q, You H, Zhu F (2018) Baseline sensitivity and control efficacy of propiconazole against *Sclerotinia sclerotiorum*. Crop Prot 114:208–214

Zubrod JP, Bundschuh M, Feckler A, Englert D, Schulz R (2011) Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. Environ Toxicol Chem 30:2718–2724

Zubrod JP, Englert D, Wolfram J, Wallace D, Schnetzer N, Baudy P, Konschak M, Schulz R, Bundschuh M (2015) Waterborne toxicity and diet-related effects of fungicides in the key leaf shredder *Gammarus fossarum* (Crustacea: Amphipoda). Aquat Toxicol 169:105–112

Zubrod JP, Bundschuh M, Arts G, Brühl CA, Imfeld G, Knäbel A, Payraudeau S, Rasmussen JJ, Rohr J, Scharmüller A, Smalling K, Stehle S, Schulz R, Schäfer RB (2019) Fungicides: an overlooked pesticide class? Environ Sci Technol 53:3347–3365

## SUPPLEMENTARY DATA

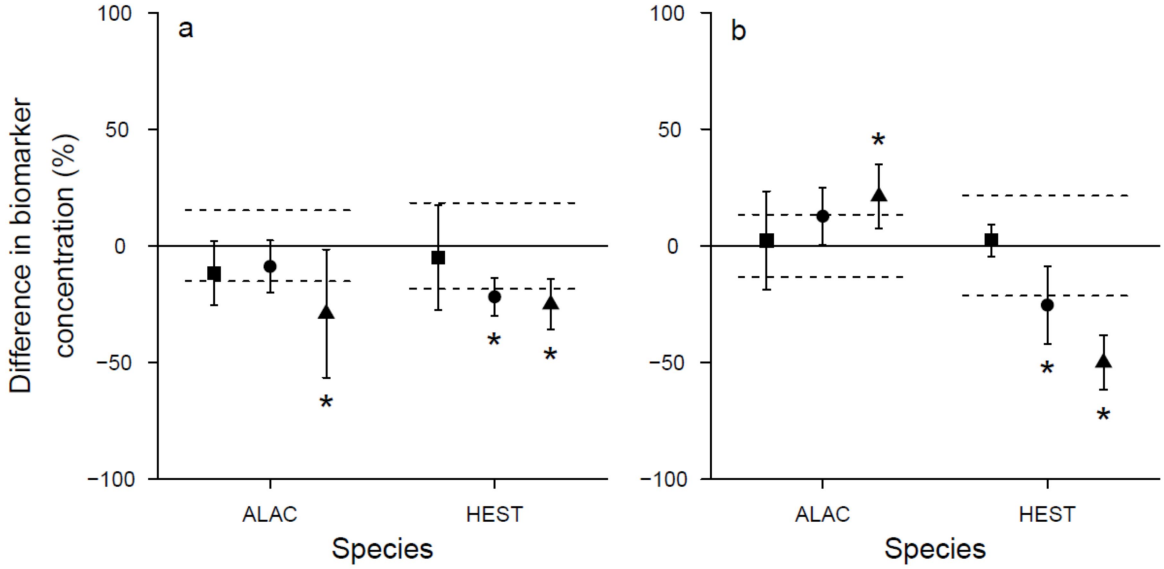



Fig. S1 Mean percentage difference in ergosterol (a) and DNA concentrations (b) in *Alatospora acuminata* (ALAC) and *Heliscella stellata* (HEST) between treatments exposed to tebuconazole concentrations of 0.7 (squares), 7 (circles), and 35 µg/L (triangles) and controls (solid lines). 95% confidence intervals for controls and tebuconazole treatments are represented by dashed lines and error bars, respectively. Asterisks indicate significant differences between treatments and the control (as judged by Dunnett's tests; $p<0.05$)

Table S1 Nominal and measured tebuconazole concentrations in dilution water and stock solutions. The low stock solution was used to spike treatment microcosms.

| Medium | Nominal concentration (mg/L) | Measured concentration (mg/L) | Recovery (% of nominal concentration) |
|---|---|---|---|
| Dilution water | 0.00 | < LOQ | n.a. |
| Low stock solution | 0.21 | 0.21 (± 0.01) | 99.95 |
| High stock solution | 105.00 | 111.53 (± 2.67) | 106.22 |

Abbreviations: LOQ: limit of quantification (0.0005 mg/L); n.a.: not applicable

**APPENDIX A.3**

## Fungal-fungal and fungal-bacterial interactions in aquatic decomposer communities: bacteria promote fungal diversity

Patrick Baudy, Jochen P. Zubrod, Marco Konschak, Sara Kolbenschlag, Annika Pollitt, Christiane Baschien, Ralf Schulz, Mirco Bundschuh

Ecology 102, e03471
Impact Factor (2020): 5.499

**ABSTRACT**

Fungi produce a variety of extracellular enzymes, making recalcitrant substrates bioavailable. Thus, fungi are central for the decomposition of dead organic matter such as leaf litter. Despite their ecological importance, our understanding of relationships between fungal species diversity and ecosystem functioning is limited, especially with regard to aquatic habitats. Moreover, fungal interactions with other groups of microorganisms such as bacteria are rarely investigated. This lack of information may be attributed to methodological limitations in tracking the biomass of individual fungal species in communities, impeding a detailed assessment of deviations from the overall performance expected from the sum of individual species' performances, so-called net diversity effects (NDEs). We used fungal species-specific biomolecular tools to target fungal-fungal and fungal-bacterial interactions on submerged leaves using four cosmopolitan aquatic fungal species and a stream microbial community dominated by bacteria. In microcosms, we experimentally manipulated fungal diversity and bacterial absence/presence and assessed functional performances and fungal community composition after 14 d of incubation. Fungal community data were used to evaluate NDEs on leaf colonization. The individual fungal species were functionally distinct and fungal cultures were on average more efficient than the bacterial culture. In absence of bacteria, NDEs correlated with growth rate (negatively) and genetic divergence (positively), but were predominantly negative, suggesting that higher fungal diversity led to a lower colonization success (niche overlap). In both absence and presence of bacteria, the overall functional performances of the communities were largely defined by their composition (i.e., no interactions at the functional level). In the presence of bacteria, NDEs correlated with genetic divergence (positively) and were largely positive, suggesting higher fungal diversity stimulated colonization (niche complementarity). This stimulation may be driven by a bacteria-induced inhibition of fungal growth, alleviating competition among fungi. Resulting feedback loops eventually promote fungal coexistence and synergistic interactions. Nonetheless, overall functional performances are reduced compared to bacteria-free cultures. These findings highlight the necessity to conduct future studies, investigating biodiversity-ecosystem functioning relationships using artificial systems, without exclusion of key organisms naturally co-occurring in the compartment of interest. Otherwise, study outcomes

might not reflect true ecological relationships and ultimately misguide conservation strategies.

**KEYWORDS**

Antagonism – Biodiversity-ecosystem functioning – Cellulolytic enzymes – Community composition – Complementarity – Diversity effects – Fungal interactions – Fungal-bacterial interactions – Ligninolytic enzymes – Microbial decomposers – Saprotrophs – Traits

## INTRODUCTION

The decomposition and recycling of leaf litter is a key process in ecosystems, which is realized by microbial (bacteria and fungi) and invertebrate decomposers. In particular fungi play a pivotal role in this process (Dighton and White 2017). Using a repertory of extracellular enzymes, they have the capability of converting a broad range of non-utilizable low- (e.g., phosphate esters and oligosaccharides) and high-molecular-weight organic compounds (e.g., cellulose, hemicellulose, and lignin) to bioavailable substances (e.g., inorganic nutrients or monomeric carbohydrates [Evans and Hedger 2001, Kubicek and Druzhinina 2007]). Yet, our (mechanistic) understanding of the relationship between fungal community structure (e.g., diversity and composition) and ecosystem functioning (i.e., biodiversity-ecosystem functioning; B-EF) is still at the beginning (De Laender et al. 2016, Grossart and Rojas-Jimenez 2016). Consequently, impacts of human-induced biodiversity loss on ecosystem-level processes, such as leaf litter decomposition, remain difficult to predict (Bell et al. 2009, Canhoto et al. 2016, Wright et al. 2017).

In aquatic and terrestrial ecosystems, there is vast potential for positive diversity effects on fungal-mediated leaf litter decomposition, which is considered to be largely based on resource partitioning (Gessner et al. 2010). In fungal assemblages, resource partitioning is realized via complementary extracellular enzyme inventories and varying activity patterns (i.e., functional divergence; Gessner et al. 2010). The extent of complementarity and thus decomposition efficiency is assumed to increase with increasing genetic divergence (Eichlerová et al. 2015, Andrade et al. 2016). Because of differences in biotic as well as abiotic drivers of diversity, abundance, and activity, however, the relative importance of mechanisms underlying B-EF relationships can vary between aquatic and terrestrial habitats

(Gessner et al. 2010, Bärlocher and Boddy 2016). Because of a higher degree of functional redundancy, the scope for complementarity is assumed to be much lower in aquatic compared to terrestrial leaf decomposing fungal communities (Bell et al. 2009, Gessner et al. 2010, Gonçalves et al. 2015). It is instead assumed that competitive and productive species dominate leaf decomposition in aquatic ecosystems (i.e., dominance or selection effect [Bärlocher and Corkum 2003, Ferreira and Chauvet 2012, Gonçalves et al. 2015]). Yet, the relative importance of complementarity and dominance effects for fungal B-EF relationships as well as the fundamental mechanisms supporting fungal species co-occurrence in aquatic ecosystems are poorly understood (Bärlocher and Corkum 2003, Krauss et al. 2011, Bärlocher 2016). Most findings on interactions in aquatic fungi were generated under controlled conditions, excluding other groups of organisms, such as bacteria, which, together with fungi, dominate leaf decomposition in aquatic habitats (Hieber and Gessner 2002). Therefore, and as these organism groups inevitably co-occur and closely interact with each other (Baschien et al. 2009, Deveau et al. 2018), the universality of findings on fungal interactions, generated under restrictive laboratory conditions, is questionable.

A key to investigating fungal B-EF relationships is the assessment of net diversity effects (NDEs). NDEs are measured as the deviation between observed productivity in multispecies assemblages and productivity predicted from individual species, weighted by their initial contribution to an assemblage, under the null hypothesis that there are no interactions between species (Loreau and Hector 2001). Assessing the mechanisms underlying NDEs (e.g., complementarity or dominance), however, necessitates tracking the biomass of individual species in complex assemblages not only at the initial but also at later successional stages (Bärlocher and Corkum 2003, Duarte et al. 2006, Grossart and Rojas-Jimenez 2016). This has just recently been enabled via TaqMan® quantitative real-time PCR (qPCR) assays specific to widespread aquatic fungal species (Baudy et al. 2019).

In order to address current knowledge gaps, we conducted a multifactorial laboratory microcosm experiment to assess fungal-fungal and fungal-bacterial interactions on submerged leaves in the absence and presence of stream bacteria. As model organisms, four widely distributed aquatic fungi – belonging to a polyphyletic fungal group adapted to submerged leaf litter (so called aquatic hyphomycetes) – and a natural community of stream bacteria co-occurring with

aquatic hyphomycetes in the wild, were used. We experimentally manipulated fungal diversity and bacterial absence/presence and analyzed functional variables (i.e., leaf mass loss, total fungal biomass, and enzyme activities) and community composition after 14 d of incubation.

We expected fungal (bacteria-free) monocultures to show varying functional performances (i.e., different functional traits; sensu Aguilar-Trigueros et al. [2015]). Furthermore, we hypothesized that fungal–fungal interactions in mixed cultures would be species-dependent and would change from antagonistic (competitive) to synergistic (complementary) with increasing functional or genetic diversity of the component species (Andrade et al. 2016). Lastly, we anticipated fungal-bacterial interactions to be species dependent, but antagonistic in nature (Mille-Lindblom and Tranvik 2003, Romaní et al. 2006), resulting in negative NDEs on the investigated variables.

## MATERIAL AND METHODS

### MICROORGANISMS AND LEAF SUBSTRATE

Strains of the aquatic hyphomycete species *Alatospora acuminata* (DSM 104360), *Heliscella stellata* (DSM 104386), *Neonectria lugdunensis* (DSM 104361), and *Tetracladium marchalianum* (DSM 104373) were selected as model fungi. This selection covers ascomycete species frequently detected in aquatic decomposer communities of the temperate latitudes and exhibits a gradient of sensitivities to anthropogenic stress (e.g., Solé et al. [2008], Fernández et al. [2016]). The strains were isolated from German streams and deposited in the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany [Baudy et al. 2019]). In the laboratory, isolated strains were exclusively handled under sterile conditions. Axenic cultures of the fungi were grown on 15 mL of malt extract agar (10 g malt extract/L, 20 g agar/L) in Petri dishes at 16°C in darkness for 21 d. Cylindrical agar plugs (5 mm diameter) were cut from the growing edges of the colonies – to sample young, metabolically active mycelium – serving as fungal inoculum of leaf disks in microcosms.

The stream inoculum, harboring the bacterial community considered as a factor (absence or presence) in this study, originated from the near-natural stream Hainbach, Germany (49.24202° N; 8.04586° E). Therefore, stream water and submerged leaf litter were collected in a pre-autoclaved 1-L Schott glass bottle, a day

before the start of the experiment, in December 2017. In the laboratory, using a sterilized filtration apparatus, the stream water was passed through glass fiber filters (1.2 µm nominal pore size; GF/C; Whatman GmbH, Dassel, Germany) to obtain approximately 500 mL of stream inoculum (for details see Danger et al. [2013]). A 250-mL aliquot was sterilized for use in bacteria-free treatments ensuring equivalent nutrient conditions across all treatments. Sterilization was realized by filtration through a polycarbonate filter (0.2 µm nominal pore size; Isopore™; Merck KGaA, Darmstadt, Germany) to remove microbial biomass, and autoclavation to halt any microbial activity. Both stream inoculum and sterilized stream water were kept in pre-autoclaved 250-mL Schott glass bottles at 4°C under permanent stirring until use in the experiment. It was presumed that the size-fractionated stream inoculum (i.e., ≤ 1.2 µm) excludes larger eukaryotic microorganisms (Morris and Nunn 2013) and is dominated by a taxonomically and functionally diverse bacterial community (Leflaive et al. 2008), whereas other unicellular organisms smaller than 1.2 µm, such as archaea, only contribute a minor fraction to the microbial biomass in the inoculum (e.g., Manerkar et al. [2008], Uyaguari-Diaz et al. [2016], Lambirth et al. [2018]). Therefore, the stream inoculum will in the following be referred to as “bacterial inoculum” and associated observations are solely discussed with respect to bacterial activity as done elsewhere (cf. Mille-Lindblom et al. [2006], Danger et al. [2013]).

Leaves of *Alnus glutinosa* (L.) Gaertn. (black alder) – a widespread European riparian tree species (Copolovici et al. 2014) – were used as model substrate. Only leaves with no visible signs of damage or symptoms of disease were handpicked from trees near Landau, Germany (49.20116° N; 8.09331° E) shortly before abscission in 2016 and stored at −20°C until further use (for ~ 1 yr). After thawing, disks (16 mm diameter) were cut from the leaves, avoiding the midvein. Leaf disks were subsequently processed in packs of 20. To account for variability associated with initial mass losses due to physical leaching of soluble leaf components (Petersen and Cummins 1974), leaf disks were leached for 48 h in 50 mL of nutrient medium (adjusted to pH 7; for composition see Dang et al. [2005]). Subsequently, leaf disks were dried at 60°C for 24 h and their initial dry weight was determined to the nearest 0.01 mg. On the day prior to the experiment, each test vessel – a 100-mL Erlenmeyer flask – was provided with a pre-weighed leaf disk pack, covered with aluminum foil, autoclaved, and kept overnight in a sealed ethanol-disinfected box. At the beginning of the experiment, nine leaf disk packs were directly sampled, dried at

60°C for 24 h, and weighed again to quantify leaf mass losses related to the autoclaving procedure.

EXPERIMENTAL DESIGN

The microcosm experiment followed a 12 × 2 factorial design. Twelve fungal treatments, comprising no fungi, four monocultures, six binary, and one quaternary combination of fungal species, were cultivated in absence and presence of bacteria. Each treatment was replicated six times ($n$ = 6), which resulted in a total number of 144 microcosms ($N$ = 144).

The microcosms were prepared under sterile conditions in a laminar flow cabinet (UV-treated for 30 min prior use; NU-437-500E; Nuaire, Plymouth, MN, USA) using autoclaved materials. A microcosm consisted of a 100-mL Erlenmeyer flask containing 20 leaf disks (see Microorganisms and leaf substrate), 60 mL of nutrient medium, four agar plugs, and 1 mL of bacterial inoculum or sterile stream water, respectively. Procedures for fungal and bacterial inoculation followed the methods described in Andrade et al. (2016) and Danger et al. (2013), respectively. Microcosms designated to contain one, two, or four fungal species were equipped with four, two, or one cultivated agar plug(s) per species, respectively, while microcosms with no fungi were equipped with four sterile agar plugs. This procedure followed the assumption that one colonized agar plug already constitutes an excess of fungal inoculum, by which the leaf colonization rate of each species is maximized (cf. Bärlocher and Corkum [2003]). Although initial microbial biomass was not further characterized, the setup of microcosms containing fungal and bacterial inoculum likely resulted in a high fungal/bacterial biomass ratio ($\gg$1), which is typical for decomposing leaves throughout various successional stages (Findlay et al. 2002, Hieber and Gessner 2002, Manerkar et al. 2008). All microcosms were sealed with cellulose plugs, randomly distributed on a horizontal shaker (model VKS 75 B control; Edmund Bühler GmbH, Bodelshausen, Germany) set at 110 rpm, and incubated at 16°C in darkness. To minimize location effects all microcosms were shuffled daily. After 7 d, the nutrient medium was renewed; after 14 d, the experiment was terminated and the leaf disks were recovered. Per microcosm, one leaf disk was preserved in a capped 12-mL round-bottom plastic centrifuge tube and stored at -20°C for later enzyme activity analyses. Two leaf disks were conserved in 2% formaldehyde/0.1% sodium pyrophosphate solution and stored at 4°C for analysis of

bacterial densities, and a further two leaf disks were preserved for potential future analysis. The remaining leaf disks were preserved in pre-weighed 2-mL Eppendorf tubes, frozen (-20°C) before freeze drying for 24 h, weighed to the nearest 0.01 mg, and stored at -20°C for later ergosterol and deoxyribonucleic acid (DNA) analysis. Two and one replicates of the binary cultures *A. acuminata-H. stellata* (*n* = 4 remaining) and *H. stellata-N. lugdunensis* (*n* = 5 remaining), respectively, were contaminated with waste medium of various cultures during the medium renewal and were therefore excluded from further analyses (*N* = 141 remaining).

ERGOSTEROL ANALYSIS

Total fungal biomass was analyzed as the amount of the fungal membrane molecule ergosterol, following the procedure described in Gessner (2005). Ergosterol was extracted from 30 to 50 mg of lyophilized leaf material, weighed to the nearest 0.01 mg, in 10 mL of alkaline methanol at 80°C. Solubilized ergosterol was purified via solid phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent; Waters, Milford, MA, USA), eluted in isopropanol, and stored at -20°C until analysis. After reaching room temperature, the extracts were quantified for ergosterol using a high-performance liquid chromatography (HPLC) system (1200 Series; Agilent Technologies, Santa Clara, CA, USA) as detailed in Zubrod et al. (2011). One sample of the *H. stellata* monoculture vaporized during ergosterol extraction and could therefore not be used for further analyses (*n* = 5).

ENZYME ACTIVITY ASSAYS

Using artificial substrates, activities of hydrolases and oxidases were analyzed fluorometrically and colorimetrically, respectively, as described in DeForest (2009) modified for leaf samples. The assessed degradative enzymes were phosphatases (PHO; EC 3.1.3.1 and 3.1.3.2; targeting phosphate esters), α-1,4-glucosidase (AGL; EC 3.2.1.20; targeting starch and maltose), β-1,4-glucosidase (BGL; EC 3.2.1.21; targeting cellulose), cellobiohydrolase (CEL; EC 3.2.1.91; targeting cellulose), β-1,4-xylosidase (XYL; EC 3.2.1.37; targeting hemicellulose), peroxidase (PER; EC 1.11.1.7; targeting lignin), and phenol oxidase (PHE; EC 1.10.3.2; targeting lignin). Leaf disks were thawed and homogenized in 350 mL of nutrient medium. Hydrolase and oxidase activities in sample homogenates were analyzed in 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) using a microplate reader

(Infinite 200; Tecan Group, Männedorf, Switzerland). All sample preparation and analysis steps were performed in a temperature-controlled laboratory room at approximately 18°C. For a detailed description of used artificial substrates, assay procedures, dispensing volumes, plate setups, and fluorescence/absorbance calculations, see Appendix S1. Ultimately, 13 out of 987 enzyme activity measurements apparently resulted from handling errors during sample processing and were thus excluded from further analyses. This concerned single replicates of the bacteria-free cultures *A. acuminata* (PER), *T. marchalianum* (BGL and XYL), *A. acuminata-N. lugdunensis* (PHE), *A. acuminata-T. marchalianum* (CEL and PHO), and in the control (PER and PHE) as well as the bacteria-containing cultures *H. stellata* (CEL), *N. lugdunensis* (CEL), *A. acuminata-H. stellata* (PHE), *H. stellata-N. lugdunensis* (PHE), and all fungi (XYL).

## FUNGAL SPECIES-SPECIFIC QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qPCR)

Individual biomasses of the four model fungi were estimated via DNA concentrations obtained from species-specific qPCR analyses, following Baudy et al. (2019). DNA was extracted from 10 to 20 mg of lyophilized leaf material, weighed to the nearest 0.01 mg, using the FastDNA® Spin Kit for Soil in conjunction with the FastPrep™-24 5G Instrument (MP Biomedicals Germany GmbH, Eschwege, Germany) as per manufacturer's instructions. Ten times diluted extracts were used to quantify DNA of the model fungi in species-specific TaqMan® qPCR reactions (Applied Biosystems, Waltham, MA, USA) performed in a Mastercycler® ep gradient S (Eppendorf, Hamburg, Germany). For a detailed description of the used species-specific TaqMan® assays, reaction compositions, cycling conditions, and data analysis see Baudy et al. (2019).

## BACTERIAL DENSITIES

Following the procedure described in Buesing (2005), bacterial densities were analyzed in all replicates, which received bacterial inoculum. Bacteria were detached from the leaf substrate via ultrasonication, filtered onto an inorganic filter membrane (0.2 µm nominal pore size; Anodisk™; Whatman GmbH, Dassel, Germany), and stained using the fluorophore SYBRGreen II (Molecular Probes, Eugene, OR, USA). Digital pictures of filter sections were captured with an epifluorescence microscope

(Axio Scope.A1; Carl Zeiss, Jena, Germany) using a 1,000× magnification. Afterwards, following the procedure described in Zubrod et al. (2011), cells were systematically counted by image analysis software (Axio Vision 4.8.2; Carl Zeiss, Jena, Germany).

## GENERAL CALCULATIONS

Leaf mass loss ($L$) was expressed in percent and calculated as follows:

$$L = \frac{(w_i * 0.924 - w_f * 1.333)}{(w_i * 0.924)} * 100,$$

where $w_i$ and $w_f$ refer to the initial and final dry weight of 20 and 15 leaf disks, respectively, and 0.924 and 1.333 are empirical factors controlling for leaf mass losses due to the autoclaving procedure and the destructive sampling of five leaf disks, respectively. Ergosterol and DNA concentrations were expressed as µg/g leaf dry weight and were calculated as described in Baudy et al. (2019). Fungal growth rates were calculated from ergosterol concentrations in bacteria-free fungal monocultures and were expressed as µg ergosterol/g leaf dry weight/d. Enzyme activities were expressed as µmol/(h × g) leaf dry weight and calculated as described in Appendix S1: Eqs. S1-S6. All data were normalized to the control (i.e., no fungi and no bacteria present) and any resulting negative values were replaced by zero (concerning 115 out of 987 enzyme activity measurements).

## PREDICTION OF FUNCTIONAL PERFORMANCES

Biomass-specific functional performances (B-SFPs) were calculated from fungal monocultures in absence of bacteria and from the bacteria-only treatment, by normalizing overall functional performances (OFPs) to ergosterol, DNA, or bacterial cell concentrations. In order to characterize functional traits of the fungi, BSFPs were based on ergosterol (Appendix S2: Table S1). Compared to DNA, ergosterol concentrations show less interspecific variability (Baudy et al. 2019), which is why B-SFPs based on this biomarker are more readily comparable among species. To predict OFPs in mixed cultures following the assumption that biomasses of component species are not equal, B-SFPs were based on individual fungal species' DNA concentrations and bacterial cell densities. These predictions were calculated as the sum of B-SFPs of the component fungi and bacteria (Appendix S2: Table S2) multiplied by species-specific DNA concentrations and bacterial cell densities

measured in mixed cultures. Observed and predicted OFPs were tested against the null hypothesis of additivity (Bärlocher and Corkum 2003).

## CALCULATION OF GENETIC DIVERGENCES

The mean genetic divergence of fungal strains in mixed cultures served – besides species number – as a measure of diversity and was based on the internal transcribed spacer (ITS) region of the ribosomal DNA operon – the most frequently used barcoding region for fungi (Schoch et al. 2012). ITS sequences of the model fungi (GenBank accession numbers MH930815, MH930820, MH930822, and MH930823) were trimmed to a common region (i.e., partial ITS1, 5.8S, and ITS2) and aligned, whereupon genetic divergences for combinations of two and four species were calculated as pairwise distances and overall mean distance, respectively, using the bioinformatics software MEGA X (Kumar et al. 2018) with default settings (maximum composite likelihood substitution model).

## CALCULATION OF DIVERSITY EFFECTS ON LEAF COLONIZATION

To assess interactions between co-occurring fungi on leaf colonization, which requires information on individual species' biomasses, NDEs based on species-specific DNA concentrations in monocultures and mixed cultures were calculated applying a modified Price equation (i.e., tripartite partition [Fox 2005]). Based on the assumption that the fungi perform in mixed cultures equally well as in monocultures (i.e., additivity), NDEs on DNA concentrations ($\Delta Y$) were calculated as

$$\Delta Y = NE_{\mathrm{uw}}(M)E_{\mathrm{uw}}(\Delta RY) + N\mathrm{Cov}_{\mathrm{uw}}\left(M, \frac{RY_O}{RYT_O} - RY_E\right) + N\mathrm{Cov}_{\mathrm{uw}}\left(M, RY_O - \frac{RY_O}{RYT_O}\right),$$

which allows discrimination between trait-independent complementarity (expectation term), dominance (first covariance term), and trait-dependent complementarity effects (second covariance term; for a detailed description of these effects, see Fox [2005]). $N$ is the total number of fungal species in a mixture (i.e., 2 or 4), $E_{\mathrm{uw}}$ is the unweighted mean of the $N$ species in a mixture, and $M$ is the DNA concentration of each species in monoculture. $RY_O$ is the observed relative DNA concentration of a species in a mixture (i.e., its observed DNA concentration in the mixture divided by $M$) and $RY_E$ is the expected relative DNA concentration of a species in a mixture, which is its proportion to the inoculum (i.e., 0.5 or 0.25 for binary or quaternary species combinations, respectively). $\Delta RY$ is the deviation from expected relative DNA concentrations of a species in a mixture (i.e., the difference between $RY_O$ and $RY_E$)

and $RYT_O$ is the total observed relative DNA concentration of a mixture (i.e., the sum of all species' $RY_O$ in a mixture).

NDEs are an indicator of general synergistic (positive values) or antagonistic interactions (negative values). Trait-independent complementarity effects are indicative of interactions resulting in a mutual benefit (e.g., niche partitioning; positive values) or in a mutual expense (e.g., niche overlap; negative values). Dominance effects are indicative of rapidly growing species dominating at the expense of slowly growing species (positive values) or slowly growing species dominating at the expense of rapidly growing species (negative values), while in both cases species occupy similar niches. Trait-dependent complementarity effects are indicative of interactions benefiting rapidly (positive values) or slowly growing species (negative values), but in both cases not at the expense of other species (Fox 2005).

STATISTICAL ANALYSES

Prior to statistical testing, extreme values were identified by visual inspection of box plots (values deviating from the box by more than three times the interquartile range) and excluded from further analyses if values were identified at both the treatment level ($n$ = 6) and the variable level ($N$ = 141). Accordingly, one replicate of the *H. stellata*-*T. marchalianum* culture treatment was excluded from analysis of NDEs. Normality and homogeneity of variances was tested using the Shapiro-Wilk and Levene's test, respectively.

To assess the effects of fungal diversity, species combination (nested within diversity) and bacterial presence as well as their interactions on response variables, three-way nested analysis of variance (ANOVA) was performed on the original data if the presumptions for parametric testing were met. Otherwise ANOVAs were performed on square-root-transformed or rank-transformed data (Conover and Iman [1981]; but see also Seaman et al. [1994] for critique). To assess whether B-SFPs based on ergosterol concentrations represent ecologically meaningful functional traits (i.e., significantly different from zero) of individual fungi, one-sample t-tests and Wilcoxon signed-rank tests (as nonparametric alternative) were performed. Single comparisons between observations and predictions and between observations in bacterial presence and absence were performed using paired and unpaired two-sample tests, respectively (i.e., *t*-tests and Wilcoxon rank-sum tests as nonparametric alternative). Multiple comparisons between DNA concentrations of fungal mono- and

mixed cultures were performed using ANOVAs followed by Dunnett's tests, or if the presumptions for parametric testing were not met, by Wilcoxon rank-sum tests with Bonferroni correction for multiple comparisons. To assess associations between two variables, Pearson's correlation and Spearman's rank correlation (as nonparametric alternative) were used.

Multivariate data were log(x + 1)-transformed and min-max normalized to decrease the discriminatory power of enzymes with high activities and to enable the simultaneous analysis of different types of variables (i.e., leaf mass loss, growth rates, and enzyme activities). Replicates with missing values were excluded from multivariate analysis. To assess the effects of fungal diversity, species combination (nested within diversity) and bacterial presence as well as their interactions on enzyme activity profiles, permutational multivariate analysis of variance (PERMANOVA) was performed on enzyme activity profiles of all cultures. Functional dissimilarities between fungal species (i.e., in bacteria-free monocultures) were calculated from biomass-specific functional performances (i.e., leaf mass loss, enzyme activities, and growth rates, based on ergosterol concentrations) using Bray–Curtis dissimilarity. For the visualization of dissimilarities of the enzyme activity profiles as well as species-specific functional performances, data were displayed via non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity. All statistics and figures were prepared using R version 3.5.2 (R Development Core Team 2018) as well as the add-on packages "multcomp", "plotrix", and "vegan". The term "significant(ly)" is exclusively used with regard to statistical significance throughout this study.

## RESULTS

### LEAF MASS LOSS

Generally, leaf decomposition efficiency was shaped by complex interactions between fungal species number, fungal species identity, and bacterial presence (Fig. 1a; Appendix S2: Table S3). The model fungi differed in their degradative capabilities with mean leaf mass losses ranging between 0.5 and 21.0% (Fig. 1a), and biomass-specific leaf mass losses were nearly identical for *A. acuminata*, *N. lugdunensis*, and *T. marchalianum* (Appendix S2: Table S1). Nonetheless, changes in biomass-specific leaf mass loss significantly contributed to the functional

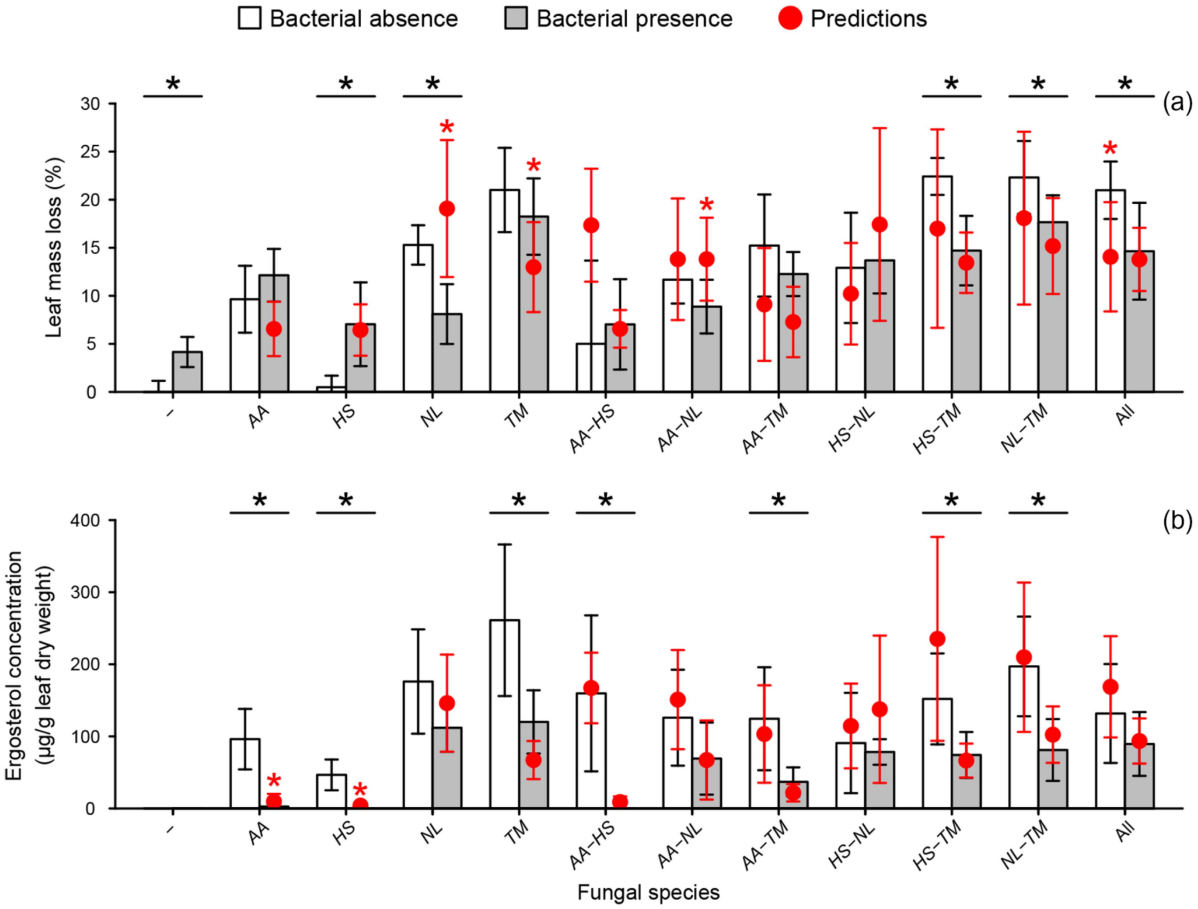



Fig. 1. (a) Mean leaf mass loss (in percent; relative to the initial leaf dry weight) and (b) ergosterol concentrations (in µg/g leaf dry weight; with 95% confidence intervals, respectively; *n* = 6) in fungal cultures in absence (white bars) and presence of bacteria (gray bars). Red points indicate predictions based on fungal and bacterial biomass–specific leaf mass loss or ergosterol concentrations. Red asterisks indicate significant differences between observed and predicted leaf mass loss or ergosterol concentrations of fungal treatments containing more than one organism (i.e., presence of bacteria or further fungi). Black asterisks indicate significant differences in leaf mass loss or ergosterol concentrations between bacteria-free and bacteria-containing cultures (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*).

separation of the species (Fig. 2a). Observed leaf mass losses in bacteria-free fungal mixed cultures (6.4%-22.4%) did hardly exceed the performance of the most productive fungal monoculture (i.e., *T. marchalianum*: 21.0%) and largely matched predictions (Fig. 1a). Only in the quaternary species combination observations were significantly higher than predictions, indicating ecologically meaningful interactions (Fig. 1a). The bacteria-only culture was capable of degrading a significant amount of leaf mass (4.2%) but was 2.8 times less productive than the average of all fungal monocultures (11.6%; Fig. 1a). Leaf mass loss in bacteria-containing fungal cultures

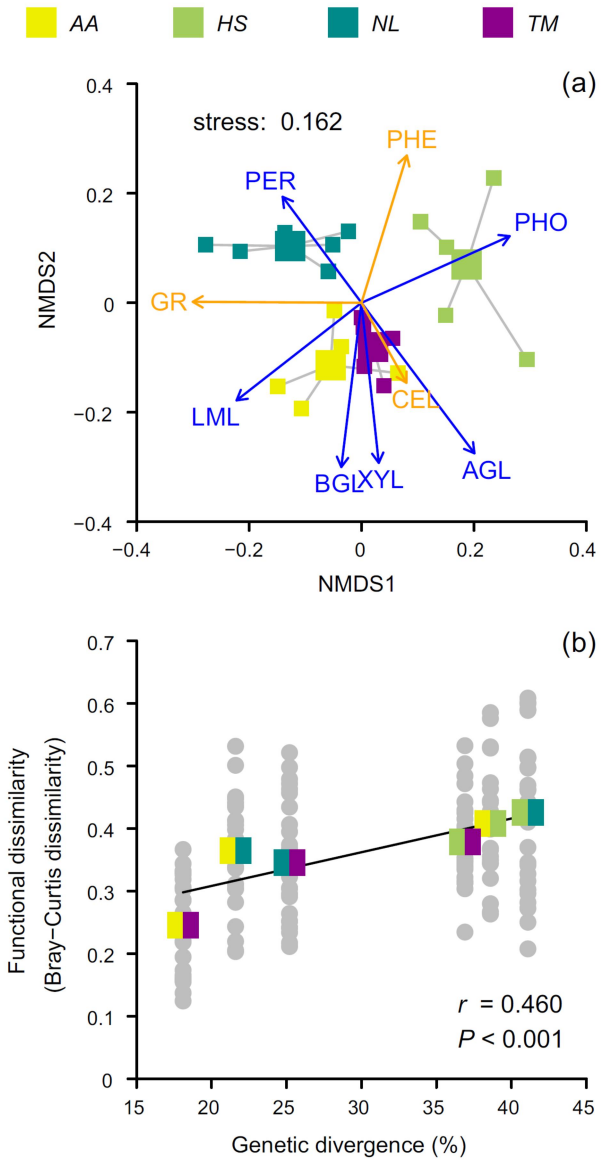



Fig. 2. (a) Non-metric multidimensional scaling (NMDS) plot for biomass-specific functional performances of the fungi (i.e., leaf mass loss [LML], enzyme activities [for abbreviations see below], and growth rates [GR] based on ergosterol concentrations) using Bray-Curtis dissimilarity. Group centroids and replicates are displayed as large and small squares, respectively. Blue and orange arrows display functional performances significantly and non-significantly contributing to the separation between the species, respectively (LML: $P$ = 0.002; GR: $P$ = 0.096; PHO: $P$ = 0.001; AGL: $P$ = 0.001, BGL: $P$ = 0.001; CEL: $P$ = 0.521; XYL: $P$ = 0.001; PER: $P$ = 0.011; PHE: $P$ = 0.122). The provided stress value indicates a reasonable fit (i.e., <0.2; Clarke 1993). (b) Correlation between functional dissimilarity (Bray-Curtis dissimilarity) and genetic divergence of pairs of fungi (grey circles; dissimilarities were calculated for all possible replicate combinations of two cultures). Mean functional dissimilarities between two fungi are displayed as colored squares. Pearson's correlation coefficient ($r$) and $P$-value indicate a weak but statistically significant positive correlation (Hinkle et al. 2003). The regression line was added to visualize the relationship between the variables (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*; PHO, phosphatase; AGL, α-1,4-glucosidase; BGL, β-1,4-glucosidase; CEL, cellobiohydrolase; XYL, β-1,4-xylosidase; PER, peroxidase; PHE, phenol oxidase). Note the reduced sample size for *A. acuminata*, *H. stellata*, and *T. marchalianum* due to missing ergosterol or enzyme activity data for one replicate, respectively ($n$ = 5).

was generally lower than in bacteria-free ones, except in the monocultures of the two least productive fungi *A. acuminata* and *H. stellata* (Fig. 1a). Observed leaf mass loss in bacteria-containing fungal mono- and mixed cultures in large part matched predictions (Fig. 1a). Observations only deviated significantly from predictions in the bacteria-containing fungal monocultures *N. lugdunensis* (overpredicted) and *T. marchalianum* (underpredicted) and the binary culture *A. acuminata*-*N. lugdunensis* (overpredicted), indicating ecologically meaningful interactions (Fig. 1a).

ERGOSTEROL

Ergosterol concentrations (i.e., total fungal biomass) were the result of complex interactions between fungal species number, fungal species identity (/identities), and bacterial presence (Fig. 1b; Appendix S2: Table S3). Biomass production capacity of the four model fungi, expressed as ergosterol concentrations, ranged from 47 to 261 µg/g leaf dry weight (Fig. 1b) and followed the same pattern as the fungi's degradative capacities (Fig. 1a; Appendix S2: Fig. S1). Growth rate ranged, on average, from 3.33 to 18.65 µg ergosterol/g leaf dry weight/d (Appendix S2: Table S1) and tended to be a critical determinant for the functional separation between the species, though not statistically significant (Fig. 2a). Ergosterol concentrations in bacteria-free fungal mixed cultures (90.8-197.0 µg/g leaf dry weight) did not exceed the performance of the most productive monoculture (i.e., *T. marchalianum*: 261.1 µg/g leaf dry weight) and matched predictions (Fig. 1b). Ergosterol concentrations in bacteria-containing fungal cultures were constantly lower than in those being bacteria-free (Fig. 1b) and largely met predictions. The only exceptions were the bacteria-containing fungal monocultures *A. acuminata* and *H. stellata*, which showed significantly lower ergosterol concentrations (2.7 and 0.0 µg/g leaf dry weight, respectively) than predicted (9.61 and 3.28 µg/g leaf dry weight, respectively; Fig. 1b). Likely these deviations are not the result of ecological interactions. In fact, such low ergosterol concentrations – as predicted by amounts of DNA – could possibly be below the HPLCs' detection limit. In bacteria-only cultures, no ergosterol was detected (Fig. 1b), which indicates that the bacterial inoculum was not contaminated with true fungi (cf. Mille-Lindblom et al. [2006], Danger et al. [2013]).

ENZYME ACTIVITIES

The enzyme activities varied substantially depending on the treatment and changes in activities of all enzymes significantly contributed to the functional separation between cultures (Fig. 3; Appendix S2: Tables S4, S5). Mean activities ranged among the different cultures from 3.42 to 42.74 (PHO), 0.45 to 4.86 (AGL), 1.54 to 54.68 (BGL), 0.43 to 2.27 (CEL), 0.17 to 3.95 (XYL), 0.01 to 0.37 (PER), and 0.01 to 0.17 μmol/(h × g) leaf dry weight (PHE; detailed information on enzyme activities is provided in Appendix S2: Fig. S2). Note that absolute activities do not necessarily reflect the relative importance of an enzyme compared to others (e.g., BGL vs. PHE; Appendix S2: Fig. S2), because sample storage conditions and duration as well as differences in experimental and analysis temperature (16°C vs. 18°C) can affect activities in an enzyme-specific manner (German et al. 2011). Significant impacts of independent variables on enzyme activities were identified as follows: fungal diversity impacted PHO, BGL, XYL, and PER activities; fungal species identity impacted AGL, BGL, CEL, XYL, PER, and PHE activities; bacterial presence impacted AGL, CEL, XYL, PER, and PHE activities; the interaction of fungal diversity and bacterial presence only impacted AGL activity; and the interaction of fungal species identity and bacterial presence impacted AGL, BGL, XYL, and PER activities (Appendix S2: Table S4). The fungi were capable of producing all of the investigated enzymes (Fig. 3; Appendix S2: Fig. S2), whereas the enzymatic capabilities of the fungi considered as ecologically meaningful functional trait (for definition see Statistical analyses) varied among species (Appendix S2: Table S1). Functional separation of the species was primarily driven by changes in activities of PHO, AGL, BGL, XYL, and PER (significant) and to a lesser extent by CEL and PHE (non-significant; Fig. 2a). Enzyme activities in bacteria-free fungal mixed cultures did not exceed performances of the most productive monocultures (i.e., *N. lugdunensis*; Appendix S2: Fig. S2f, g; and *T. marchalianum*; Appendix S2: Fig. S2a-e), and only deviated in approximately 10% of the comparisons from the predictions (Appendix S2: Fig. S2), indicating the prevalence of additive relationships. Also, the bacteria-only culture was capable of producing all of the investigated enzymes, whereas only AGL, BGL, CEL, and XYL were significantly different from the sterile control (Appendix S2: Fig. S2). The average enzyme activity in the bacteria-only culture was 2.4-fold lower compared to the average of the four fungal monocultures (Appendix S2: Fig. S2), which approximately corresponds to the observed difference

in leaf decomposition performance between these organism groups (i.e., a 2.8-fold difference). Compared to bacteria-free cultures, bacterial presence induced a shift in enzyme profiles towards higher AGL, BGL, CEL, and XYL activities and lower PHO and oxidase activities (PER and PHE; Fig. 3). In approximately 20% of comparisons, enzyme activities in bacteria-containing fungal cultures significantly deviated from predictions, indicating a higher frequency of interactions in bacteria-containing fungal cultures than in bacteria-free fungal mixed cultures (Appendix S2: Fig. S2). The majority of these significant deviations was identified for PER and PHE activities (12 out of 17), which generally showed – along with PHO activity – the highest variability among enzymes (Appendix S2: Fig. S2a, f, g).

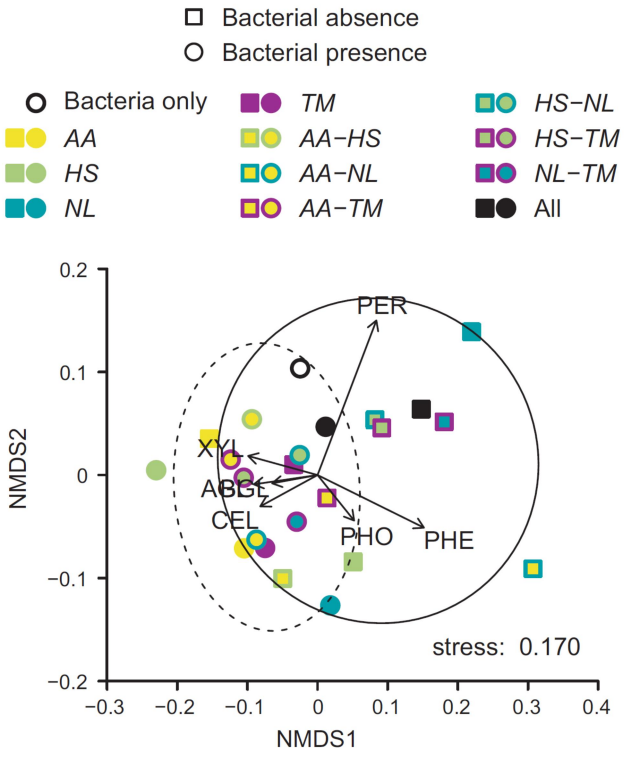



Fig. 3. Non-metric multidimensional scaling (NMDS) plot for enzyme activities using Bray–Curtis dissimilarity. Group centroids are displayed as colored symbols (for individual replicates see Appendix S2: Fig. S4). Squares and circles indicate bacterial absence and presence, respectively. Solid and dashed ellipses indicate group clusters of cultures in absence and presence of bacteria, respectively. Arrows display enzyme activities significantly contributing to the separation between treatments ($P$ = 0.001-0.003). The provided stress value indicates a reasonable fit (i.e., <0.2; Clarke 1993; *AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*; PHO, phosphatase; AGL, α-1,4- glucosidase; BGL, β-1,4-glucosidase; CEL, cellobiohydrolase; XYL, β-1,4-xylosidase; PER, peroxidase; PHE, phenol oxidase).

## GENETIC DIVERGENCE AND FUNCTIONAL DISSIMILARITY

Genetic divergences based on base substitutions in the ITS region ranged from 18% to 41%. Functional dissimilarities based on Bray-Curtis dissimilarity indices ranged from 0.25 to 0.43 (Fig. 2b; Table 1). The correlation between genetic divergence and functional dissimilarity among the strains was weak but significantly positive (Fig. 2b).

Table 1. Genetic divergence (GD) and functional dissimilarities (FD) between the fungal species. Estimates of genetic divergence between the fungi are based on ITS sequences. The percentage of base substitutions between sequences is shown. Analyses were conducted using the Maximum Composite Likelihood model. Mean functional dissimilarities based on biomass-specific functional performances were calculated using Bray-Curtis dissimilarity.

| Species | *A. acuminata* | | *H. stellata* | | *N. lugdunensis* | | *T. marchalianum* | |
|---|---|---|---|---|---|---|---|---|
| | GD | FD | GD | FD | GD | FD | GD | FD |
| *A. acuminata* | 0 | 0.00 | | | | | | |
| *H. stellata* | 39 | 0.41 | 0 | 0.00 | | | | |
| *N. lugdunensis* | 22 | 0.36 | 41 | 0.43 | 0 | 0.00 | | |
| *T. marchalianum* | 18 | 0.25 | 37 | 0.38 | 25 | 0.35 | 0 | 0.00 |

## COMMUNITY COMPOSITION AND LEAF COLONIZATION

Fungal diversity, species identity (of co-occurring species), and bacterial presence were significant determinants for DNA concentrations of all fungi (Fig. 4; Appendix S2: Table S6). Only for *H. stellata*, effects on DNA concentrations did result from an interaction of fungal diversity and bacterial presence (Fig. 4b; Appendix S2: Table S6). For all fungi but *T. marchalianum*, effects on DNA concentrations were affected by the interaction of species identity and bacterial presence (Fig. 4d; Appendix S2: Table S6). Irrespective of bacterial absence or presence, DNA concentrations of each fungus were in mixed cultures lower than in monoculture, except for *A. acuminata* in the bacteria-free binary culture *A. acuminata-H. stellata* (Fig. 4a). Compared to monocultures, DNA concentrations of *A. acuminata*, *H. stellata*, *N. lugdunensis*, and *T. marchalianum* in fungal mixed cultures were, in the absence of bacteria, on average 56%, 90%, 30%, and 69% lower, respectively, and in the presence of bacteria on average 47%, 58%, 52%, and 23% lower, respectively (Fig. 4). Generally, compared to bacteria-free cultures, the presence of bacteria resulted in reduced DNA concentrations in fungal monocultures and mixed cultures with average reductions of 44%, 73%, 36%, and 53% for *A. acuminata*, *H. stellata*, *N. lugdunensis*, and *T. marchalianum*, respectively (Fig. 4).

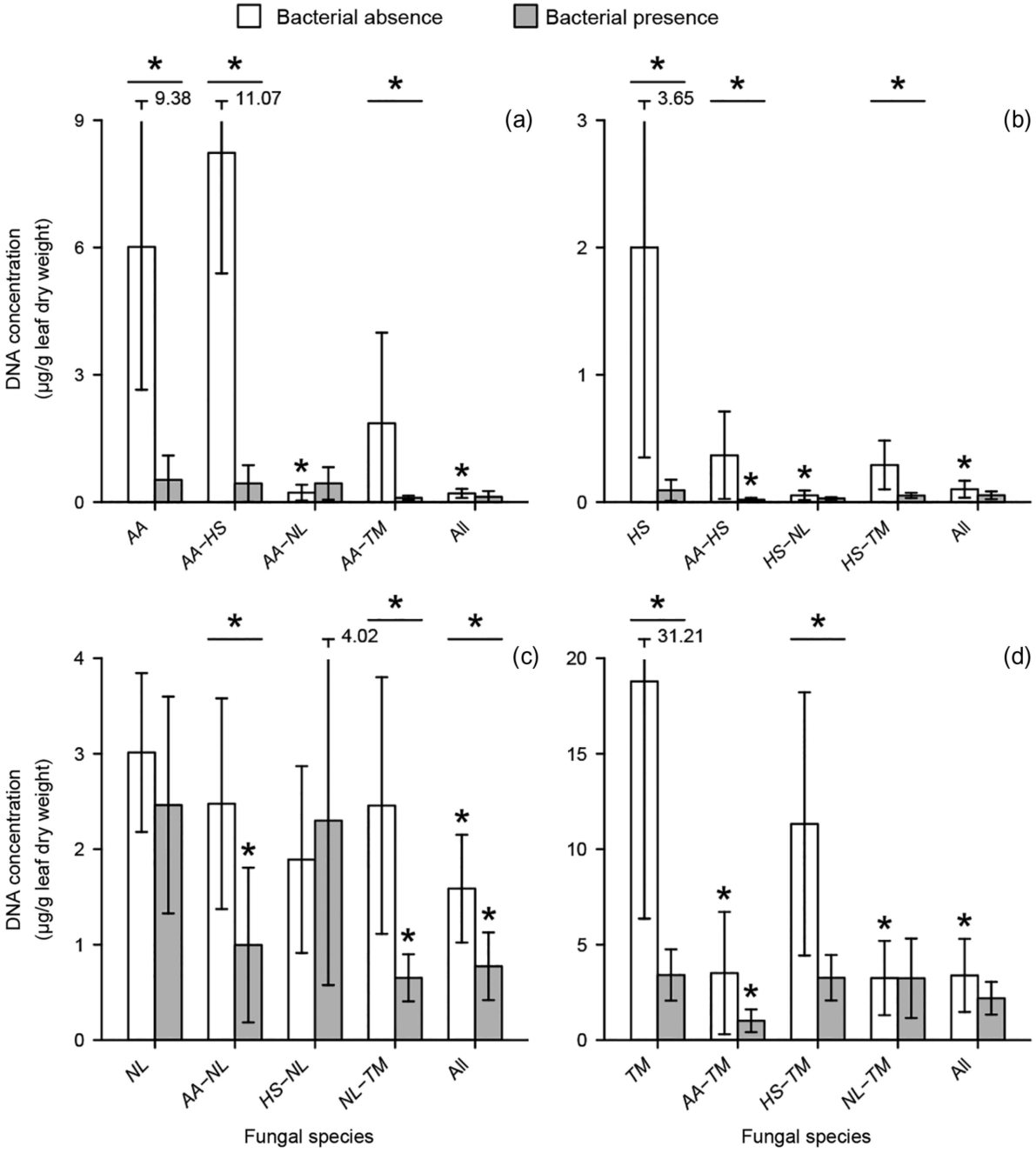



Fig. 4. Mean species-specific DNA concentrations (µg DNA/g leaf dry weight) of (a) *A. acuminata*, (b) *H. stellata*, (c) *N. lugdunensis*, and (d) *T. marchalianum* (with 95% confidence intervals) in mono, binary, and quaternary cultures in the absence (white bars) and presence of bacteria (gray bars). Asterisks above bars indicate significant differences to the monoculture within the same bacteria treatment and asterisks above horizontal lines indicate significant differences between bacteria-free and bacteria-containing cultures within the same fungal combination (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*).

Fungal leaf colonization, expressed as NDEs on DNA concentrations, was, in bacteria-free fungal mixed cultures, on average negative (-13.5%) and ranged from -56.7% to 114.6% among the cultures (Fig. 5). NDEs were mainly composed of trait-independent complementarity effects (explaining 113.8% of NDEs), whereas

dominance and trait-dependent complementarity effects were opposed to NDEs (explaining -7.6% and -6.2% of NDEs, respectively; Fig. 5). NDEs significantly correlated with the average growth rate and genetic divergence of the mixed cultures (negatively and positively, respectively; Fig. 6a, b). Pairwise genetic distances between the fungi ranged from 18% to 41% (Table 1) and the overall mean distance was 30%. With one exception (i.e., the binary culture *A. acuminata-H. stellata*), NDEs were (partially) significantly higher in bacteria-containing than in bacteria-free fungal mixed cultures and were on average positive (42.8%), ranging between -43.4% and 93.8% among cultures (Fig. 5). These NDEs were, in the presence of bacteria, mainly composed of trait-independent complementarity and dominance effects (explaining 50.6% and 33.7% of NDEs, respectively; Fig. 5), prevailing over trait-dependent complementarity effects (explaining 15.6% of NDEs; Fig. 5). In the

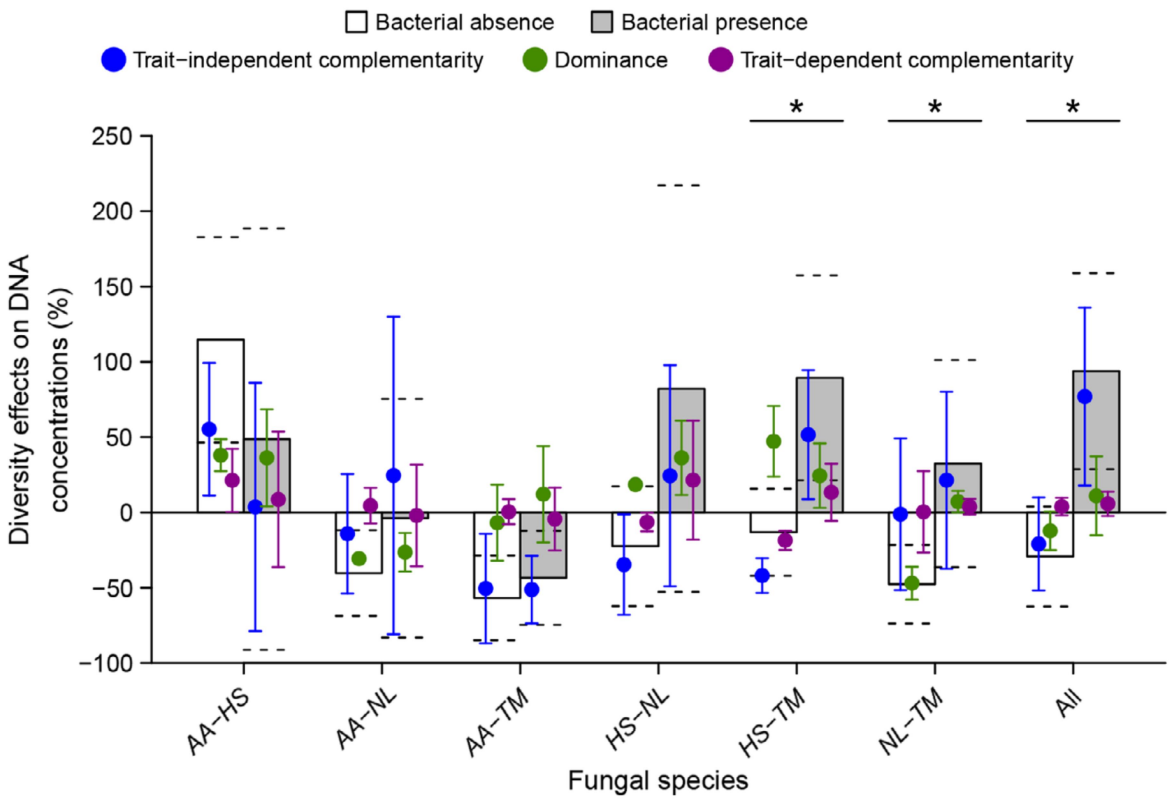



Fig. 5. Mean diversity effects on DNA concentrations (in percent; with 95% confidence intervals; *n* = 6) in fungal mixed cultures based on a modified Price equation (i.e., tripartite partition; Fox [2005]). Bars indicate mean net diversity effects on fungal leaf colonization in absence (white bars) and presence of bacteria (gray bars), which are composed of the sum of trait-independent complementarity (blue points), dominance (green points), and trait-dependent complementarity effects (purple points). Asterisks indicate significant differences in net diversity effects between bacteria-free and bacteria-containing cultures (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*).

presence of bacteria, NDEs were not correlated with cultures' average growth rate, but still showed a slight positive dependence on genetic divergence (Fig. 6c, d).

Finally, bacterial densities ranged from 66 × $10^9$ to 113 × $10^9$ cells per mg leaf dry weight among cultures that received bacterial inoculum (Appendix S2: Fig. S3). Fungal diversity and species identity had no significant impact on bacterial cell densities (Appendix S2: Table S7).

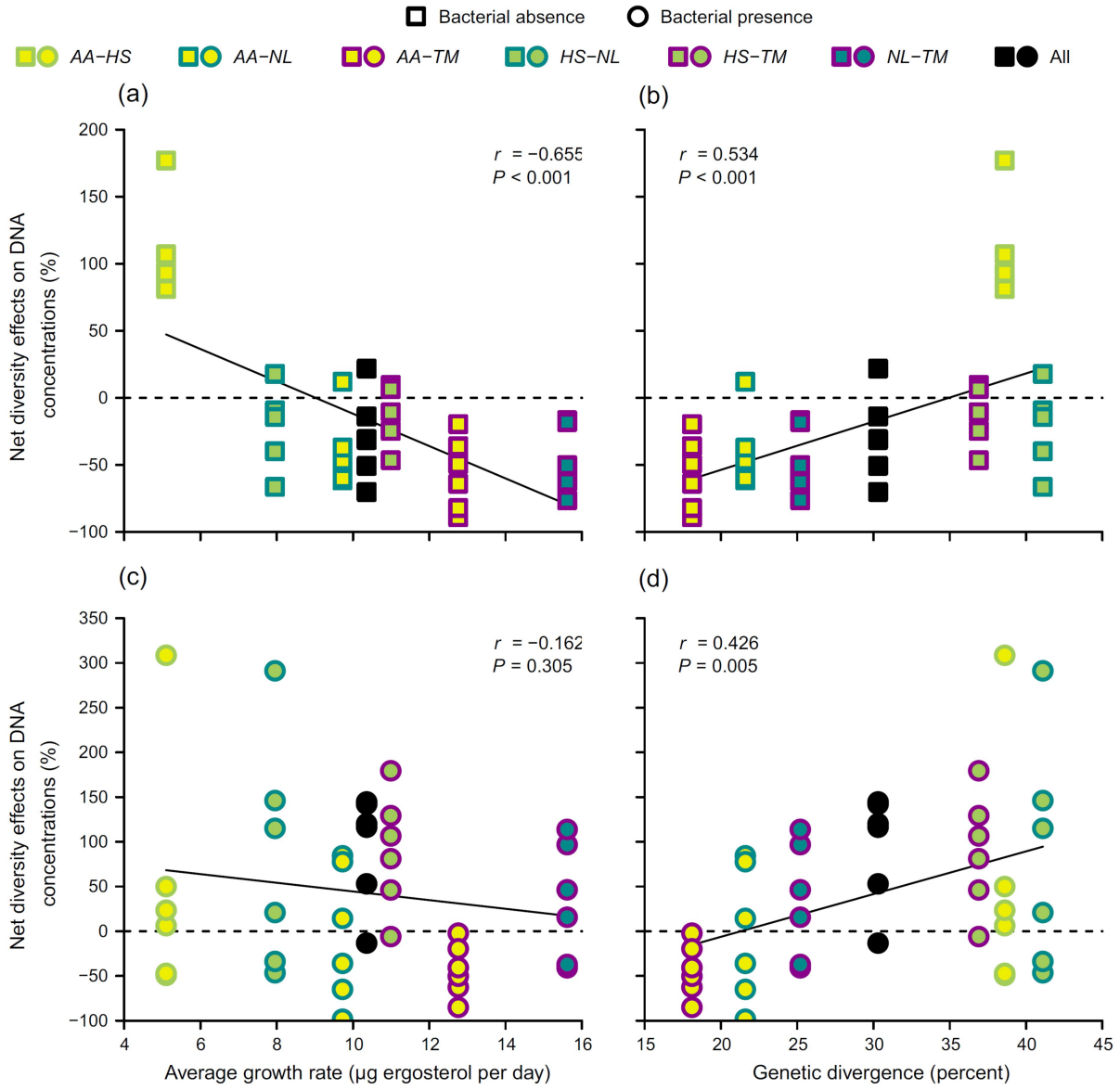



Fig. 6. Correlations between net diversity effects on DNA concentrations and average growth rates (a), (c) or genetic divergence (b), (d) of fungal mixed cultures in bacterial absence (a), (b) and presence (c), (d) with Pearson's correlation coefficients (*r*) and *P* values. The regression lines were added to visualize the relationships between the variables (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*).

## DISCUSSION

### FUNCTIONAL CHARACTERIZATION OF THE FUNGI AND BACTERIAL COMMUNITY

Although both fungi and bacteria were capable of decomposing leaf material and producing the investigated enzymes, fungal cultures were on average more productive (Fig. 1a; Appendix S2: Fig. S2). Our results indicate that both organism groups exploit similar resources (Fig. 3; Appendix S2: Fig. S2), potentially causing antagonistic (e.g., competitive) interactions, which are predominantly reported on submerged plant material (Gulis and Suberkropp 2003, Mille-Lindblom and Tranvik 2003, Mille-Lindblom et al. 2006, Romaní et al. 2006).

As observed earlier, fungi show substantial differences in leaf mass loss (Fig. 1a; e.g., Andrade et al. [2016], Bärlocher and Corkum [2003]), which are largely reflected by fungal biomass production (Fig. 1b; Appendix S2: Fig. S1). This suggests the species' growth rate as a major trait determining their OFP (Gessner et al. 2010, Aguilar-Trigueros et al. 2015). Furthermore, marked differences in enzyme activities among fungi (Fig. 2a; Appendix S2: Table S1) suggest different functional roles supporting synergistic (e.g., complementary) interactions. Although the leaf decomposition efficiency was nearly identical for *A. acuminata*, *N. lugdunensis*, and *T. marchalianum*, the different patterns in enzyme activities point to a qualitative difference among species and the occupation of different ecological niches (Fig. 2a). *Alatospora acuminata* and *T. marchalianum*, however, exhibit a very similar pattern in enzyme activities dominated by hydrolytic enzymes degrading phosphate esters, oligosaccharides, cellulose, and hemicellulose (Fig. 2a; Appendix S2: Fig. S2, Table S1). Consequently, both species are assumed to exploit similar resources (i.e., niche similarity), which is also reflected by their low genetic divergence and functional dissimilarity (Fig. 2, Table 1; Andrade et al. 2016) as well as their placement in the same taxonomic class (i.e., Leotiomycetes [Baschien et al. 2013]). Leaf decomposition by *N. lugdunensis*, in contrast, is based on a low and high activity of hydrolytic and oxidative enzymes, respectively (Fig. 2a; Appendix S2: Fig. S2, Table S1). The latter degrade lignin, the most recalcitrant plant polymer (Kaltschmitt et al. 2016), accounting for up to 18% of dry mass in black alder leaves (Lecerf and Chauvet 2008). However, contrary to our findings, oxidative enzymes are assumed to play a minor role in enzyme inventories of aquatic fungi colonizing leaves (Gessner et al. 2010). The functional traits of these three species indicate niche differentiation

and thus synergistic interactions among *N. lugdunensis* and *A. acuminata* or rather *T. marchalianum* (Fig. 2). Relative to the other three fungal species, the leaf decomposition efficiency of *H. stellata* was 10 times lower (Appendix S2: Table S1). This observation suggests that *H. stellata* primarily utilizes other energy resources, such as dissolved organic compounds, for growth instead of solid substrate (Fig. 1a). Moreover, *H. stellata* shows only pronounced activity of those enzymes that are involved in the degradation of less recalcitrant low-molecular-weight organic compounds (Fig. 2a; Appendix S2: Fig. S2, Table S1), such as phosphate esters or maltose, which are readily leached from leaves or released during advancing leaf senescence (Wildman and Parkinson 1981, Baldwin 1999, Duan et al. 2014). This functional differentiation of *H. stellata* from the other fungi (Appendix S2: Table S1) suggests niche differentiation, which is supported by a high genetic divergence and functional dissimilarity (Fig. 2b; Table 1).

All in all, these data highlight substantial differences in the enzymatic capacities among the fungal species, despite this being generally considered rare in aquatic fungi (Gessner et al. 2010). These findings are highly relevant for B-EF research but require further attention to understand transferability to other environmental conditions and a broader range of species.

## CHARACTERIZATION OF FUNGAL-FUNGAL INTERACTIONS

Fungal mixed cultures did not (or barely) surpass OFPs in terms of leaf mass loss, fungal biomass production, and enzyme activities of the most productive monocultures (i.e., *N. lugdunensis* and *T. marchalianum*; Fig. 1; Appendix S2: Fig. S2). This observation is in line with earlier B-EF studies on aquatic fungi (e.g., Bärlocher and Corkum [2003], Duarte et al. [2006], Andrade et al. [2016]). However, earlier studies were not able to quantify individual species' biomasses, making it difficult to unravel the underlying processes (complementarity vs. dominance or additivity vs. interactions). As a consequence of methodological developments (Baudy et al. 2019), this study demonstrates that interactions (i.e., NDEs) occur during leaf colonization and highly depend on species identity, whereas the OFP of communities is defined by the sum of individual species' performance (i.e., additivity) and thus by community composition.

Fungal leaf colonization, expressed as NDEs on DNA concentrations, was in mixed cultures largely negative and driven by negative trait-independent

complementarity effects (Fig. 5), indicating competition for similar resources (niche overlap) at mutual expense (Fox 2005). This result is in contrast to the synergistic interactions predicted from fungal traits (see Functional characterization of the fungi and the bacterial community; Fig. 2; Appendix S2: Table S1). Nonetheless, differences in NDEs on leaf colonization among the mixed cultures may be explained by the average fungal growth rate and genetic divergence of the communities, which are significantly negatively and positively correlated with these NDEs, respectively (Fig. 6a, b). The negative relationship between NDEs on leaf colonization and fungal growth rate may result from the exclusion of environmental constraints, as for instance imposed by competition with other microorganism groups or consumption by invertebrates (Bärlocher 1980, Mille-Lindblom and Tranvik 2003), allowing rapid fungal colonization of the leaf substrate. Accordingly, species with higher growth rates may rapidly colonize leaves up to their carrying capacity, thereby preventing or limiting colonization of species with lower growth rates (Fig. 4). Such interactions – referred to as inhibitory priority effects (Fukami 2015) – may, however, be the result of the experimental conditions (i.e., exclusion of environmental constraints), possibly masking facilitative interactions occurring under field conditions (cf. Gessner et al. 2010). The positive relationship between NDEs on leaf colonization and genetic divergence supports the findings of Andrade et al. (2016), suggesting a close link between genetic and functional diversity in aquatic fungal communities (for terrestrial communities, see also Eichlerová et al. [2015]). Accordingly, and although these NDEs were mainly negative (Fig. 5), antagonistic interactions were less pronounced between genetically and functionally distinct species (see previous subsection), being thus more likely to coexist on leaves.

OFPs in terms of leaf decomposition, fungal biomass production, and enzyme activities in mixed cultures could largely be predicted by individual species' B-SFPs (Fig. 1; Appendix S2: Fig. S2), indicating that the functioning of these communities is based on additivity. However, there are exceptions. In the binary culture *A. acuminata-H. stellata*, *A. acuminata* was the only species in this experiment, showing a higher biomass in mixed cultures than in monoculture (Fig. 4a). Yet, leaf decomposition was (close to significant) more than two times lower than predicted (Fig. 1a), and enzyme activities tended to be lower as well (Appendix S2: Fig. S2). This indicates a more efficient conversion of leaf substrate (but also dissolved organic compounds, as presumed for *H. stellata*) to fungal biomass by the joint action

of these species, which might be attributed to a complementary resource use supported by their distinct enzyme inventories. In contrast, in the quaternary species combination, leaf decomposition was significantly higher than predicted (Fig. 1a), whereas the observed enzyme activities largely met the predictions (Appendix S2: Fig. S2). This points to a more efficient decomposition of leaf substrate by the joint activity of a diverse enzymatic pool provided by the four species, which can also be interpreted as complementary interaction (cf. Pascoal et al. [2010]). As indicated by the relatively low ergosterol concentration (cf. *T. marchalianum* in monoculture; Fig. 1b), however, the excess of decomposition products was seemingly not converted into fungal biomass, but may possibly have been utilized to meet increased energy demands for physiological responses involved in competitive interactions among fungi (Chan et al. 2019). Such energetically costly responses include, for instance, an enhanced nutrient uptake and metabolism or protein stabilization and recycling (Ujor et al. 2018).

CHARACTERIZATION OF FUNGAL-BACTERIAL INTERACTIONS

As hypothesized, compared to bacteria-free cultures, bacterial presence largely resulted in lower leaf decomposition and an inhibition of fungal growth (Fig. 1; Gulis and Suberkropp 2003), which may be the result of competition (Mille-Lindblom et al. 2006). The magnitude of this effect, however, highly depends on the number and identity of fungal species (Appendix S2: Table S3). Compared to bacteria-free cultures, oxidase activities were lower in the presence of bacteria (Fig. 3); although against our hypothesis, hydrolase activities were by tendency increased (Fig. 3; Appendix S2: Fig. S2). Thus, the lower leaf decomposition observed in the presence of bacteria may be based on lower oxidase activities. This indicates that ligninolytic capacities could play a greater role in leaf decomposition by aquatic fungi than previously assumed (cf. Gessner et al. [2010], Bärlocher and Boddy [2016]). These lower (overpredicted) oxidase activities in presence of bacteria (Appendix S2: Fig. S2) may possibly be explained by a release of bacterial metabolites functioning as oxidase inhibitors (Fernandes and Kerkar 2017). As bacterial presence affected biomass production of rapidly growing fungi to a lesser extent (i.e., *N. lugdunensis* and *T. marchalianum*; Fig. 1b), the growth rate appears to be a crucial trait determining the resistance of aquatic fungi in competition with bacteria (but see Mille-Lindblom and Tranvik [2003], Mille-Lindblom et al. [2006]). Accordingly, priority

effects may also play an important role in the interplay between bacteria and fungi during leaf colonization.

Although bacterial presence generally seemed to inhibit fungal OFPs, NDEs on leaf colonization in fungal mixed cultures shifted from negative – as observed in bacteria-free mixed cultures – to positive (Fig. 5). These positive NDEs are mainly driven by positive trait-independent complementarity effects (Fig. 5), suggesting the prevalence of synergistic interactions between fungi (Fox 2005), which may have been induced by the presence of bacteria. Bacterial inhibition of fungal growth may alleviate inhibitory priority effects among fungi during colonization. This enables fungal species with lower growth rates (i.e., *A. acuminata* and *H. stellata*) to colonize the substrate under lower competitive pressure exerted by species with higher growth rates (i.e., *N. lugdunensis* and *T. marchalianum*; Figs. 4 and 5). Accordingly, bacteria may have induced new fungal diversity-based feedback loops that promote coexistence and synergistic interactions as predicted from fungal traits (see Functional characterization of the fungi and the bacterial community). In support of this statement, fungal growth rates, which explained interactions in bacteria-free cultures, were in presence of bacteria no longer explanatory for differences in NDEs on leaf colonization (Fig. 6c). In contrast, the differences in these NDEs were in presence of bacteria still positively correlated with genetic divergence in fungal mixed cultures (Fig. 6d), which provides further evidence for the hypothesized link between genetic and functional diversity in aquatic fungal communities (Fig. 2; Andrade et al. 2016), also under more environmentally realistic conditions.

In the presence of bacteria, OFPs in fungal monocultures and mixed cultures were again largely determined by the sum of contributing organisms' B-SFPs (i.e., by community composition; Fig. 1; Appendix S2: Fig. S2). However, in a few instances, observations clearly deviated from predictions, mainly concerning leaf decomposition in culture combinations comprising fungal monocultures and bacteria (Fig. 1a). In the presence of bacteria, leaf decomposition in the *A. acuminata* and *T. marchalianum* cultures was substantially higher than predicted, which again points to an increased allocation of energy to physiological processes involved in competitive interactions by fungi and bacteria. In contrast, co-occurrence of bacteria and *N. lugdunensis* – the model fungus most resistant to bacteria (Fig. 1b) – resulted in a more than two times lower leaf decomposition than predicted (Fig. 1a). This indicates synergistic

interactions between the fungus and bacteria, which may be expressed by a higher efficiency in the assimilation of leaf substrate by *N. lugdunensis*.

## CONCLUSION

The application of novel qPCR assays uncovered that fungal-fungal interactions are determined by functional and genetic divergence. Specifically, niche complementarity does not necessarily lead to positive biodiversity effects on substrate colonization or functioning within fungal communities. The presence of bacteria likely resulted in new fungal diversity-based feedback loops. These eventually promote fungal coexistence and complementary functioning, irrespective of the apparent antagonistic relationship between bacteria and fungi. Although fungi are considered as suitable model organisms to address key questions in community ecology (Zubrod et al. 2019), our study highlights the necessity to conduct future B-EF microcosm studies on fungi not under exclusion of naturally co-occurring key organism groups within the microbial compartment of interest. Otherwise, study outcomes might likely not reflect true ecological relationships as they occur in the environment and ultimately lead to misguided strategies for the conservation of biodiversity and ecosystem processes.

Although in this experiment the culture with the highest diversity (i.e., quaternary fungal species combination in presence of bacteria) was outperformed by a range of cultures with a lower diversity, species-rich communities are assumed to bear a higher capacity to buffer effects of environmental change on microbial functioning than species-poor communities do (Fernandes et al. 2011, Gonçalves et al. 2015). In the light of climate change and biodiversity loss, however, this buffering capacity might be substantially reduced, which poses a risk to the integrity of aquatic ecosystems. To be able to conserve these systems, a mechanistic understanding of relationships between biodiversity, resilience, and ecosystem functions in microbial decomposer communities facing anthropogenic stress is essential and should be pursued in future studies.

## ACKNOWLEDGEMENTS

We thank the two anonymous reviewers for valuable comments and suggestions. Furthermore, we thank Therese Bürgi, Lisa Friedrichs, Thu Huyen Nguyen, Sebastian Pietz, and Britta Wahl-Ermel for assistance in the laboratory. This

study was funded by the German Research Foundation (DFG): Project AQUA-REG (DFG; SCHU 2271/14-1) and BIO2FUN (DFG; BU 2439/5-1). The authors declare no conflict of interest.

## LITERATURE CITED

Aguilar-Trigueros, C. A., et al. 2015. Branching out: Towards a trait-based understanding of fungal ecology. Fungal Biology Reviews 29:34–41.

Andrade, R., C. Pascoal, and F. Cássio. 2016. Effects of inter and intraspecific diversity and genetic divergence of aquatic fungal communities on leaf litter decomposition – a microcosm experiment. FEMS Microbiology Ecology 92: fiw102.

Baldwin, D. S. 1999. Dissolved organic matter and phosphorus leached from fresh and 'terrestrially' aged river red gum leaves: implications for assessing river-floodplain interactions. Freshwater Biology 4:675–685.

Bärlocher, F. 1980. Leaf-eating invertebrates as competitors of aquatic hyphomycetes. Oecologia 47:303–306.

Bärlocher, F. 2016. Aquatic hyphomycetes in a changing environment. Fungal Ecology 19:14–27.

Bärlocher, F., and L. Boddy. 2016. Aquatic fungal ecology – How does it differ from terrestrial? Fungal Ecology 19:5–13.

Bärlocher, F., and M. Corkum. 2003. Nutrient enrichment overwhelms diversity effects in leaf decomposition by stream fungi. Oikos 101:247–252.

Baschien, C., G. Rode, U. Bockelmann, P. Gotz, and U. Szewzyk. 2009. Interactions between hyphosphere-associated bacteria and the fungus *Cladosporium herbarum* on aquatic leaf litter. Microbial Ecology 58:642–650.

Baschien, C., C.-K.-M. Tsui, V. Gulis, U. Szewzyk, and L. Marvanová. 2013. The molecular phylogeny of aquatic hyphomycetes with affinity to the Leotiomycetes. Fungal Biology 117:660–672.

Baudy, P., J. P. Zubrod, N. Röder, C. Baschien, A. Feckler, R. Schulz, and M. Bundschuh. 2019. A glance into the black box: Novel species-specific quantitative real-time PCR assays to disentangle aquatic hyphomycete community composition. Fungal Ecology 42:100858.

Bell, T., M. O. Gessner, R. I. Griffiths, J. R. McLaren, P. J. Morin, M. van der Heijden, and W. H. van der Putten. 2009. Microbial biodiversity and ecosystem

functioning under controlled conditions and in the wild. Pages 121–133 in S. Naeem, D. E. Bunker, A. Hector,M. Loreau, and C. Perrings, editors. Biodiversity, ecosystem functioning, and human wellbeing: an ecological and economic perspective. Oxford University Press, Oxford, UK.

Buesing, N. 2005. Bacterial counts and biomass determination by epifluorescence microscopy. Pages 203–208 in M. A. S. Graça, F. Bärlocher, and M. O. Gessner, editors. Methods to study litter decomposition: a practical guide. Springer-Verlag, Dordrecht, the Netherlands.

Canhoto, C., A. L. Gonçalves, and F. Bärlocher. 2016. Biology and ecological functions of aquatic hyphomycetes in a warming climate. Fungal Ecology 19:201–218.

Chan, J. Y., S. P. Bonser, J. R. Powell, and W. K. Cornwell. 2019. When to cut your losses: Dispersal allocation in an asexual filamentous fungus in response to competition. Ecology and Evolution 9:4129–4137.

Clarke, K. R. 1993. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 18:117–143.

Conover, W. J., and R. L. Iman. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. American Statistician 35:124–129.

Copolovici, L., A. Kännaste, T. Remmel, and Ü. Niinemets. 2014. Volatile organic compound emissions from *Alnus glutinosa* under interacting drought and herbivory stresses. Environmental and Experimental Botany 100:55–63.

Dang, C. K., E. Chauvet, and M. O. Gessner. 2005. Magnitude and variability of process rates in fungal diversity–litter decomposition relationships. Ecology Letters 8:1129–1137.

Danger, M., J. Cornut, E. Chauvet, P. Chavez, A. Elger, and A. Lecerf. 2013. Benthic algae stimulate leaf litter decomposition in detritus-based headwater streams. A case of aquatic priming effect? Ecology 94:1604–1613.

De Laender, F., et al. 2016. Reintroducing environmental change drivers in biodiversity–ecosystem functioning research. Trends in Ecology and Evolution 31:905–915.

DeForest, J. L. 2009. The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and l-DOPA. Soil Biology and Biochemistry 41:1180–1186.

Deveau, A., et al. 2018. Bacterial–fungal interactions: ecology, mechanisms and challenges. FEMS Microbiology Reviews 42:335–352.

Dighton, J., and J. F. White, editors. 2017. The fungal community: Its organization and role in the ecosystem. CRC Press, Boca Raton, Florida, USA.

Duan, S., K. Delaney-Newcomb, S. S. Kaushal, S. E. G. Findlay, and K. T. Belt. 2014. Potential effects of leaf litter on water quality in urban watersheds. Biogeochemistry 121:61–80.

Duarte, S., C. Pascoal, F. Cássio, and F. Bärlocher. 2006. Aquatic hyphomycete diversity and identity affect leaf litter decomposition in microcosms. Oecologia 147:658–666.

Eichlerová, I., L. Homolka, L. ˇ Žifčáková, L. Lisá, P. Dobiášová, and P. Baldrian. 2015. Enzymatic systems involved in decomposition reflects the ecology and taxonomy of saprotrophic fungi. Fungal Ecology 13:10–22.

Evans, C. S., and J. N. Hedger. 2001. Degradation of plant cell wall polymers. Pages 1–26 In G. M. Gadd, editor. Fungi in bioremediation. Cambridge University Press. Cambridge, UK.

Fernandes, I., C. Pascoal, and F. Cássio. 2011. Intraspecific traits change biodiversity effects on ecosystem functioning under metal stress. Oecologia 166:1019–1028.

Fernandes, M. S., and S. Kerkar. 2017. Microorganisms as a source of tyrosinase inhibitors: a review. Annals of Microbiology 67:343–358.

Fernández, D., M. Tummala, V. C. Schreiner, S. Duarte, C. Pascoal, C. Winkelmann, D. Mewes, K. Muñoz, and R. B. Schäfer. 2016. Does nutrient enrichment compensate fungicide effects on litter decomposition and decomposer communities in streams? Aquatic Toxicology 174:169–178.

Ferreira, V., and E. Chauvet. 2012. Changes in dominance among species in aquatic hyphomycete assemblages do not affect litter decomposition rates. Aquatic Microbial Ecology 66:1–11.

Findlay, S., et al. 2002. A cross-system comparison of bacterial and fungal biomass in detritus pools of headwater streams. Microbial Ecology 43:55–66.

Fox, J. W. 2005. Interpreting the ‘selection effect’ of biodiversity on ecosystem function. Ecology Letters 8:846–856.

Fukami, T. 2015. Historical contingency in community assembly: integrating niches, species pools, and priority effects. Annual Review of Ecology, Evolution, and Systematics 46:1–23.

German, D. P., M. N. Weintraub, A. S. Grandy, C. L. Lauber, Z. L. Rinkes, and S. D. Allison. 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biology and Biochemistry 43:1387–1397.

Gessner, M. O. 2005. Ergosterol as a measure of fungal biomass. Pages 189–195 in M. A. S. Graça, F. B¨arlocher, and M. O. Gessner, editors. Methods to study litter decomposition: a practical guide. Springer-Verlag, Dordrecht, the Netherlands.

Gessner, M. O., C. M. Swan, C. K. Dang, B. G. McKie, R. D. Bardgett, D. H. Wall, and S. H¨attenschwiler. 2010. Diversity meets decomposition. Trends in Ecology and Evolution 25:372–380.

Gonçalves, A. L., M. A. S. Graça, and C. Canhoto. 2015. Is diversity a buffer against environmental temperature fluctuations? – A decomposition experiment with aquatic fungi. Fungal Ecology 17:96–102.

Grossart, H.-P., and K. Rojas-Jimenez. 2016. Aquatic fungi: targeting the forgotten in microbial ecology. Current Opinion in Microbiology 31:140–145.

Gulis, V., and K. Suberkropp. 2003. Interactions between stream fungi and bacteria associated with decomposing leaf litter at different levels of nutrient availability. Aquatic Microbial Ecology 30:149–157.

Hieber, M., and M. O. Gessner. 2002. Contribution of stream detrivores, fungi, and bacteria to leaf breakdown based on biomass estimates. Ecology 83:1026–1038.

Hinkle, D. E., W. Wiersma, and S. G. Jurs. 2003. Applied Statistics for the Behavioral Sciences. Houghton Mifflin, Boston, Massachusetts, USA.

Kaltschmitt, M., H. Hartmann, and H. Hofbauer, editors. 2016. Energie aus Biomasse. Grundlagen, Techniken und Verfahren. Springer, Berlin and Heidelberg, Germany.

Krauss, G.-J., M. Solé, G. Krauss, D. Schlosser, D. Wesenberg, and F. Bärlocher. 2011. Fungi in freshwaters: ecology, physiology and biochemical potential. FEMS Microbiology Reviews 35:620–651.

Kubicek, C. P., and I. S. Druzhinina, editors. 2007. Microbial and environmental relationships. Springer, Berlin, Germany.

Kumar, S., G. Stecher, M. Li, C. Knyaz, and K. Tamura. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35:1547–1549.

Lambirth, K., et al. 2018. Microbial community composition and antibiotic resistance genes within a North Carolina urban water system.Water 10:1539.

Lecerf, A., and E. Chauvet. 2008. Intraspecific variability in leaf traits strongly affects alder leaf decomposition in a stream. Basic and Applied Ecology 9:598–605.

Leflaive, J., M. Danger, G. Lacroix, E. Lyautey, C. Oumarou, and L. Ten-Hage. 2008. Nutrient effects on the genetic and functional diversity of aquatic bacterial communities. FEMS Microbiology Ecology 66:379–390.

Loreau, M., and A. Hector. 2001. Partitioning selection and complementarity in biodiversity experiments. Nature 412:72–76.

Manerkar, M. A., S. Seena, and F. Bärlocher. 2008. Q-RT-PCR for assessing archaea, bacteria, and fungi during leaf decomposition in a stream. Microbial Ecology 56:467–473.

Mille-Lindblom, C., H. Fischer, and L. J. Tranvik. 2006. Antagonism between bacteria and fungi: substrate competition and a possible tradeoff between fungal growth and tolerance towards bacteria. Oikos 113:233–242.

Mille-Lindblom, C., and L. J. Tranvik. 2003. Antagonism between bacteria and fungi on decomposing aquatic plant litter. Microbial Ecology 45:173–182.

Morris, R. M., and B. L. Nunn. 2013. Sample preparation and processing for planktonic microbial community proteomics. Methods in Enzymology 531:271–287.

Pascoal, C., F. Cássio, L. Nikolcheva, and F. Bärlocher. 2010. Realized fungal diversity increases functional stability of leaf litter decomposition under zinc stress. Microbial Ecology 59:84–93.

Petersen, R. C., and K. W. Cummins. 1974. Leaf processing in a woodland stream. Freshwater Biology 4:343–368.

R Development Core Team. 2018. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. www.r-project.org

Romaní, A. M., H. Fischer, C. Mille-Lindblom, and L. J. Tranvik. 2006. Interactions of bacteria and fungi on decomposing litter: differential extracellular enzyme activities. Ecology 87:2559–2569.

Schoch, C. l., et al. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America 109:6241–6246.

Seaman, J. W., S. C. Walls, S. E. Wise, and R. G. Jaeger. 1994. Caveat emptor: rank transform methods and interaction. Trends in Ecology and Evolution 9:261–263.

Solé, M., I. Fetzer, R. Wennrich, K. R. Sridhar, H. Harms, and G. Krauss. 2008. Aquatic hyphomycete communities as potential bioindicators for assessing anthropogenic stress. Science of the Total Environment 389:557–565.

Ujor, V. C., E. C. Adukwu, and C. C. Okonkwo. 2018. Fungal wars: The underlying molecular repertoires of combating mycelia. Fungal Biology 122:191–202.

Uyaguari-Diaz, M. I., et al. 2016. A comprehensive method for amplicon-based and metagenomic characterization of viruses, bacteria, and eukaryotes in freshwater samples. Microbiome 4:20.

Wildman, H. G., and D. Parkinson. 1981. Seasonal changes in water-soluble carbohydrates of *Populus tremuloides* leaves. Canadian Journal of Botany 59:862–869.

Wright, A. J., D. A. Wardle, R. Callaway, and A. Gaxiola. 2017. The overlooked role of facilitation in biodiversity experiments. Trends in Ecology and Evolution 32:383–390.

Zubrod, J. P., et al. 2019. Fungicides: an overlooked pesticide class? Environmental Science & Technology 53:3347–3365.

Zubrod, J. P., M. Bundschuh, A. Feckler, D. Englert, and R. Schulz. 2011. Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. Environmental Toxicology and Chemistry 30:2718–2724.

**SUPPLEMANTARY DATA**

APPENDIX S1

**Setup of enzyme assays**

Procedures for the analysis of fluorometric (hydrolase) and colorimetric assays (oxidase) as well as calculations of enzyme activities followed DeForest (2009) with minor modifications. As reaction buffer autoclaved nutrient medium (containing morpholino propane sulfonic acid as buffer; Dang et al. 2005), adjusted to pH 7, was used. Stock solutions of the free 4-methylumbelliferone reference standard (MUB), 4-methylumbelliferyl-linked substrates (MUF), and L-3,4-dihydroxyphenylalanine (L-DOPA) were prepared with deionized water, using 2-ethoxyethanol as additional solvent (0-10%; Table S1). MUF and L-DOPA were used as 5-mM and 25-mM solutions, ensuring substrate saturation throughout the incubation period (as determined via saturation curves). After thawing, single leaf disks were homogenized in 350 mL of nutrient medium at 24,000 rpm for 30 s, using an Ultra-turrax® blender (IKA®-Werke GmbH & Co. KG, Germany). The homogenate was stirred for the duration of the analysis (for ~2 h). To normalize enzyme activity to leaf dry weight, the remaining homogenate of each sample (i.e., ~340 mL) was filtered through pre-weighed glass fiber filters (GF/6; Whatman GmbH, Germany). After drying at 60°C for 24 h, the filters were weighed to the nearest 0.01 mg, whereupon the sample dry weight was determined as the difference of both weighings. Incubation and analysis was performed in black and clear flat-bottom 96-well 300-µL well plates (Thermo Fisher Scientific, USA) for hydrolase and oxidase assays, respectively. Dispensing of reaction components followed a strict sequence (Table S2, S3) and location on the 96-well plates (Fig. S1a, b). Hydrolase and oxidase assays were covered with a lid and incubated on a rotary shaker (model KS 15; Edmund Bühler GmbH, Germany) at 120 rpm in darkness for approximately 1 h and 2 h, respectively, while the exact time of assay initiation and termination was recorded to the nearest minute. Fluorescence and absorbance was measured using a microplate reader (Infinite 200, Tecan Group; Switzerland).

Hydrolase assays were initiated by the addition of MUF solution (Table S2). After the incubation period reactions were terminated by adding 10 µL of 1-M NaOH solution to each well. Afterwards, fluorescence was measured at 365 nm excitation and 450 nm emission and reported as the average of two reads. Fluorescence levels of technical replicates of the same reaction groups (Table S2; Fig. S1a) were

averaged, blank-corrected, and used for hydrolase activity calculations. Hydrolase activities were expressed as µmol/(h*g) and calculated as per DeForest (2009):

$$\text{Hydrolase activity } (\mu\text{mol}/(\text{h} * \text{g})) = \frac{\text{Net fluorescence} * 350 \text{ mL}}{\text{Emission coefficient} * 0.2 \text{ mL} * \text{Incubation time (h)} * \text{Sample dry weight (g)}} \quad \text{(Eq. S1)}$$

where

$$\text{Net fluorescence} = \left(\frac{\text{Sample assay} - \text{Sampl control}}{\text{Quench coefficient}}\right) - \text{Negative control} \quad \text{(Eq. S2)}$$

$$\text{Emission coefficient (fluoresence}/\mu\text{mol)} = \left(\frac{\text{Reference standard}}{5 * 10^{-4}\ \mu\text{mol}}\right) \quad \text{(Eq. S3)}$$

$$\text{Quench coefficient} = \frac{(\text{Quench standard} - \text{Sam control})}{\text{Reference standard}} \quad \text{(Eq. S4)}$$

The 350 mL and 0.2 mL refer to the total and the dispensed (per well) volume of sample homogenate, respectively. Incubation time (h) is the exact time, from assay initiation to termination. Sample dry weight (g) refers to the weight of the filtered sample homogenate and $5*10^{-4}$ µmol refers to the amount of free MUB in reference standard reactions.

Oxidase assays were initiated by the addition of L-DOPA (phenol oxidase) or hydrogen peroxide (peroxidase; Table S3). Directly after the incubation period, absorbance was measured at 450 nm and reported as the average of two reads. Absorbance levels of technical replicates of the same reaction groups (Table S3; Fig. S1b) were averaged, blank-corrected, and used for oxidase activity calculations. Oxidase activities were expressed as µmol/(h*g) and calculated as per DeForest (2009):

$$\text{Oxidase activity } (\mu\text{mol}/(\text{h} * \text{g})) = \frac{\text{Final absorbance} * 350 \text{ mL}}{(4.3\ \mu\text{mol}^{-1}) * 0.2 \text{ mL} * \text{Incubation time (h)} * \text{Sample dry weight (g)}} \quad \text{(Eq. S5)}$$

where

$$\text{Final absorbance} = \text{Sample assay} - \text{Negative control} - \text{Sample control} \quad \text{(Eq. S6)}$$

The extinction coefficient of 4.3 $\mu mol^{-1}$ was obtained from the standard absorbance curve of oxidized L-DOPA (Fig. S2).

Table S1. Artificial substrates used to estimate activities of hydrolytic and oxidative enzymes.

| Substrate | Manufacturer | Addition 2-ethoxyethanol to the stock solution (v/v) |
|---|---|---|
| 4-methylumbelliferone | Sigma-Aldrich, Germany | 10% |
| 4-methylumbelliferyl-α-D-glucopyranoside | Iris Biotech GmbH, Germany | 10% |
| 4-methylumbelliferyl-β-D-cellobioside | Iris Biotech GmbH, Germany | 0% |
| 4-methylumbelliferyl-β-D-glucopyranoside | Iris Biotech GmbH, Germany | 5% |
| 4-methylumbelliferyl-β-D-xylopyranoside | Acros Organics, Belgium | 10% |
| 4-methylumbelliferyl-phosphate | Alfa Aesar Co., Inc., USA | 5% |
| L-3,4-dihydroxyphenylalanine | Cayman Chemical Company, USA | 0% |

Table S2. Composition of reactions and dispensing sequence (number in brackets) in hydrolase assays.

| | | Volume (µL) | | | |
|---|---|---|---|---|---|
| Reaction group | Technical replicates | Reaction buffer (1) | Sample homogenate (2) | MUB solution (3) | MUF solution (4) |
| Blank | 4 | 250 | | | |
| Negative control | 6 | 200 | | | 50 |
| Reference standard | 6 | 200 | | 50 | |
| Quench control | 3 | | 200 | 50 | |
| Sample control | 2 | 50 | 200 | | |
| Sample assay | 3 | | 200 | | 50 |

Table S3. Composition of reactions and dispensing sequence (number in brackets) in oxidase assays.

| | | Volume (µL) | | | |
|---|---|---|---|---|---|
| Reaction group | Technical replicates | Reaction buffer (1) | Sample homogenate (2) | L-DOPA solution (3) | 0.3% hydrogen peroxide solution (only peroxidase assays) (4) |
| Blank | 4 | 250 | | | 10 |
| Negative control | 12 | 200 | | 50 | 10 |
| Sample control | 2 | 50 | 200 | | 10 |
| Sample assay | 3 | | 200 | 50 | 10 |

**a**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | BL | BL | SC 1 | SC 2 | SC 3 | SC 4 | SC 5 | SC 6 | SC 7 | SC 8 | SC 9 | SC 10 |
| B | BL | BL | SC 1 | SC 2 | SC 3 | SC 4 | SC 5 | SC 6 | SC 7 | SC 8 | SC 9 | SC 10 |
| C | RF | RF | QC 1 | QC 2 | QC 3 | QC 4 | QC 5 | QC 6 | QC 7 | QC 8 | QC 9 | QC 10 |
| D | RF | RF | QC 1 | QC 2 | QC 3 | QC 4 | QC 5 | QC 6 | QC 7 | QC 8 | QC 9 | QC 10 |
| E | RF | RF | QC 1 | QC 2 | QC 3 | QC 4 | QC 5 | QC 6 | QC 7 | QC 8 | QC 9 | QC 10 |
| F | NC | NC | SA 1 | SA 2 | SA 3 | SA 4 | SA 5 | SA 6 | SA 7 | SA 8 | SA 9 | SA 10 |
| G | NC | NC | SA 1 | SA 2 | SA 3 | SA 4 | SA 5 | SA 6 | SA 7 | SA 8 | SA 9 | SA 10 |
| H | NC | NC | SA 1 | SA 2 | SA 3 | SA 4 | SA 5 | SA 6 | SA 7 | SA 8 | SA 9 | SA 10 |

**b**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | BL | BL | SC 1 | SC 2 | SC 3 | SC 4 | SC 5 | SC 6 | SC 7 | SC 8 | SC 9 | SC 10 |
| B | BL | BL | SC 1 | SC 2 | SC 3 | SC 4 | SC 5 | SC 6 | SC 7 | SC 8 | SC 9 | SC 10 |
| C | NC | NC | SA 1 | SA 2 | SA 3 | SA 4 | SA 5 | SA 6 | SA 7 | SA 8 | SA 9 | SA 10 |
| D | NC | NC | SA 1 | SA 2 | SA 3 | SA 4 | SA 5 | SA 6 | SA 7 | SA 8 | SA 9 | SA 10 |
| E | NC | NC | SA 1 | SA 2 | SA 3 | SA 4 | SA 5 | SA 6 | SA 7 | SA 8 | SA 9 | SA 10 |
| F | NC | NC | SA 1 | SA 2 | SA 3 | SA 4 | SA 5 | SA 6 | SA 7 | SA 8 | SA 9 | SA 10 |
| G | NC | NC | SA 1 | SA 2 | SA 3 | SA 4 | SA 5 | SA 6 | SA 7 | SA 8 | SA 9 | SA 10 |
| H | NC | NC | SA 1 | SA 2 | SA 3 | SA 4 | SA 5 | SA 6 | SA 7 | SA 8 | SA 9 | SA 10 |

Fig. S1. Layout of 96-well plates for (a) hydrolase and (b) oxidase assays with locations of blanks (BL), reference standards (RF), negative controls (NC), sample controls (SC), quench controls (QC), and sample assays (SA). The numbers indicate reactions associated to the ten samples that can be simultaneously analyzed on a single 96-well plate. Reactions with the same designation are technical replicates.

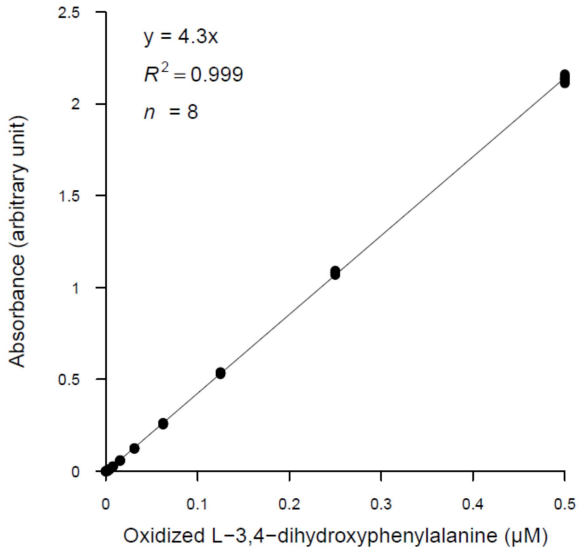



Fig. S2. Standard absorbance curve (solid line) of oxidized L-DOPA amounts ranging from $0.5*2^{-8}$ µM to 0.5 µM per well (250-µL reactions; *n*=8). Absorbance was blank-corrected in all reactions. The analyzed dilutions were prepared from a 2.5-mM L-DOPA stock solution, after it was oxidized by purified mushroom tyrosinase until increases in absorbance leveled off (i.e., after ~5 h).

**Literature Cited**

Dang, C. K., E. Chauvet, and M. O. Gessner. 2005. Magnitude and variability of process rates in fungal diversity-litter decomposition relationships. Ecology Letters 8:1129–1137.

DeForest, J. L. 2009. The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and l-DOPA. Soil Biology and Biochemistry 41:1180–1186.

APPENDIX S2

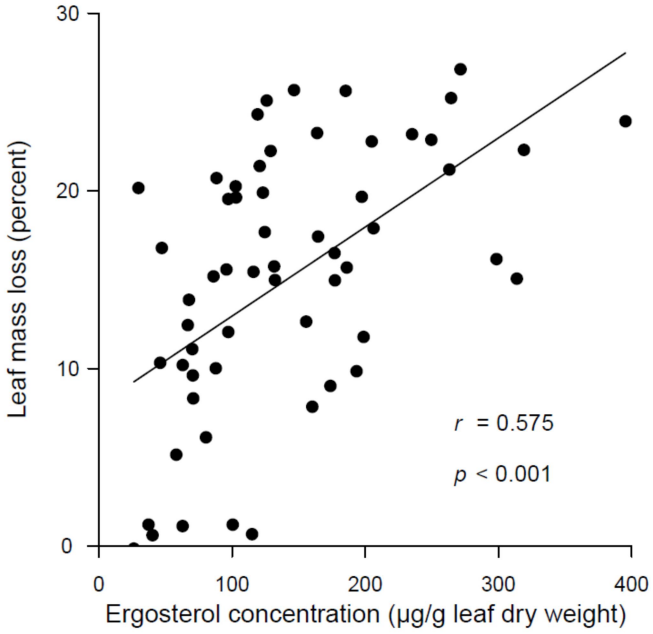



Fig. S1. Correlation between ergosterol concentrations and leaf mass loss in bacteria-free fungal monocultures and mixed cultures. Spearman's rank correlation coefficient (*r*) and *p*-value indicate a moderate statistically significant correlation. The regression line was added to visualize the positive relationship between the variables.

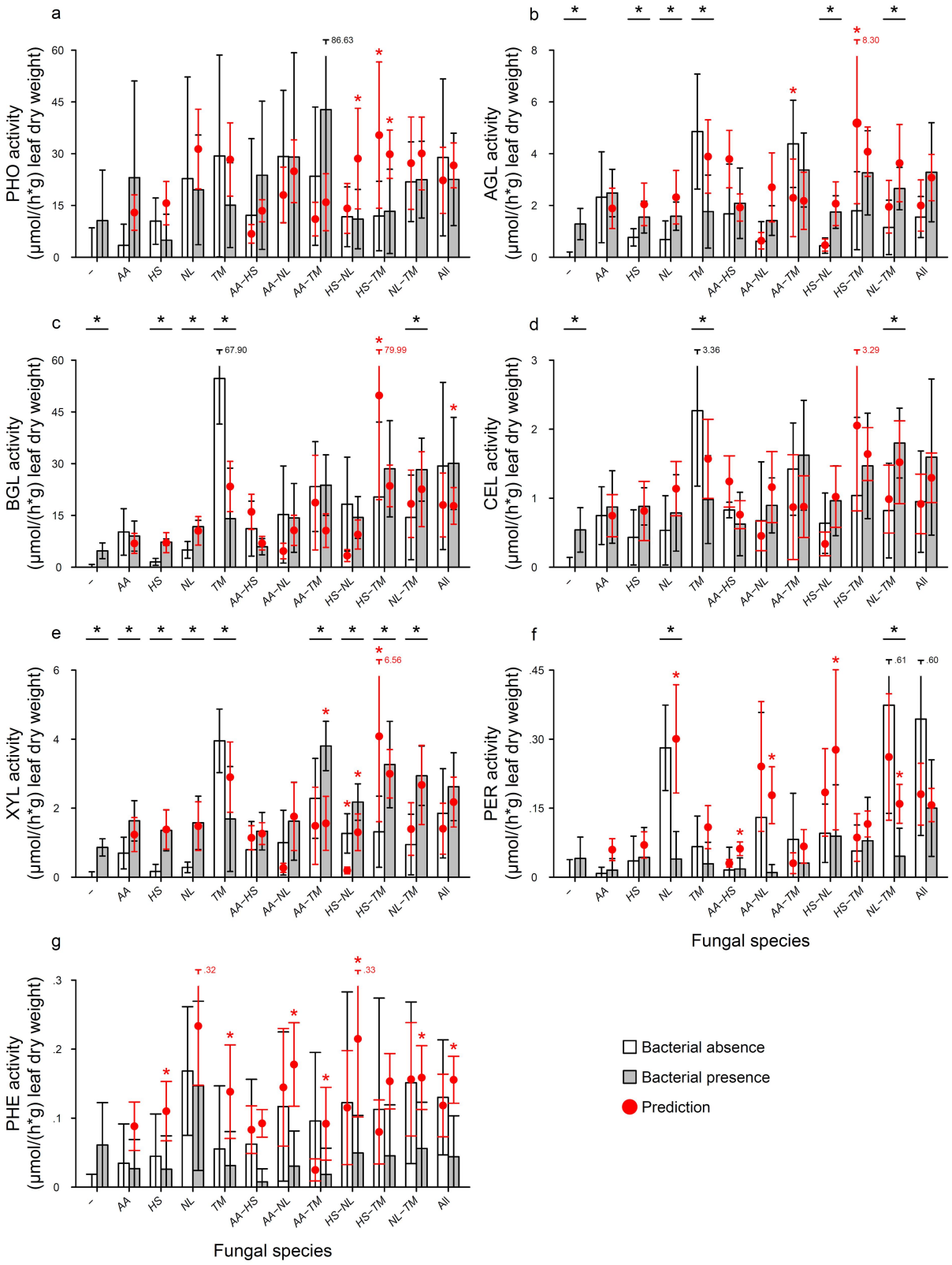



Fig. S2. For caption, see next page.

Fig. S2. Mean enzyme activities (in µmol/(h*g) leaf dry weight; with 95%-CIs; *n*=6) in fungal cultures in absence (white bars) and presence of bacteria (grey bars) for (a) PHO, (b) AGL, (c) BGL, (d) CEL, (e) XYL, (f) PER, and (g) PHE. Red points indicate predictions based on fungal and bacterial biomass-specific enzyme activities. Red asterisks indicate significant differences between observed and predicted enzyme activities of fungal treatments containing more than one organism (i.e., presence of bacteria or further fungi). Black asterisks indicate significant differences in observed enzyme activities between bacteria-free and bacteria-containing cultures (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*; PHO, phosphatase; AGL, α-1,4-glucosidase; BGL, β-1,4-glucosidase; CEL, cellobiohydrolase; XYL, β-1,4-xylosidase; PER, peroxidase; PHE, phenol oxidase).

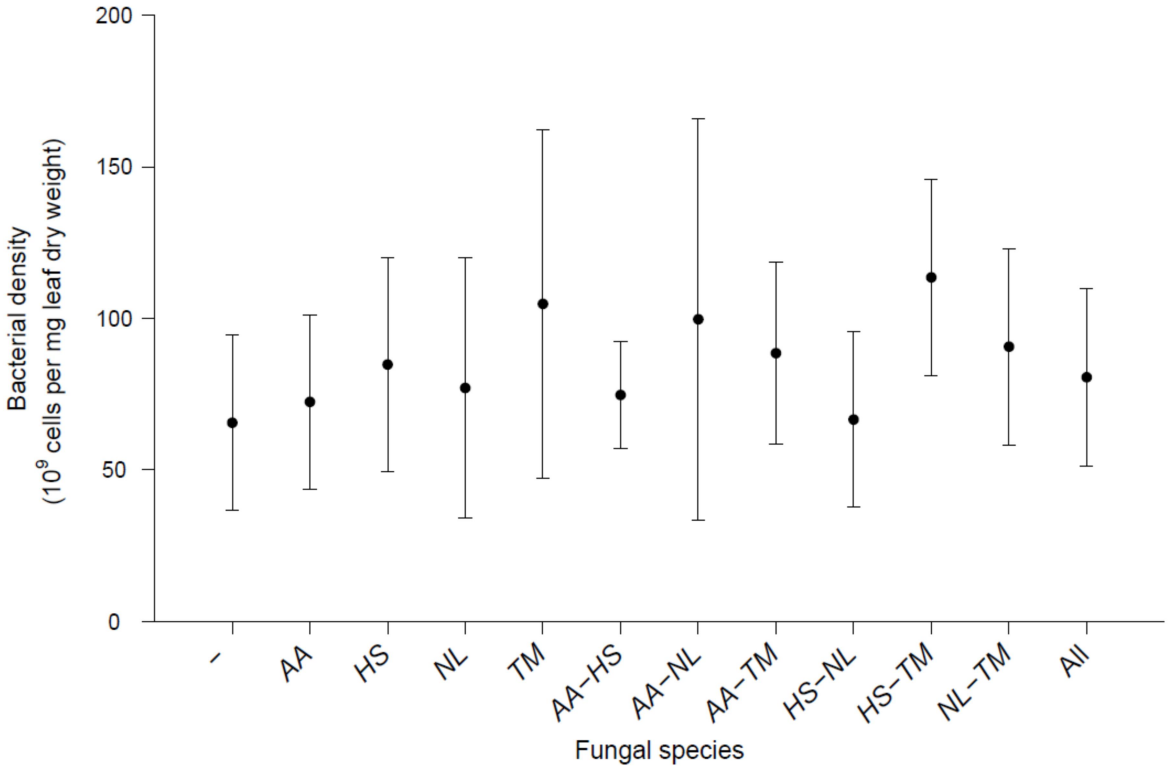



Fig. S3. Mean bacterial densities (with 95%-CIs) in bacteria-containing cultures (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*).

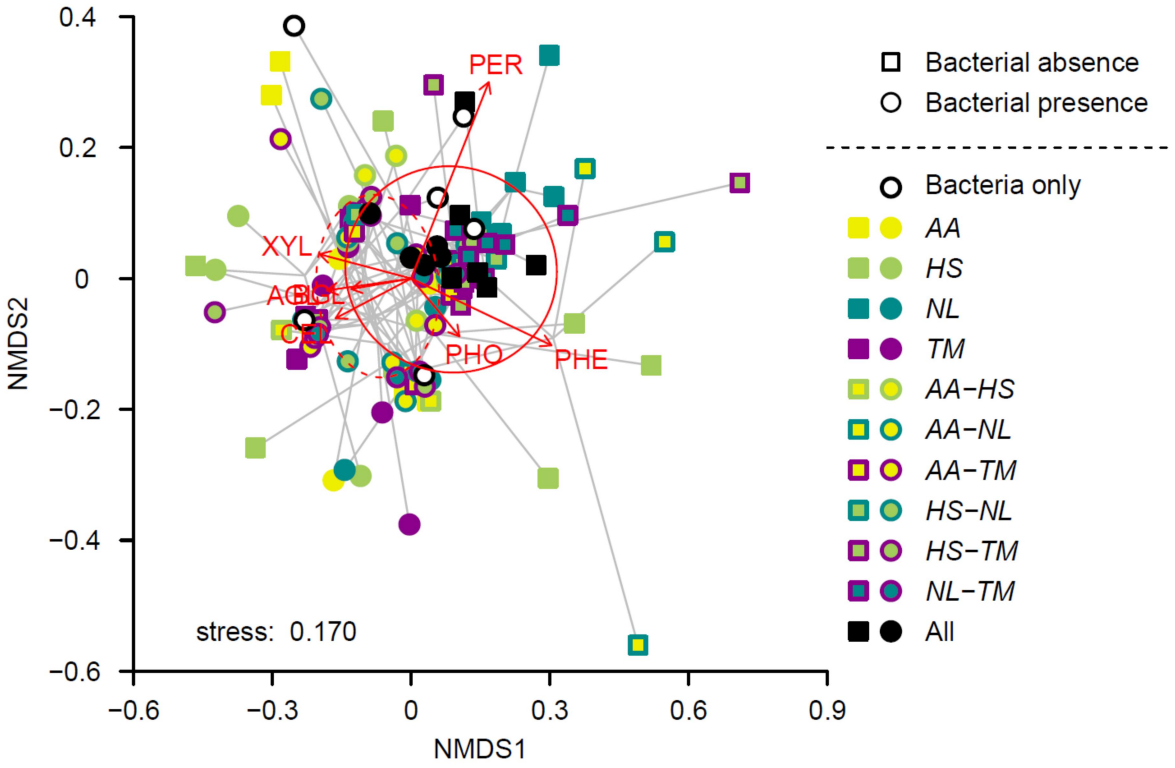



Fig. S4. Non-metric multidimensional scaling (NMDS) plot for enzyme activities using Bray-Curtis dissimilarity. Replicates are displayed as colored symbols. Squares and circles indicate bacterial absence and presence, respectively. Solid and dashed ellipses indicate group clusters of cultures in absence and presence of bacteria, respectively. Arrows display enzyme activities significantly contributing to the separation between treatments ($p$=0.001-0.003). The provided stress value indicates a reasonable fit (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*; PHO, phosphatase; AGL, α-1,4-glucosidase; BGL, β-1,4-glucosidase; CEL, cellobiohydrolase; XYL, β-1,4-xylosidase; PER, peroxidase; PHE, phenol oxidase).

Table S1. Mean growth rates and mean biomass-specific activities based on ergosterol concentrations. Values printed in bold are significantly different from zero and indicate ecologically meaningful functional traits. Note that non-parametric tests can indicate a significant difference from zero although 95%-CIs for means include zero (e.g., PHO and *T. marchalianum*; PHO, phosphatase; AGL, α-1,4-glucosidase; BGL, β-1,4-glucosidase; CEL, cellobiohydrolase; XYL, β-1,4-xylosidase; PER, peroxidase; PHE, phenol oxidase).

| | Growth rate (µg ergosterol per g of leaf dry weight per day) | Biomass-specific functional performances per g of leaf dry weight and per µg of ergosterol | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Hydrolases (nmol/h) | | | | | Oxidases (nmol/h) | |
| Species | | Leaf mass loss (%) | PHO | AGL | BGL | CEL | XYL | PER | PHE |
| *Alatospora acuminata* | **6.87 (±3.00)** | **0.11 (±0.04)** | 41.40 (±87.88) | **26.31 (±21.14)** | **109.91 (±69.07)** | **8.75 (±6.13)** | **7.88 (±6.16)** | 0.02 (±0.02) | 0.28 (±0.43) |
| *Heliscella stellata* | **3.33 (±1.52)** | 0.01 (±0.02) | **281.96 (±137.58)** | **17.93 (±15.17)** | 35.87 (±40.23) | 6.40 (±7.26) | 3.34 (±5.74) | 0.69 (±1.45) | 1.66 (±2.81) |
| *Neonectria lugdunensis* | **12.58 (±5.17)** | **0.10 (±0.04)** | 151.77 (±238.43) | **3.48 (±3.14)** | **32.50 (±23.15)** | **2.89 (±2.28)** | **1.77 (±1.33)** | **1.63 (±0.77)** | **1.11 (±0.78)** |
| *Tetracladium marchalianum* | **18.65 (±7.51)** | **0.10 (±0.06)** | **169.30 (±214.74)** | **20.15 (±10.48)** | **234.85 (±102.25)** | **10.49 (±8.79)** | **18.08 (±10.08)** | 0.25 (±0.32) | 0.17 (±0.28) |

Table S2. Biomass-specific activities based on fungal DNA concentrations and bacterial cell densities used for predictions on functional performances in mixed cultures. Note the difference in units between fungi and bacteria as well as between hydrolase and oxidase activities (PHO, phosphatase; AGL, α-1,4-glucosidase; BGL, β-1,4-glucosidase; CEL, cellobiohydrolase; XYL, β-1,4-xylosidase; PER, peroxidase; PHE, phenol oxidase).

| | | Biomass-specific functional performances per g of leaf dry weight and (for fungi) per µg of DNA or (for bacteria) per $10^{14}$ cells | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Hydrolases | | | | | Oxidases | |
| Organism | Species | Leaf mass loss (%) | Ergosterol content (µg) | PHO (µmol/h) | AGL (µmol/h) | BGL (µmol/h) | CEL (µmol/h) | XYL (µmol/h) | PER (nmol/h) | PHE (nmol/h) |
| Fungi | *Alatospora acuminata* | 2.09 (±1.46) | 18.41 (±8.85) | 0.45 (±0.85) | 0.42 (±0.24) | 1.86 (±1.03) | 0.14 (±0.09) | 0.13 (±0.09) | 2.83 (±4.41) | 5.30 (±8.88) |
| | *Heliscella stellata* | 0.44 (±0.60) | 42.41 (±39.40) | 8.38 (±8.02) | 0.96 (±1.37) | 1.90 (±3.05) | 0.27 (±0.30) | 0.20 (±0.31) | 18.40 (±22.93) | 107.70 (±220.55) |
| | *Neonectria lugdunensis* | 5.39 (±1.68) | 59.32 (±20.32) | 7.24 (±7.24) | 0.22 (±0.20) | 1.72 (±0.82) | 0.17 (±0.14) | 0.10 (±0.05) | 96.91 (±37.98) | 57.93 (±29.99) |
| | *Tetracladium marchalianum* | 1.49 (±0.88) | 19.68 (±12.07) | 2.91 (±5.17) | 0.43 (±0.43) | 4.35 (±2.53) | 0.17 (±0.15) | 0.36 (±0.32) | 7.14 (±11.43) | 4.29 (±8.15) |
| Bacteria | - | 7.54 (±4.10) | 0.00 (±0.00) | 17.55 (±25.97) | 2.30 (±1.68) | 8.17 (±5.39) | 0.93 (±0.78) | 1.61 (±0.98) | 80.53 (±106.05) | 118.01 (±131.74) |

TABLE S3. Summary of three-way ANOVAs testing the effects of diversity, bacteria, and species combination (nested within diversity) as well as their interactions on leaf mass loss and ergosterol concentrations.

| Endpoint | Data transformation | Source of variation | *df* | SS | MS | *F* | *p* |
|---|---|---|---|---|---|---|---|
| Leaf mass loss | Original | Diversity | 1 | 422.5 | 422.5 | 33.321 | **<0.001** |
| | | Bacteria | 1 | 184.0 | 184.0 | 15.378 | **<0.001** |
| | | Species combination (diversity) | 9 | 2939.2 | 326.6 | 27.299 | **<0.001** |
| | | Diversity × bacteria | 1 | 101.7 | 101.7 | 8.503 | **0.004** |
| | | Species combination (diversity) × bacteria | 9 | 500.1 | 55.6 | 4.645 | **<0.001** |
| | | Residuals | 107 | 1280.0 | 12.0 | | |
| Ergosterol concentration | Rank | Diversity | 1 | 658 | 658 | 1.369 | 0.245 |
| | | Bacteria | 1 | 49553 | 49553 | 103.146 | **<0.001** |
| | | Species combination (diversity) | 9 | 60071 | 6675 | 13.893 | **<0.001** |
| | | Diversity × bacteria | 1 | 145 | 145 | 0.303 | 0.583 |
| | | Species combination (diversity) × bacteria | 9 | 12831 | 1426 | 2.968 | **0.004** |
| | | Residuals | 106 | 50924 | 480 | | |

Table S4. Summary of three-way ANOVAs testing the effects of diversity, bacteria, and species combination (nested within diversity) as well as their interactions on activities of the investigated enzymes (PHO, phosphatase; AGL, α-1,4-glucosidase; BGL, β-1,4-glucosidase; CEL, cellobiohydrolase; XYL, β-1,4-xylosidase; PER, peroxidase; PHE, phenol oxidase).

| Endpoint | Data transformation | Source of variation | *df* | SS | MS | *F* | *p* |
|---|---|---|---|---|---|---|---|
| PHO activity | Square root | Diversity | 1 | 28.0 | 28.0 | 5.628 | **0.020** |
| | | Bacteria | 1 | 0.5 | 0.5 | 0.107 | 0.745 |
| | | Species combination (diversity) | 9 | 83.4 | 9.3 | 1.866 | 0.065 |
| | | Diversity × bacteria | 1 | 0.0 | 0.0 | 0.001 | 0.971 |
| | | Species combination (diversity) × bacteria | 9 | 41.6 | 4.6 | 0.930 | 0.503 |
| | | Residuals | 106 | 526.5 | 5.0 | | |
| AGL activity | Square root | Diversity | 1 | 0.266 | 0.266 | 1.549 | 0.216 |
| | | Bacteria | 1 | 2.183 | 2.183 | 12.722 | **<0.001** |
| | | Species combination (diversity) | 9 | 12.656 | 1.406 | 8.197 | **<0.001** |
| | | Diversity × bacteria | 1 | 0.898 | 0.898 | 5.232 | **0.024** |
| | | Species combination (diversity) × bacteria | 9 | 6.087 | 0.676 | 3.942 | **<0.001** |
| | | Residuals | 107 | 18.357 | 0.172 | | |
| BGL activity | Rank | Diversity | 1 | 24646 | 24646 | 33.649 | **<0.001** |
| | | Bacteria | 1 | 1280 | 1280 | 1.748 | 0.189 |
| | | Species combination (diversity) | 9 | 41150 | 4572 | 6.242 | **<0.001** |
| | | Diversity × bacteria | 1 | 486 | 486 | 0.663 | 0.417 |
| | | Species combination (diversity) × bacteria | 9 | 29551 | 3283 | 4.483 | **<0.001** |
| | | Residuals | 106 | 77639 | 732 | | |
| CEL activity | Square root | Diversity | 1 | 0.243 | 0.243 | 2.199 | 0.141 |
| | | Bacteria | 1 | 0.687 | 0.687 | 6.227 | **0.014** |
| | | Species combination (diversity) | 9 | 3.186 | 0.354 | 3.208 | **0.002** |
| | | Diversity × bacteria | 1 | 0.314 | 0.314 | 2.841 | 0.095 |
| | | Species combination (diversity) × bacteria | 9 | 1.957 | 0.217 | 1.970 | 0.050 |
| | | Residuals | 104 | 11.478 | 0.110 | | |

Table S4 continued.

| Endpoint | Data transformation | Source of variation | *df* | SS | MS | *F* | *p* |
|---|---|---|---|---|---|---|---|
| XYL activity | Rank | Diversity | 1 | 10485 | 10485 | 17.243 | **<0.001** |
| | | Bacteria | 1 | 27641 | 27641 | 45.460 | **<0.001** |
| | | Species combination (diversity) | 9 | 41409 | 4601 | 7.567 | **<0.001** |
| | | Diversity × bacteria | 1 | 361 | 361 | 0.593 | 0.443 |
| | | Species combination (diversity) × bacteria | 9 | 26938 | 2993 | 4.923 | **<0.001** |
| | | Residuals | 105 | 63844 | 608 | | |
| PER activity | Rank | Diversity | 1 | 15381 | 15381 | 17.991 | **<0.001** |
| | | Bacteria | 1 | 14092 | 14092 | 16.484 | **<0.001** |
| | | Species combination (diversity) | 9 | 26568 | 2952 | 3.453 | **<0.001** |
| | | Diversity × bacteria | 1 | 0 | 0 | 0.000 | 0.998 |
| | | Species combination (diversity) × bacteria | 9 | 19142 | 2127 | 2.488 | **0.013** |
| | | Residuals | 105 | 89765 | 855 | | |
| PHE activity | Rank | Diversity | 1 | 2473 | 2473 | 2.461 | 0.120 |
| | | Bacteria | 1 | 16773 | 16773 | 16.690 | **<0.001** |
| | | Species combination (diversity) | 9 | 31269 | 3474 | 3.457 | **<0.001** |
| | | Diversity × bacteria | 1 | 2630 | 2630 | 2.617 | 0.109 |
| | | Species combination (diversity) × bacteria | 9 | 2858 | 318 | 0.316 | 0.968 |
| | | Residuals | 104 | 104515 | 1005 | | |

TABLE S5. Summary of three-way PERMANOVA testing the effects of diversity, bacteria, and species combination (nested within diversity) as well as their interactions on enzyme activity profiles.

| Endpoint | Data transformation | Source of variation | *df* | SS | MS | *F* | $R^2$ | *p* |
|---|---|---|---|---|---|---|---|---|
| Enzyme activities | Log (x+1) followed by min-max normalization | Diversity | 1 | 0.0978 | 0.0978 | 6.4723 | 0.0398 | **0.001** |
| | | Bacteria | 1 | 0.2290 | 0.2290 | 15.1554 | 0.0933 | **0.001** |
| | | Species combination (diversity) | 10 | 0.3417 | 0.0342 | 2.2620 | 0.1392 | **0.001** |
| | | Diversity × bacteria | 1 | 0.0198 | 0.0198 | 1.3121 | 0.0081 | 0.261 |
| | | Species combination (diversity) × bacteria | 9 | 0.2250 | 0.0250 | 1.6544 | 0.0917 | **0.038** |
| | | Residuals | 102 | 1.5410 | 0.0151 | | 0.6279 | |

Table S6. Summary of three-way ANOVAs testing the effects of diversity, bacteria, and species combination (nested within diversity) as well as their interactions on DNA concentrations of the four model fungi (*AA*, *Alatospora acuminata*; *HS*, *Heliscella stellata*; *NL*, *Neonectria lugdunensis*; *TM*, *Tetracladium marchalianum*)

| Endpoint | Data transformation | Source of variation | *df* | SS | MS | *F* | *p* |
|---|---|---|---|---|---|---|---|
| *AA* DNA quantity | Rank | Diversity | 1 | 3474 | 3474 | 33.745 | **<0.001** |
| | | Bacteria | 1 | 3422 | 3422 | 33.239 | **<0.001** |
| | | Species combination (diversity) | 3 | 1611 | 537 | 5.217 | **0.003** |
| | | Diversity × bacteria | 1 | 256 | 256 | 2.482 | 0.122 |
| | | Species combination (diversity) × bacteria | 3 | 2551 | 850 | 8.259 | **<0.001** |
| | | Residuals | 48 | 4941 | 103 | | |
| *HS* DNA quantity | Rank | Diversity | 1 | 622 | 622 | 8.383 | **0.006** |
| | | Bacteria | 1 | 6506 | 6506 | 87.753 | **<0.001** |
| | | Species combination (diversity) | 3 | 3143 | 1048 | 14.131 | **<0.001** |
| | | Diversity × bacteria | 1 | 364 | 364 | 4.906 | **0.032** |
| | | Species combination (diversity) × bacteria | 3 | 1310 | 437 | 5.888 | **0.002** |
| | | Residuals | 47 | 3484 | 74 | | |
| *NL* DNA quantity | Rank | Diversity | 1 | 2963 | 2963 | 19.287 | **<0.001** |
| | | Bacteria | 1 | 3456 | 3456 | 22.490 | **<0.001** |
| | | Species combination (diversity) | 3 | 1399 | 466 | 3.036 | **0.038** |
| | | Diversity × bacteria | 1 | 56 | 56 | 0.361 | 0.551 |
| | | Species combination (diversity) × bacteria | 3 | 1708 | 569 | 3.704 | **0.018** |
| | | Residuals | 49 | 7529 | 154 | | |
| *TM* DNA quantity | Rank | Diversity | 1 | 1690 | 1690 | 11.474 | **0.001** |
| | | Bacteria | 1 | 3315 | 3315 | 22.509 | **<0.001** |
| | | Species combination (diversity) | 3 | 4572 | 1524 | 10.348 | **<0.001** |
| | | Diversity × bacteria | 1 | 230 | 230 | 1.564 | 0.217 |
| | | Species combination (diversity) × bacteria | 3 | 823 | 274 | 1.862 | 0.148 |
| | | Residuals | 50 | 7364 | 147 | | |

Table S7. Summary of the two-way ANOVA testing the effects of diversity and species combination on bacterial cell density.

| Endpoint | Data transformation | Source of variation | *df* | SS | MS | *F* | *p* |
|---|---|---|---|---|---|---|---|
| Bacterial cell density | Original | Diversity | 1 | $5.198*10^{20}$ | $5.198*10^{20}$ | 0.396 | 0.531 |
| | | Species combination (diversity) | 10 | $1.463*10^{22}$ | $1.463*10^{21}$ | 1.115 | 0.367 |
| | | Residuals | 59 | $7.740*10^{22}$ | $1.312*10^{21}$ | | |

**APPENDIX A.4**

## Environmentally relevant fungicide levels modify fungal community composition and interactions but not functioning

Patrick Baudy, Jochen P. Zubrod, Marco Konschak, Nina Röder, Thu Huyen Nguyen, Verena C. Schreiner, Christiane Baschien, Ralf Schulz, Mirco Bundschuh

Environmental Pollution 285, 117234
Impact Factor (2020): 8.071

## ABSTRACT

Aquatic hyphomycetes (AHs), a group of saprotrophic fungi adapted to submerged leaf litter, play key functional roles in stream ecosystems as decomposers and food source for higher trophic levels. Fungicides, controlling fungal pathogens, target evolutionary conserved molecular processes in fungi and contaminate streams via their use in agricultural and urban landscapes. Thus fungicides pose a risk to AHs and the functions they provide. To investigate the impacts of fungicide exposure on the composition and functioning of AH communities, we exposed four AH species in monocultures and mixed cultures to increasing fungicide concentrations (0, 5, 50, 500, and 2500 µg/L). We assessed the biomass of each species via quantitative real-time PCR. Moreover, leaf decomposition was investigated. In monocultures, none of the species was affected at environmentally relevant fungicide levels (5 and 50 µg/L). The two most tolerant species were able to colonize and decompose leaves even at very high fungicide levels (≥500 µg/L), although less efficiently. In mixed cultures, changes in leaf decomposition reflected the response pattern of the species most tolerant in monocultures. Accordingly, the decomposition process may be safeguarded by tolerant species in combination with functional redundancy. In all fungicide treatments, however, sensitive species were displaced and interactions between fungi changed from complementarity to competition. As AH community composition determines leaves' nutritional quality for consumers, the data suggest that fungicide exposures rather induce bottom-up effects in food webs than impairments in leaf decomposition.

## KEYWORDS

Biodiversity-ecosystem functioning – Diversity effects – Leaf litter decomposition – DMI fungicides - Strobilurins

## INTRODUCTION

Aquatic fungi adapted to colonize and degrade submerged leaf litter (mainly aquatic hyphomycetes; ¼ AHs; Baschien et al., 2013; Dighton and White, 2017) are key to ecosystems fueled by allochthonous organic matter. Making use of a diverse set of extracellular enzymes, AHs efficiently catalyze leaf litter decomposition, thereby converting non-utilizable biopolymers to bioavailable substances (Evans and

Hedger, 2001). Through the simultaneous accumulation of fungal biomass, they additionally increase leaves' nutritional quality (i.e., conditioning; Cummins and Klug, 1979) for consumers, such as shredders.

Globally, the majority of streams are subject to human impact, including pollution with xenobiotics (Vörösmarty et al., 2010). Synthetic fungicides represent a group of xenobiotics applied to control fungal pathogens and enter streams following their use in agricultural and urban landscapes (as reviewed by Zubrod et al., 2019). Since fungicides target evolutionary conserved molecular processes of close relatives to AHs (Stenersen, 2004), these compounds pose a risk for aquatic fungi and the functions they provide. Moreover, an overlap of measured field and effect concentrations of fungicides, which is particularly large for the groups of demethylation inhibitors and strobilurins (e.g., tebuconazole and azoxystrobin, respectively; Zubrod et al., 2019), causes further concern. Changes in the functioning of AH communities, mostly assessed via leaf mass loss or total fungal biomass, are predominantly induced at high fungicide concentrations (Pimentão et al., 2020; Zubrod et al., 2019). In contrast, structural changes (i.e., diversity or community composition) may already appear at low (field relevant) concentrations (Fernández et al., 2015; Pimentão et al., 2020; Zubrod et al., 2019). This discrepancy may be explained by the replacement of sensitive by tolerant AH species in combination with functional redundancy (Pascoal et al., 2005). While ecological mechanisms (e.g., selection or complementarity effects) linking biodiversity and ecosystem functioning (i.e., B-EF relationships) in AH communities remain poorly understood (Bärlocher, 2016; Grossart and Rojas-Jimenez, 2016), it is assumed that resource partitioning constitutes a major type of interaction in undisturbed communities (e.g., via complementary extracellular enzyme inventories; Gessner et al., 2010). Assessing impacts of fungicide exposures on B-EF relationships requires the ability to track the biomass of individual AH species within a community throughout various successional stages, which is offered by species-specific quantitative real-time PCR (qPCR; Duarte et al., 2006; Grossart and Rojas-Jimenez, 2016). To the best of our knowledge, only one study exists that applied species-specific qPCR assays to assess B-EF relationships in AH assemblages under chemical stress (i.e., Fernandes et al., 2011). In this study, cadmium exposure altered the relative abundance of AH species and induced shifts from complementary towards competitive interactions, while leaf decomposition was maintained by cadmium-tolerant strains due to

dominance effects (i.e., selective pressure of cadmium favored tolerant species at the expense of others).

Using a similar experimental setup and novel species-specific TaqMan® qPCR assays (Baudy et al., 2019), the present study aimed at investigating B-EF relationships in AH communities under fungicide exposure. Employing a factorial microcosm experiment, leaves were colonized by four AH monocultures, six binary, and one quaternary combination (i.e., eleven different cultures). Each culture was exposed to five concentrations of a five-component fungicide mixture, reflecting environmentally relevant (i.e., chronic exposure to fungicide mixtures in the low µg/L range) to worst-case exposure scenarios (exposure in the mg/L range; Zubrod et al., 2019). To assess the functional performance of these communities, leaf mass loss, fungal biomass production (via ergosterol), and the activity of seven extracellular enzymes were analyzed. AH community composition was investigated via species-specific qPCR (Baudy et al., 2019).

We expected that the AH species (in monocultures) differ in their fungicide tolerance, as has been reported for aquatic fungi exposed to different concentrations of various fungicides (Dijksterhuis et al., 2011). We additionally hypothesized that cultures would be more resistant with increasing diversity and maintain leaf decomposition up to high fungicide concentrations, due to functional redundancy and the presence of tolerant species (Biggs et al., 2020; Pascoal et al., 2005). Community composition, on the other hand, would already be affected at relatively low concentrations (Zubrod et al., 2019). We finally hypothesized that fungicide exposure alters the interactions between AH species (e.g., complementarity and dominance effects) occurring during leaf colonization (cf. Fernandes et al., 2011), which would be most pronounced in assemblages comprising species with marked differences in fungicide tolerance.

## MATERIAL AND METHODS

### CHEMICALS

The fungicide mixture comprised the synthetic fungicides azoxystrobin, carbendazim, cyprodinil, quinoxyfen, and tebuconazole applied at equal concentrations. These fungicides cover a broad range of modes of action (Table 1) currently used in agriculture and were already detected in the same streams within one year (Landesamt für Umwelt Rheinland-Pfalz, 2016). The fungicide sum

**Table 1.** Information on origin, mode of action and concentrations of the applied fungicides

| Fungicide | Product | Manufacturer | Mode of action [a] | Analytical limit of quantification (µg/L) | Fungicide concentrations (µg/L) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 5 µg/L fungicide treatment | | | 2,500 µg/L fungicide treatment | | |
| | | | | | Nominal | Measured in fresh nutrient medium [b] (*n* = 3) | Measured in old nutrient medium [b] (*n* = 3) | Nominal | Measured in fresh nutrient medium [b] (*n* = 3) | Measured in old nutrient medium [b] (*n* = 3) |
| Azoxystrobin | Ortiva® | Syngenta Agro GmbH, Germany | Inhibition of respiration | 0.4 | 1 | 0.68 ± 0.03 (68.0%) | 0.50 ± 0.06 (50.3%) | 500 | 481.3 ± 58.8 (96.3%) | 256.0 ± 22.3 (51.2%) |
| Carbendazim | Derosal® | Bayer CropScience, Germany | Inhibition of mitosis and cell division | 0.1 | 1 | 1.04 ± 0.30 (104.3%) | 0.16 ± 0.07 (15.7%) | 500 | 447.7 ± 78.0 (89.5%) | 295.7 ± 34.6 (59.1%) |
| Cyprodinil | Chorus® | Syngenta Agro GmbH, Germany | Inhibition of amino acids and protein synthesis | 0.1 | 1 | 0.84 ± 0.03 (84.0%) | < LOQ [c] | 500 | 453.0 ± 67.4 (90.6%) | 38.3 ± 5.7 (7.7%) |
| Quinoxyfen | Fortress™ 250 | Dow AgroSciences GmbH, Germany | Inhibition of signal transduction | -- | 1 | NA [d] | NA [d] | 500 | NA [d] | NA [d] |
| Tebuconazole | Folicur® | Bayer CropScience, Germany | Inhibition of sterol biosynthesis in membranes | 0.5 | 1 | 1.41 ± 0.34 (140.7%) | 0.79 ± 0.01 (79.0%) | 500 | 373.3 ± 49.4 (74.7%) | 156.7 ± 10.1 (31.3%) |
| Mixture | All of the above | All of the above | All of the above | -- | 5 | 3.97 ± 0.36 (99.3% [e]) | 1.45 ± 0.01 (36.3% [e]) | 2,500 | 1,755.3 ± 250.9 (87.8% [e]) | 746.7 ± 67.6 (37.3% [e]) |

[a] Fungicide Resistance Action Committee (2020)
[b] Mean concentration and standard deviation (percent recovery from nominal concentration)
[c] Limit of quantification
[d] Not analyzed
[e] Recovery based on nominal sum concentrations adapted to the proportion of the measurable four fungicides (i.e., 4 and 2,000 µg/L), excluding quinoxyfen

concentrations of 0, 5, 50, 500, and 2500 µg/L were chosen based on an earlier study (Zubrod et al., 2015a), and to cover field-relevant (i.e., 5 and 50 µg/L) to worst-case exposure scenarios (i.e., 500 and 2500 µg/L; Rabiet et al., 2010; Zubrod et al., 2019). Fungicides were applied as commercial products (Table 1), making the use of further solvents redundant. The products were diluted in autoclaved (at 121 °C for 20 min; Systec DE-65®, Systec, Germany) nutrient medium (for composition see Dang et al., 2005) to obtain the respective nominal sum concentrations. To verify exposure concentrations, volumes of 10 mL were sampled in the 0, 5, and 2500 µg/L fungicide treatments (*n* = 1/3/3) at test initiation and at the time of the first medium renewal (i.e., after 7 days). Samples were taken from fresh medium in additionally prepared microcosms at test initiation and old medium of quaternary cultures at the time of medium renewal on day 7 of the experiment. Samples were stored frozen at -20 °C until chemical analysis. After thawing, fungicides were measured by direct injection into a liquid chromatography high-resolution mass-spectrometry (LC-HRMS) Orbitrap system (Thermo Fisher Scientific, Germany) using matrix-matched standards for calibration. The limit of quantification (LOQ) was set to the lowest concentration reliably distinguishable from blanks and ranged from 0.1 to 0.5 µg/L among fungicides (Table 1; for details see Fernández et al., 2016). Fungicide concentrations in the controls were below the LOQ. Due to technical reasons, concentrations of quinoxyfen could not be quantified. However, as the measured sum concentrations of the other four fungicides deviated less than 15% of the nominal (Table 1), a proper dosing with all five fungicides at test initiation is assumed. Thus, nominal sum concentrations are used throughout the present manuscript.

## FUNGI AND LEAF SUBSTRATE

Strains of the AH species *Alatospora acuminata*, *Heliscella stellata*, *Neonectria lugdunensis*, and *Tetracladium marchalianum* were used as model fungi (for strain specifications see Table S1). These species are representatives of decomposer communities in temperate streams and are assumed to vary in their tolerance to the fungicide mixture used in this study (Bundschuh et al., 2011; Zubrod et al., 2015a). The strains were isolated in 2015 and 2016 from small streams in Germany and deposited at the German Collection for Microorganisms and Cell Cultures (Leibniz-Institute DSMZ; Baudy et al., 2019). Cultures were grown on Petri dishes containing 15 mL of 1% malt extract agar (10 g/L malt extract, 20 g/L agar) at

16 °C in darkness for 21 days. Agar plugs (diameter: 5 mm) were cut from the growing edges of colonies and served as fungal inoculum. Leaves of *Alnus glutinosa* (L.) GAERTN. (black alder), a wide-spread European riparian tree species (Copolovici et al., 2014), with no visible signs of damage or symptoms of diseases, were handpicked from trees near Landau, Germany (49.20116 °N; 8.09331 °E) shortly before abscission in 2015 and stored frozen at -20 °C (instead of dry storage) for practical reasons. It is noted that frozen storage can impact leaching and conditioning of leaves (Bärlocher, 1992), which does, however, not impede comparability across treatments (see below for details). Upon thawing, disks (diameter: 16 mm) were cut, excluding the midrib. In the test vessels (i.e., 100-mL Erlenmeyer flasks), 20 leaf disks were leached in 20 mL of autoclaved nutrient medium for 48 h. Subsequently (i.e., one day prior to the experiment), leachates were decanted, whereas flasks and leaf disks therein were autoclaved to eliminate unwanted microbial activity. The sterile test vessels were kept overnight in a sealed ethanol-disinfected box.

EXPERIMENTAL DESIGN

The microcosm experiment was employed as an 11 × 5-factorial design. Eleven fungal treatments – comprising four monocultures, six binary, and a quaternary combination of the four species – were exposed to five fungicide sum concentrations (i.e., 0, 5, 50, 500, and 2500 µg/L) leading to 55 treatments. Additionally, two sterile (fungus-free) treatments receiving either no fungicides or a fungicide sum concentration of 2500 µg/L were prepared. These treatments served as references for later analyses (see below for details). All 57 treatments were replicated five times ($n$ = 5). Inoculation of the microcosms largely followed the methods described in Andrade et al. (2016). Microcosms were initiated under sterile conditions in a laminar flow cabinet (UV-treated for 30 min prior to use; NU-437-500 E; Nuaire, USA). Each microcosm consisted of a 100-mL Erlenmeyer flask containing 20 leaf disks, four agar plugs, and 60 mL of nutrient medium spiked with the respective fungicide concentration. Microcosms receiving one, two, or four species were equipped with four, two, or one cultivated agar plug(s) per species. Reference microcosms containing no fungi were equipped with four sterile agar plugs. The test vessels were closed with gas-permeable cellulose stoppers. Incubation of the microcosms was carried out on a horizontal shaker (model

VKS 75 B control; Edmund Bühler GmbH, Germany) at 115 rpm at 16 °C in darkness. The microcosms were randomly distributed on the shaker and shuffled daily to avoid location effects. After 7 days of incubation, the nutrient medium and the respective fungicide concentrations were renewed in all microcosms (cf. Andrade et al., 2016). After the total incubation time of 14 days, leaf disks from all microcosms were sampled for later analyses as follows: for analysis of enzyme activity, one leaf disk was preserved in a 12-mL plastic centrifuge tube and stored at -20 °C; for assessment of bacterial contamination (this was largely prevented; see Supporting Information), two leaf disks were preserved in 10 mL of a 2% formaldehyde / 0.1% sodium pyrophosphate solution and stored at 4 °C; for potential analysis of sporulation, which was, however, not within the scope of this study, two leaf disks were agitated in deionized water for 96 h, fixed using formaldehyde (resulting in a 2% formaldehyde solution), and stored at room temperature; the remaining 15 leaf disks were preserved in pre-weighed 2-mL Eppendorf tubes, lyophilized, analyzed for dry mass to the nearest 0.01 mg and afterwards used for ergosterol and DNA analyses.

ENZYME ACTIVITY ANALYSES

Activities of hydrolytic and oxidative enzymes were assessed based on DeForest (2009) with a detailed overview of the modifications being highlighted in Baudy et al. (accepted). Briefly, leaf disks were homogenized in 350 mL of sterile nutrient medium using an Ultra-turrax® blender (24000 rpm for 30 s; IKA®-Werke GmbH & Co. KG, Germany). Using fluorescence- and absorbance-based reporter substrates, leaf homogenates were analyzed for activities of phosphatases (EC 3.1.3.1 and 3.1.3.2; targeting phosphate esters), a-1,4-glucosidase (EC 3.2.1.20; targeting starch and maltose), b-1,4-glucosidase (EC 3.2.1.21; targeting cellulose), cellobiohydrolase (EC 3.2.1.91; targeting cellulose), b-1,4-xylosidase (EC 3.2.1.37; targeting hemicellulose), peroxidase (EC 1.11.1.7; targeting lignin), and phenol oxidase (EC 1.10.3.2; targeting lignin). Reactions were performed in 96-well 300-mL well plates (Thermo Fisher Scientific, USA), which were incubated on a rotary shaker (120 rpm; KS 15; Edmund Bühler GmbH, Germany) in darkness for approximately 1 h (hydrolases) or 2 h (oxidases). Fluorescence and absorbance were measured using a microplate reader (Infinite 200, Tecan Group, Switzerland). The remaining leaf homogenate of each sample (~340 mL) was filtered through pre-weighed glass

fiber filters (GF/6; Whatman GmbH, Germany), which were subsequently dried at 60 °C for 24 h and weighed to the nearest 0.01 mg. The difference of the filter weights was used to normalize enzyme activity to leaf dry mass.

ERGOSTEROL ANALYSIS

Total fungal biomass was estimated as the fungal-specific membrane molecule ergosterol as described in Gessner (2005), without any further conversion (e.g., the average conversion factor of 5.5 µg ergosterol/mg fungal dry mass; Gessner and Chauvet, 1993). Briefly, ergosterol was extracted from 30 to 50 mg of lyophilized leaf sample in 10 mL of alkaline methanol. Extracts were purified via solid-phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent, Waters, USA), and eluted in isopropanol. Ergosterol was finally quantified using a high-performance liquid chromatography (HPLC) system (1200 Series, Agilent Technologies, USA).

FUNGAL BIOMASS QUANTIFICATION VIA DNA ANALYSIS

To estimate the biomass of individual fungi, DNA was extracted and species-specifically quantified (Baudy et al., 2019). Since the relationship between DNA concentrations and fungal dry mass is subjected to considerable interspecific variability (Baudy et al., 2019), DNA concentrations between species are not readily comparable. Briefly, genomic DNA was extracted from 25 to 50 mg of lyophilized leaf sample using the FastDNA® Spin Kit for Soil in conjunction with the FastPrep™-24 5G Instrument (MP Biomedicals, Germany). Extracted DNA amounts of individual species were quantified via TaqMan® qPCR reactions (Applied Biosystems, USA) performed in a Mastercycler® ep gradient S (Eppendorf, Germany).

DATA ANALYSIS

Leaf mass loss (*L*; in percent) was calculated as follows:

$$L = \frac{\left(\overline{w}_{sterile} - w_{fungi}\right)}{\left(\overline{w}_{sterile}\right)} \times 100$$

where $w_{sterile}$ is the mean final dry mass of leaf disks in sterile reference microcosms and $w_{fungi}$ is the final dry mass of leaf disks in individual microcosms containing fungi. Ergosterol and DNA concentrations (expressed as mg/g leaf dry mass, respectively) were calculated as described in Baudy et al. (2019). Enzyme activities (expressed as mmol/(h*g leaf dry mass); for calculation see Baudy et al. (accepted)) were

normalized to pooled fungicide-free and fungicide-treated (2500 µg/L) sterile reference microcosms, if variables in both treatments were not significantly different (as judged by t-tests). Otherwise, each treatment was normalized individually. In this case, reference values for the fungicide sum concentrations of 5, 50, and 500 µg/L were interpolated from linear regression curves based on the available reference data. Predictions for leaf mass loss ($P_{lml}$) and ergosterol concentrations ($P_{erg}$) in mixed cultures were calculated as follows:

$$P_{lml/erg} = \sum_{i=1}^{n} F_{lml/erg\ species\ i} * c_{DNA\ species\ i}$$

where $F_{lml/erg\ species\ i}$ is the mean specific leaf mass loss or mean specific ergosterol concentration (Table S1) of component species *i* in the mixed culture, $c_{DNA\ species\ i}$ is the DNA concentration of component species *i* in the mixed culture and *n* is the number of species in the mixed culture (i.e., 2 or 4).

Multiple comparisons of leaf mass loss, ergosterol and DNA concentrations, as well as diversity effects between fungicide-free controls and fungicide treatments were performed via analysis of variance (ANOVA) followed by Dunnett's tests. Comparisons between observed and predicted leaf mass loss and ergosterol concentrations were performed using paired t-tests. For multivariate analysis, enzyme activities were log(x+1) transformed and min-max normalized to decrease the discriminatory power of enzymes with high activities. To assess the effects of fungal diversity, species combination (nested within diversity) and fungicide exposure as well as their interactions on enzyme activity profiles, permutational multivariate analysis of variance (PERMANOVA) was performed on enzyme activity profiles of all cultures. Distances between enzyme activity profiles were calculated using Euclidean distance. For the visualization of dissimilarities of the enzyme activity profiles, the distance matrix was subjected to non-metric multidimensional scaling (NMDS). All statistics and figures were prepared using R version 3.5.2 (R Core Team, 2018) as well as the add-on packages "multcomp", "plotrix" and "vegan". The term "significant(ly)" is only used with regard to statistical significance ($p < 0.05$) throughout this study.

## DIVERSITY EFFECTS ON LEAF COLONIZATION

To assess interactions between AH species during the colonization of leaf substrate, diversity effects were calculated from species-specific DNA concentrations

in monocultures and mixed cultures within the same fungicide treatments, applying a modified Price equation (Fox, 2005). The calculation of these effects (in percent) is based on the assumption that species perform in mixed cultures equally well as in monocultures (i.e., additivity). Calculated net diversity effects can be partitioned into three component effects comprising trait-independent complementarity, dominance, and trait-dependent complementarity.

Net diversity effects are deviations between observed DNA concentrations in mixed cultures and DNA concentrations predicted from monocultures, weighted by the initial proportion of inoculum of each species in the mixture. Trait-independent complementarity is positive if the majority of species show higher DNA concentrations in mixed culture than expected based on their performance in monoculture, indicating synergistic interactions. It is negative if the majority of species show the opposite response, thus indicating antagonistic interactions. Dominance provides insights into the dominance relation between species. Dominance is positive if species with high DNA concentrations in monoculture perform better in mixed cultures, but at the expense of species with lower DNA concentrations in monoculture. It is negative if species with low DNA concentrations in monoculture perform better in mixed cultures, but at the expense of other species. Trait-dependent complementarity is positive if species with high DNA concentrations in monocultures perform better in mixed cultures, but not at the expense of other species. It is negative if species with low DNA concentrations in monoculture perform better in mixed cultures, but not at the expense of other species (for more details see Fox, 2005).

## RESULTS AND DISCUSSION

### RESPONSES OF MONOCULTURES

Since virtually no leaf mass loss was observed for *A. acuminata* and *H. stellata* under control conditions (2.6% and -4.4%, respectively; Fig. 1a), potential effects of fungicide exposure on these species' degradative capacity could not be detected. In contrast, *N. lugdunensis* and *T. marchalianum* efficiently decomposed leaves (up to 25% in the control), while this functional variable was only for *N. lugdunensis* significantly affected at a fungicide sum concentration of 2500 µg/L (Fig.1a). Generally, as indicated by ANCOVA (Table S2), among all monocultures

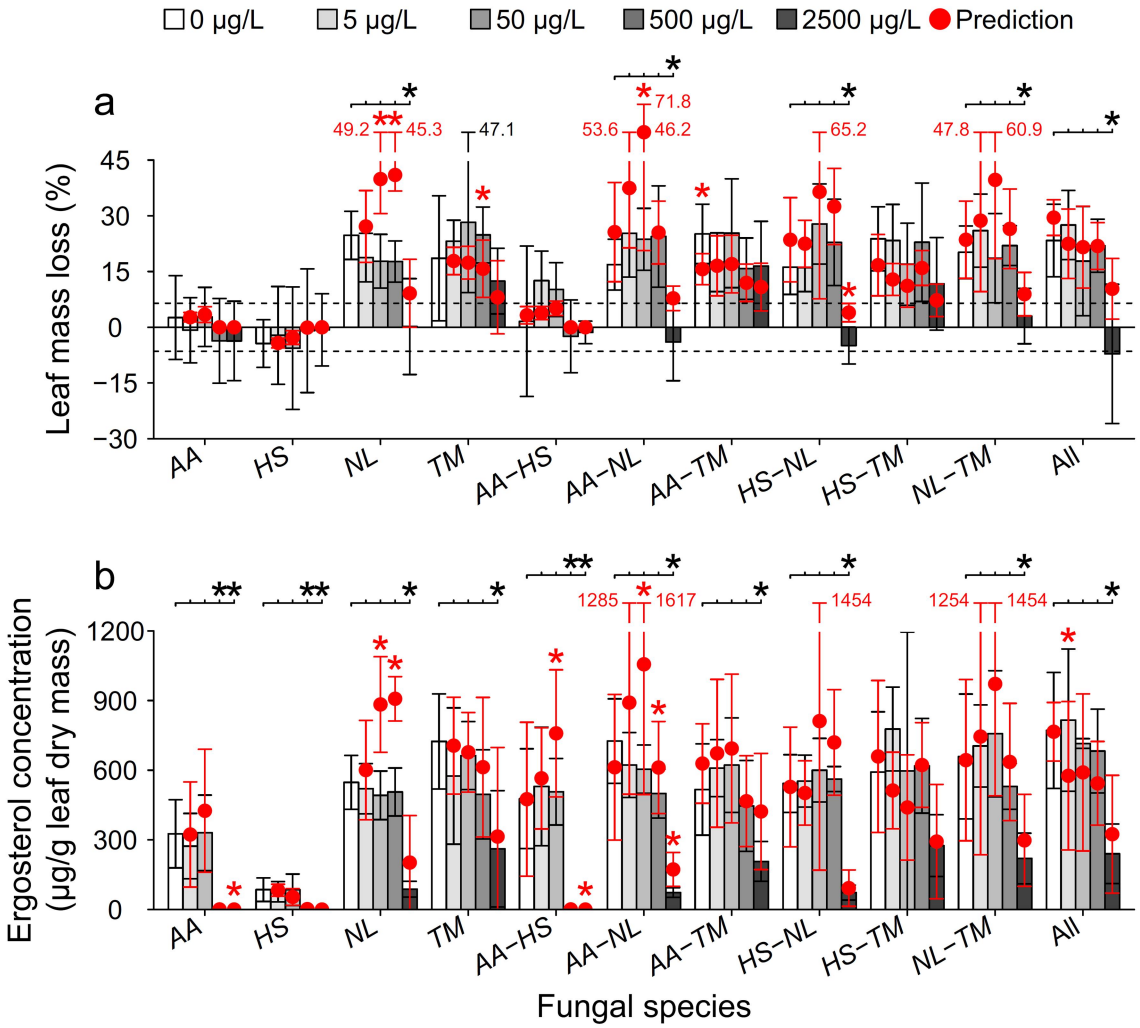



Fig. 1. Mean leaf mass loss (a) and ergosterol concentrations (b; with 95%-confidence intervals, respectively) in fungal species treatments exposed to fungicide sum concentrations of 0, 5, 50, 500, and 2500 µg/L (gray-scale bars) and mean predictions (red points with 95%-confidence intervals) based on biomass-specific leaf mass loss or ergosterol concentrations. Solid and dashed horizontal lines indicate the mean and 95%-confidence interval, respectively, of the sterile control (a). Black asterisks indicate significant differences between fungicide treatments and fungicide-free controls within fungal species treatments (Dunnett's test; $p < 0.05$; $n = 5$). Red asterisks indicate significant differences between observed and predicted variables (paired t-test; $p < 0.05$; $n = 5$). Note that predictions for each species are based on biomass-specific leaf mass loss and ergosterol concentrations in fungicide-free monocultures (therefore, mean predictions equal mean observations in these treatments; *AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*).

and mixed cultures, there was a strong linear relationship between leaf mass loss and cumulative enzyme activities. This relationship was not significantly modified by fungicide exposure (Table S2), which generally has the potential to affect the substrate affinity of enzymes (Artigas et al., 2012). In line with earlier studies (e.g., Suberkropp et al., 1983; Abdel-Raheem and Shearer, 2002; Baudy et al., accepted), the degradative capacity of the investigated AH species seems to be based on

distinct enzyme activity profiles (Fig. 2a-d). While *A. acuminata* and *T. marchalianum* show a similar profile, characterized by hydrolases (Fig. 2a, d), the profile of *N. lugdunensis* is characterized by oxidases (Fig. 2c). The profile of *H. stellata*, however, does not seem to include the investigated enzymes (Fig. 2b), which may be explained by the low biomass of this species observed in this experiment, resulting in lower productivity (Fig. 1b) or a different leaf colonization strategy (Baudy et al., accepted). Similar to leaf mass loss, fungicide exposure affected enzyme activity profiles only at high concentrations of 500 and 2500 µg/L (Fig. 2a-d), indicating no pronounced alterations in the regulation of the investigated enzymes at environmentally relevant fungicide concentrations.

Fungal biomass (independent whether assessed as ergosterol or DNA) of *A. acuminata* and *H. stellata* was maintained at constant levels up to a fungicide sum concentration of 50 µg/L. At higher fungicide concentrations, no or very low biomasses could be detected (Fig. 1b; Fig. 3a and b). Again, *N. lugdunensis* and *T. marchalianum* had measurable ergosterol contents, even at the highest fungicide sum concentration, which were, however, significantly lower compared to controls (-84% and -64%, respectively; Fig. 1b). This pattern was also reflected by DNA concentrations of *T. marchalianum* (Fig. 3d). In contrast, *N. lugdunensis* showed a substantial increase (up to 65%) in DNA levels at fungicide sum concentrations of 50 and 500 µg/L, while ergosterol remained constant (Fig. 1b; Fig. 3c), leading to an over-prediction of DNA-based ergosterol concentrations (Fig. 1b). This pattern is likely triggered by a direct effect of one fungicide of the mixture, namely tebuconazole, on the production of both biomarkers (Baudy et al., 2020). Such an effect can result in altered biomarker levels, not reflecting true mycelial biomass (Baudy et al., 2020). Nonetheless, *A. acuminata* and *H. stellata* can be considered as more sensitive compared to *N. lugdunensis* and *T. marchalianum*. This sensitivity pattern is in line with previous laboratory studies assessing AH species' productivity via morphological identification and quantification of asexual spores termed conidia (Bundschuh et al., 2011; Zubrod et al., 2015b). In these studies, it was observed that increasing fungicide concentrations reduced sporulation of *A. acuminata* and *H. stellata* and maintained or even increased sporulation of *N. lugdunensis* and *T. marchalianum* in near-natural decomposer communities.

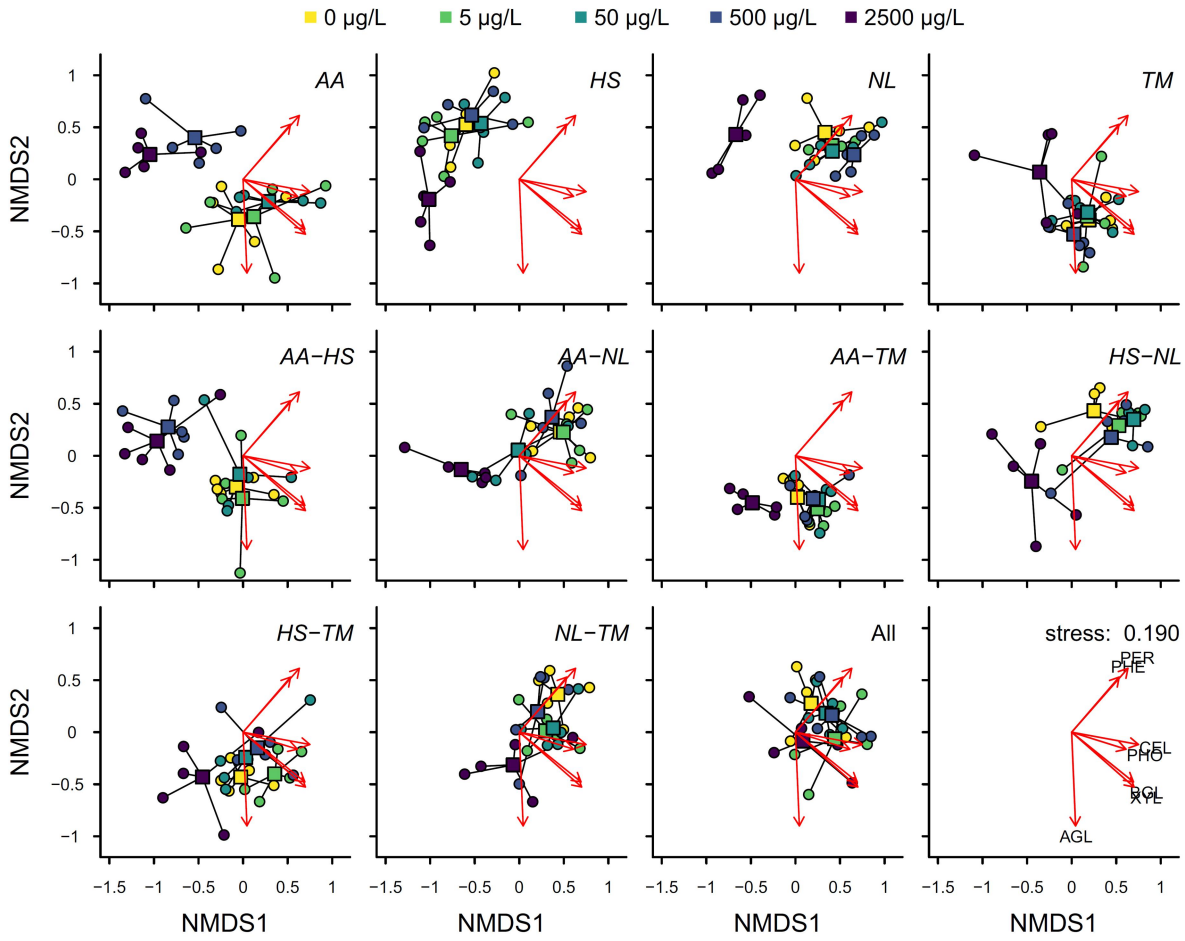



Fig. 2. Non-metric multidimensional scaling (NMDS) plots for enzyme activity profiles using Euclidean distance. Data shown in panels (a-l) originate from a common distance matrix. The panels show enzyme profiles for monocultures and mixed cultures exposed to fungicide sum concentrations of 0, 5, 50, 500, and 2500 µg/L. Group centroids and replicates are displayed as squares and circles, respectively. Red arrows display enzyme activities. The provided stress value indicates a reasonable fit (i.e., <0.2; Clarke, 1993). For the impact of the treatment factors on enzyme activity profiles, see Table S3 (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, *TM*: *T. marchalianum*, PHO: phosphatase, AGL: a-1,4-glucosidase, BGL: b-1,4-glucosidase, CEL: cellobiohydrolase, XYL: b-1,4-xylosidaseperoxidase, PER: peroxidase, and PHE: phenol oxidase).

In the field, *N. lugdunensis* and *T. marchalianum* frequently dominate AH communities inhabiting aquatic ecosystems severely contaminated with metals or xenobiotics (Solé et al., 2008; Sridhar et al., 2000, 2005). Therefore, and considering the stressors applied in this study specifically target fungi, the remarkable tolerance of these species to fungicides can be assumed to be based on highly sophisticated detoxification systems. Detoxification processes in AHs have, however, mostly been investigated with respect to metals (Krauss et al., 2011) and increases in glutathione synthesis are suspected to play a major role in the metal tolerance of *N. lugdunensis*

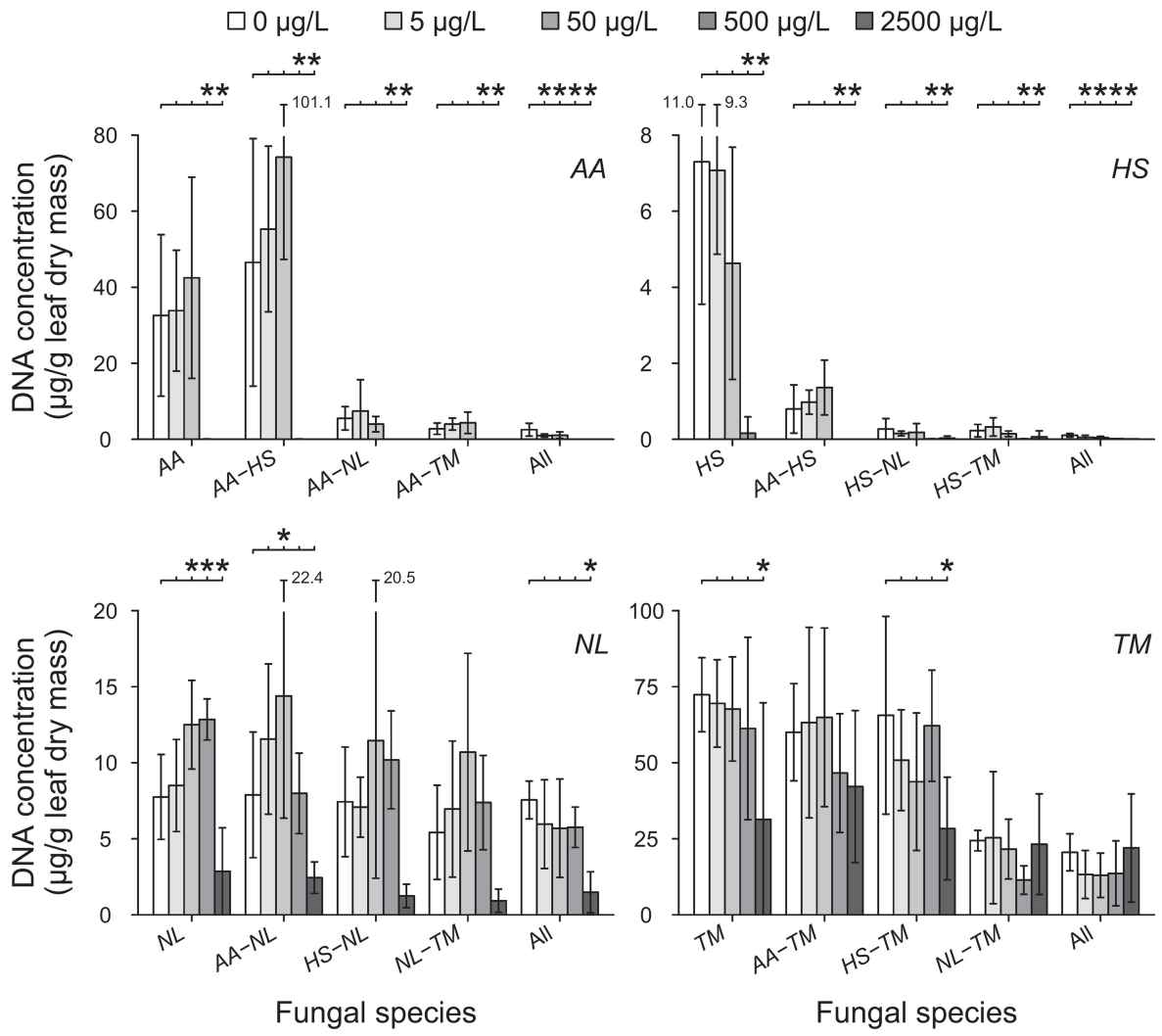



Fig. 3. Mean DNA concentrations (with 95%-confidence intervals) of *A. acuminata*, *H. stellata*, *N. lugdunensis*, and *T. marchalianum* in fungal species treatments exposed to fungicide sum concentrations of 0, 5, 50, 500, and 2500 µg/L (gray-scale bars). Asterisks indicate significant differences between fungicide treatments and fungicide-free controls (Dunnett's test; $p < 0.05$; $n = 5$; *AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*).

and *T. marchalianum* (Braha et al., 2007; Miersch et al., 2005). Yet, this detoxification pathway may also explain the tolerance of these species to synthetic fungicides, as glutathione is involved in the elimination of reactive oxygen species formatted upon xenobiotic action as well as in the conjugation of xenobiotics during phase II biotransformation reactions (Pócsi et al., 2004). Moreover, fungal ligninolytic enzymes (e.g., peroxidase and phenol oxidase) can metabolize aromatic xenobiotics (Harms et al., 2011), mitigating their toxicity (Artigas et al., 2017). However, as the most tolerant AH species of the present study had no elevated oxidase activities (i.e., *T. marchalianum*; Fig. 2d) this degradation pathway seems less relevant. To unravel the underlying biotransformation processes, the combination of "-omics" (e.g., a well-

coordinated use of genomics, transcriptomics and proteomics; Tsui et al., 2016) and chemical analyses (internal vs. external concentrations of fungicides and their metabolites) is a promising approach.

Predictions of leaf mass loss based on DNA concentrations largely matched the observations in fungicide-exposed monocultures (Fig. 1a). Accordingly, fungicide exposure does not seem to affect biomass-specific functional performances. However, three out of these 16 comparisons indicated significant differences (Fig. 1a): Two cases are likely the result of fungicide-altered DNA concentrations (as discussed above) over-estimating leaf decomposition by *N. lugdunensis* (Baudy et al., 2020). Additionally, at a fungicide sum concentration of 500 µg/L, *T. marchalianum* decomposed more leaf mass than predicted (Fig. 1a). As this effect seems independent of fungicide mixture concentrations and could not be explained by enzyme activity or other variables (Fig. 1b; Fig. 2d), this observation may be the result of chance.

RESPONSE OF MIXED CULTURES

Irrespective of the fungicide concentration, leaf mass loss, fungal biomass production and enzyme activity profiles in binary cultures and the quaternary culture show similar patterns as observed in the respective most productive and tolerant species in monoculture (Fig. 1; Fig 2). Accordingly and as hypothesized, functions are maintained in mixed cultures up to high fungicide concentrations (Pimentão et al., 2020; Zubrod et al., 2015a). For both functional variables, predictions significantly deviating from observations mainly concerned mixed AH cultures containing *N. lugdunensis* (6 out of 70 comparisons; Fig. 1), which is discussed above. Again, the few significant deviations concerning other mixed cultures (i.e., 3) are likely not the result of fungicide impacts, as these responses were not concentration-dependent and could not be explained by other variables (Fig. 1b; Fig. 2). Accordingly, and as observed in monocultures, fungicide exposure did not affect biomass-specific functional performances in mixed cultures.

In binary cultures, fungicide exposure resulted in a similar response pattern of individual species' DNA concentrations as observed in monocultures (Fig. 3). In the quaternary culture, however, exposure to already the lowest fungicide sum concentration (i.e., 5 µg/L) significantly reduced DNA concentrations of *A. acuminata* and *H. stellata* by 60% and 50%, respectively (Fig. 3a and b). This may be explained

by an intensified resource competition in the quaternary culture further increasing sensitivity to fungicides in sensitive species (i.e., synergistic effects of multiple stress factors; Steinberg, 2012). While trade-offs between somatic (mycelial) growth and (asexual) reproduction cannot be ruled out (i.e., increased conidial production at the expense of mycelial growth on the leaf substrate), it has been shown previously that fungicide exposure overall counteracts conidial production in AHs (Bundschuh et al., 2011; Dimitrov et al., 2014; Zubrod et al., 2015). Nonetheless and despite the low biomass of *A. acuminata* and *H. stellata* in the quaternary culture (compared to *N. lugdunensis* and *T. marchalianum*; Fig. 3), the present study confirms pronounced fungicide effects on community composition at environmentally relevant concentrations up to 50 µg/L. This shift in community composition is accompanied by alterations in species interactions during leaf colonization. Net diversity effects, which are positively correlated with genetic divergence of the communities (i.e., synergistic interactions increase with genetic distance; Fig. S1; cf. (Baudy et al., accepted)), remained largely unaffected in binary cultures (Fig. 4). Yet, in the quaternary culture, net diversity effects were (partially significantly) reduced from 3% in the fungicide-free control to -32% and -38% at fungicide sum concentrations of 5 and 50 µg/L, respectively (Fig. 4g). These reductions are driven by significant alterations of trait-independent complementarity effects from positive, in the fungicide-free control (i.e., 35%), to negative, at fungicide sum concentrations of 5 and 50 µg/L (-7% and -32%, respectively; Fig. 4g). Accordingly and as hypothesized, fungicide stress apparently induced a shift from complementary towards competitive interactions at the mutual expense of the community (Fox, 2005). Although in binary cultures net diversity effects remained unaffected, fungicide exposure lead to significant alterations of dominance between sensitive and tolerant species (Fig. 4c and d) as well as between the two tolerant species (Fig. 4f). The dominance of *T. marchalianum* over *A. acuminata* was slightly reduced from 31% in the control to 26% and 19% at fungicide sum concentrations of 5 and 50 µg/L, respectively (Fig. 4c). In contrast, the dominance of *N. lugdunensis* over *H. stellata* was substantially increased from 3% in the control to 9% and 43% at fungicide sum concentrations of 5 and 50 µg/L, respectively (Fig. 4d). In the binary culture comprising of the two most tolerant species, the dominance of N. lugdunensis over *T. marchalianum* seems to be maintained up to a fungicide sum concentration of 500 µg/L (-32% to -25%; Fig. 4f),

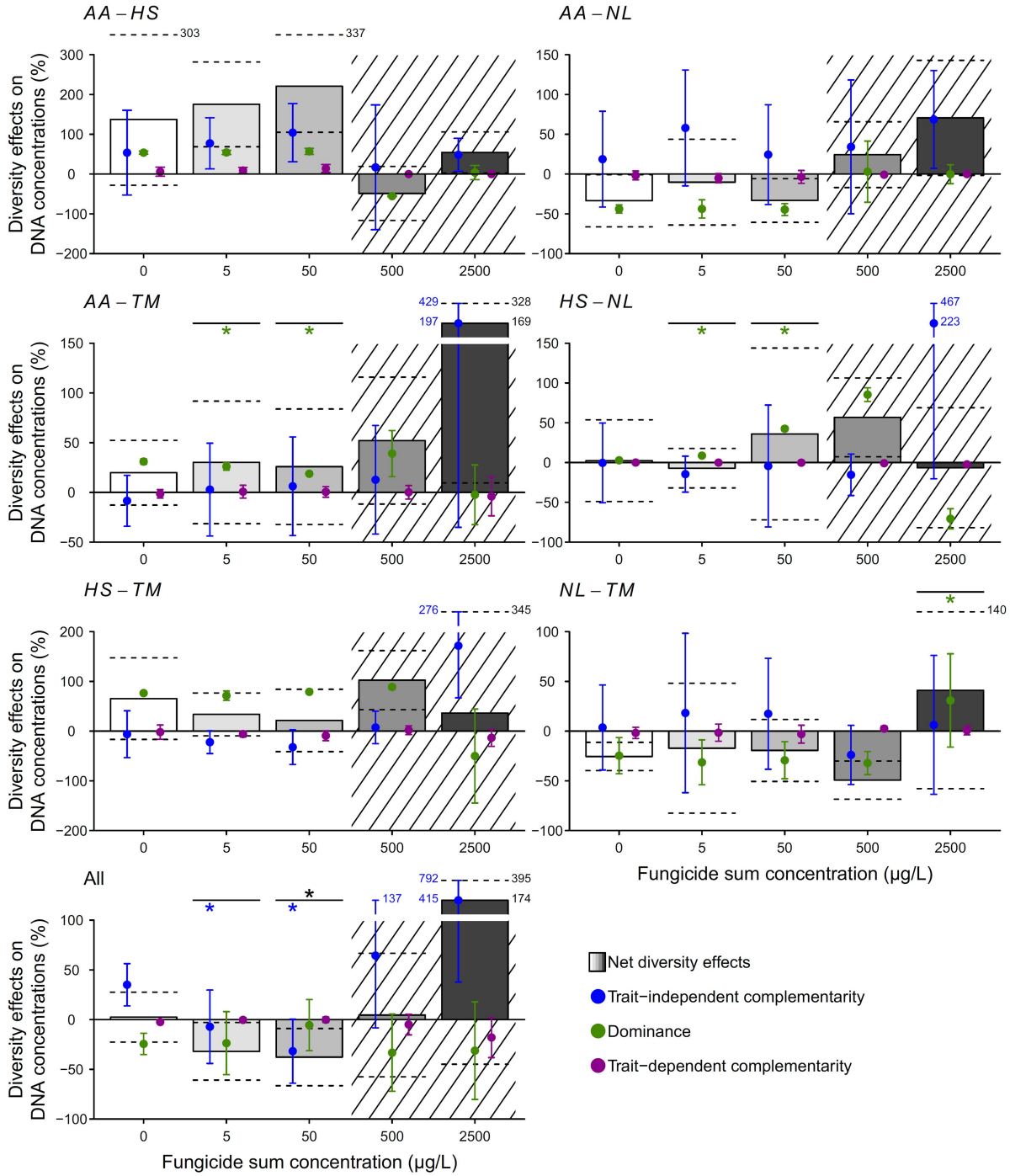



Fig. 4. Mean diversity effects on DNA concentrations (with 95%-confidence intervals) of mixed cultures exposed to fungicide sum concentrations of 0, 5, 50, 500, and 2500 µg/L. Net diversity effects (gray-scale bars) are composed of the sum of trait-independent complementarity (blue points), dominance (green points), and trait-dependent complementarity effects (purple points; for an explanation of these effects see section Diversity effects on leaf colonization). Black and colored asterisks indicate significant differences in net diversity effects or component interaction effects, respectively, between fungicide treatments and fungicide-free controls (Dunnett's test; $p < 0.05$; $n = 5$). Treatments plotted within shaded areas contain species, apparently incapable of surviving under the applied fungicide concentration, as no ergosterol could be detected in the respective monoculture treatment (Fig. 1b). Thus, these treatments likely do not reflect true interactions and are therefore excluded from statistical analysis (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*).

retrospectively characterizing N. lugdunensis under environmentally relevant conditions as the most competitive species. As this relationship is reversed at a fungicide sum concentration of 2500 µg/L (31%; Fig. 4f), *T. marchalianum* seems to be the most tolerant species to fungicide stress (cf. Zubrod et al., 2015a).

## IMPLICATIONS FOR ECOSYSTEM FUNCTIONING IN STREAMS

The results of this microcosm study indicate that AH-mediated leaf litter decomposition may not be affected by environmental fungicide exposures (Fig. 1a). As already low fungicide concentrations can have pronounced effects on community composition (Fig. 3) and diversity (Fernández et al., 2015), the maintenance of the decomposition process (in quantitative terms) seems to be safeguarded by highly tolerant species such a *N. lugdunensis* and *T. marchalianum* (Fig.1), which together cover all of the investigated hydrolytic and oxidative enzymes involved in leaf degradation (Fig. 2c and d). Yet, the universality of these findings needs to be further validated, which may be pursued in future experiments using the tools applied in this study in combination with higher-tier test systems (e.g., indoor or outdoor stream mesocosms), involving different AH species, higher fungal diversity, leaf substrates of varying toughness and fluctuating test conditions.

Analogous to leaf litter decomposition, it could be concluded that AH-mediated leaf conditioning in streams remains unaffected by environmental fungicide exposures, when considering solely total fungal biomass (i.e., ergosterol concentrations) as a surrogate variable for this process (Fig. 1b; Foucreau et al., 2013). However, the leaves' nutritional quality for consumers depends on fungal community composition (Danger et al., 2016). Assessment of community composition in the quaternary culture under fungicide exposure reveals a substantial decline of *A. acuminata* (Fig. 3a), an AH species known to be preferably consumed by stream invertebrates (Arsuffi and Suberkropp, 1989). AH species rather rejected by these consumers, on the contrary, persisted in this community, that is *N. lugdunensis* and *T. marchalianum* (Arsuffi and Suberkropp, 1989; Rong et al., 1995). Moreover, these fungicide-induced alterations of AH community composition reflect those observed in earlier studies, demonstrating the above-discussed feeding preferences in an invertebrate leaf consumer (Bundschuh et al., 2011; Zubrod et al., 2015a). While mechanisms behind this selective feeding are not fully understood, a possible explanation might be fungal interspecific differences in the composition of

macronutrients such as amino acids or essential fatty acids (as reviewed by Danger et al., 2016). Accordingly, chemical stress-induced alterations of AH community composition may affect the physiological fitness of consumers if alternative food sources are lacking (cf. Konschak et al., 2019, 2020).

## CONCLUSION

Leaf mass loss and total fungal biomass production are, amongst others, the most widely used variables to assess the functional integrity of detritus-based stream ecosystems (Colas et al., 2019; Gessner and Chauvet, 2002; Graça et al., 2005). Although the stressors applied in this study (i.e., fungicides) specifically target organisms playing key functional roles in these ecosystems (i.e., fungi), no adverse functional effects were indicated at environmentally relevant concentrations. The presence of highly tolerant species combined with functional redundancy (with respect to these variables), however, masks alterations of AH community composition. Although previous studies provide a strong indication of a close link between AH community composition and leaf consumers' physiological fitness (Konschak et al., 2019, 2020), the mechanistic basis of this relationship is not well understood and requires further research. Accordingly, filling this knowledge gap may foster the assessment of potential (fungicide) stress-induced cascading effects on detritus-based stream food webs.

## CREDIT AUTHOR STATEMENT

Patrick Baudy: Methodology, Project administration, Formal analysis, Visualization, Writing – original draft; Jochen P. Zubrod: Conceptualization, Methodology, Writing – review & editing, Supervision; Marco Konschak: Methodology, Investigation, Writing –review & editing; Nina Röder: Methodology, Investigation, Writing – review & editing; Thu Huyen Nguyen: Methodology, Investigation, Writing – review & editing; Verena C. Schreiner: Investigation, Resources, Writing – review & editing; Christiane Baschien: Resources, Methodology, Formal analysis, Writing – review & editing; Ralf Schulz: Funding acquisition, Conceptualization, Methodology, Resources, Writing – review & editing; Mirco Bundschuh: Funding acquisition, Conceptualization, Methodology, Writing – review & editing, Supervision.

## DECLARATION OF COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## ACKNOWLEDGEMENTS

The authors thank T. Bürgi, D. Englert, S. Lüderwald, A. Pollitt, B. Wahl-Ermel, and S. Wendland for assistance in the laboratory. This study was funded by the German Research Foundation, Project AQUA- REG (DFG; SCHU227/14-1) and Bio2Fun (DFG; BU2439/5-1; 411020793).

## REFERENCES

Abdel-Raheem, A., Shearer, C.A., 2002. Extracellular enzyme production by freshwater ascomycetes. Fungal Diversity 11, 1-19.

Andrade, R., Pascoal, C., Cassio, F., 2016. Effects of inter and intraspecific diversity and genetic divergence of aquatic fungal communities on leaf litter decomposition-a microcosm experiment. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 92.

Arsuffi, T.L., Suberkropp, K., 1989. Selective feeding by shredders on leaf-colonizing stream fungi: comparison of macroinvertebrate taxa. Oecologia 79, 30e37.

Artigas, J., Rossi, F., Gerphagnon, M., Mallet, C., 2017. Sensitivity of laccase activity to the fungicide tebuconazole in decomposing litter. Sci. Total Environ. 584-585, 1084-1092.

Artigas, J., Majerholc, J., Foulquier, A., Margoum, C., Volat, B., Neyra, M., Pesce, S., 2012. Effects of the fungicide tebuconazole on microbial capacities for litter breakdown in streams. Aquat. Toxicol. 122-123, 197-205.

Bärlocher, F., 2016. Aquatic hyphomycetes in a changing environment. Fungal Ecology 19, 14-27.

Bärlocher, F., 1992. Effects of drying and freezing autumn leaves on leaching and colonization by aquatic hyphomycetes. Freshw. Biol. 28, 1e7.

Baschien, C., Tsui, C.K.-M., Gulis, V., Szewzyk, U., Marvanová, L., 2013. The molecular phylogeny of aquatic hyphomycetes with affinity to the Leotiomycetes. Fungal Biology 117, 660-672.

Baudy, P., Zubrod, J.P., Konschak, M., Kolbenschlag, S., Pollitt, A., Baschien, C., Schulz, R., Bundschuh, M., accepted. Fungal-fungal and fungal-bacterial interactions in aquatic decomposer communities: bacteria promote fungal diversity. Ecology.

Baudy, P., Konschak, M., Sakpal, H., Baschien, C., Schulz, R., Bundschuh, M., Zubrod, J.P., 2020. The fungicide tebuconazole confounds concentrations of molecular biomarkers estimating fungal biomass. Bull. Environ. Contam. Toxicol. 105, 620-625.

Baudy, P., Zubrod, J.P., Röder, N., Baschien, C., Feckler, A., Schulz, R., Bundschuh, M., 2019. A glance into the black box: novel species-specific quantitative real-time PCR assays to disentangle aquatic hyphomycete community composition. Fungal Ecology 42, 100858.

Biggs, C.R., Yeager, L.A., Bolser, D.G., Bonsell, C., Dichiera, A.M., Hou, Z., Keyser, S.R., Khursigara, A.J., Lu, K., Muth, A.F., Negrete, B., Erisman, B.E., 2020. Does functional redundancy affect ecological stability and resilience? A review and meta-analysis. Ecosphere 11, 1.

Braha, B., Tintemann, H., Krauss, G., Ehrman, J., Bärlocher, F., Krauss, G.-J., 2007. Stress response in two strains of the aquatic hyphomycete *Heliscus lugdunensis* after exposure to cadmium and copper ions. Biometals 20, 93-105.

Bundschuh, M., Zubrod, J.P., Kosol, S., Maltby, L., Stang, C., Duester, L., Schulz, R., 2011. Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. Aquat. Toxicol. 104, 32-37.

Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18, 117-143.

Colas, F., Woodward, G., Burdon, F.J., Guérold, F., Chauvet, E., Cornut, J., Cébron, A., Clivot, H., Danger, M., Danner, M.C., Pagnout, C., Tiegs, S.D., 2019. Towards a simple global-standard bioassay for a key ecosystem process: organic-matter decomposition using cotton strips. Ecol. Indicat. 106, 105466.

Copolovici, L., Kännaste, A., Remmel, T., Niinemets, Ü., 2014. Volatile organic compound emissions from Alnus glutinosa under interacting drought and herbivory stresses. Environ. Exp. Bot. 100, 55-63.

Cummins, K.W., Klug, M.J., 1979. Feeding ecology of stream invertebrates. Annu. Rev. Ecol. Systemat. 10, 147-172.

Dang, C.K., Chauvet, E., Gessner, M.O., 2005. Magnitude and variability of process rates in fungal diversity-litter decomposition relationships. Ecol. Lett. 8, 1129-1137.

Danger, M., Gessner, M.O., Bärlocher, F., 2016. Ecological stoichiometry of aquatic fungi: current knowledge and perspectives. Fungal Ecology 19, 100-111.

DeForest, J.L., 2009. The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and l-DOPA. Soil Biol. Biochem. 41, 1180-1186.

Dighton, J., White, J.F. (Eds.), 2017. The Fungal Community: its Organization and Role in the Ecosystem. CRC Press, Boca Raton, USA.

Dijksterhuis, J., van Doorn, T., Samson, R., Postma, J., 2011. Effects of seven fungicides on non-target aquatic fungi. Water Air Soil Pollut. 222, 421-425.

Dimitrov, M.R., Kosol, S., Smidt, H., Buijse, L., van den Brink, P.J., Van Wijngaarden, R.P.A., Brock, T.C.M., Maltby, L., 2014. Assessing effects of the fungicide tebuconazole to heterotrophic microbes in aquatic microcosms. Sci. Total Environ. 490, 1002-1011.

Duarte, S., Pascoal, C., Cássio, F., Bärlocher, F., 2006. Aquatic hyphomycete diversity and identity affect leaf litter decomposition in microcosms. Oecologia 147, 658-666.

Evans, C.S., Hedger, J.N., 2001. Degradation of plant cell wall polymers. In: Gadd, G.M. (Ed.), Fungi in Bioremediation. British Mycological Society Symposium Series V. 23. Cambridge University Press, Cambridge, UK.

Fernandes, I., Pascoal, C., Cassio, F., 2011. Intraspecific traits change biodiversity effects on ecosystem functioning under metal stress. Oecologia 166, 1019-1028.

Fernández, D., Tummala, M., Schreiner, V.C., Duarte, S., Pascoal, C., Winkelmann, C., Mewes, D., Munoz, K., Schäfer, R.B., 2016. Does nutrient enrichment compensate fungicide effects on litter decomposition and decomposer communities in streams? Aquat. Toxicol. 174, 169-178.

Fernández, D., Voss, K., Bundschuh, M., Zubrod, J.P., Schäfer, R.B., 2015. Effects of fungicides on decomposer communities and litter decomposition in vineyard streams. Sci. Total Environ. 533, 40-48.

Foucreau, N., Puijalon, S., Hervant, F., Piscart, C., 2013. Effect of leaf litter characteristics on leaf conditioning and on consumption by *Gammarus pulex*. Freshw. Biol. 58, 1672-1681.

Fox, J.W., 2005. Interpreting the 'selection effect' of biodiversity on ecosystem function. Ecol. Lett. 8, 846-856.

Fungicide Resistance Action Committee, 2020. FRAC Code List 2020: Fungal Control Agents Sorted by Cross Resistance Pattern and Mode of Action (Including FRAC Code Numbering).

Gessner, M.O., 2005. Ergosterol as a measure of fungal biomass. In: Graça, M.A.S., Bärlocher, F., Gessner, M.O. (Eds.), Methods to Study Litter Decomposition: a Practical Guide. Springer-Verlag, Dordrecht, Netherlands, pp. 189-195.

Gessner, M.O., Chauvet, E., 2002. A case for using litter breakdown to assess functional stream integrity. Ecol. Appl. 12, 498-510.

Gessner, M.O., Chauvet, E., 1993. Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. Appl. Environ. Microbiol. 59, 502-507.

Gessner, M.O., Swan, C.M., Dang, C.K., McKie, B.G., Bardgett, R.D., Wall, D.H., Hättenschwiler, S., 2010. Diversity meets decomposition. Trends Ecol. Evol. 25, 372-380.

Graça, M.A.S., Bärlocher, F., Gessner, M.O. (Eds.), 2005. Methods to Study Litter Decomposition: a Practical Guide. Springer-Verlag, Dordrecht, Netherlands.

Grossart, H.-P., Rojas-Jimenez, K., 2016. Aquatic fungi: targeting the forgotten in microbial ecology. Curr. Opin. Microbiol. 31, 140-145.

Harms, H., Schlosser, D., Wick, L.Y., 2011. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nat. Rev. Microbiol. 9, 177-192.

Konschak, M., Zubrod, J.P., Baudy, P., Englert, D., Herrmann, B., Schulz, R., Bundschuh, M., 2019. Waterborne and diet-related effects of inorganic and organic fungicides on the insect leaf shredder *Chaetopteryx villosa* (Trichoptera). Aquat. Toxicol. 206, 33-42.

Konschak, M., Zubrod, J.P., Baudy, P., Fink, P., Kenngott, K., Lüderwald, S., Englert, K., Jusi, C., Schulz, R., Bundschuh, M., 2020. The importance of diet-related effects of the antibiotic ciprofloxacin on the leaf-shredding invertebrate *Gammarus fossarum* (Crustacea; Amphipoda). Aquat. Toxicol. 222, 105461.

Krauss, G.-J., Solé, M., Krauss, G., Schlosser, D., Wesenberg, D., Bärlocher, F., 2011. Fungi in freshwaters: ecology, physiology and biochemical potential. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 35, 620-651.

Landesamt für Umwelt Rheinland-Pfalz, 2016. Pflanzenschutz- und Arzneimittelwirkstoffe in ausgewählten rheinland-pfälzischen Fließgewässern. Auswertung relevanter organischer Spurenstoffe 2014.

Miersch, J., Neumann, D., Menge, S., Bärlocher, F., Baumbach, R., Lichtenberger, O., 2005. Heavy metals and thiol pool in three strains of *Tetracladium marchalianum*. Mycol. Prog. 4, 185-194.

Pascoal, C., Cássio, F., Marvanov_a, L., 2005. Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. Arch. Hydrobiol. 162, 481-496.

Pimentão, A.R., Pascoal, C., Castro, B.B., Cássio, F., 2020. Fungistatic effect of agrochemical and pharmaceutical fungicides on non-target aquatic decomposers does not translate into decreased fungi- or invertebrate-mediated decomposition. Sci. Total Environ. 712, 135676.

Pócsi, I., Prade, R.A., Penninckx, M.J., 2004. Glutathione, altruistic metabolite in fungi. In: Poole, R.K. (Ed.), Advances in Microbial Physiology, Advances in Microbial Physiology, vol. 49. Elsevier/Academic Press, Amsterdam, pp. 1-76.

R Core Team, 2018. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Rabiet, M., Margoum, C., Gouy, V., Carluer, N., Coquery, M., 2010. Assessing pesticide concentrations and fluxes in the stream of a small vineyard catchment. Effect of sampling frequency. Environ. Pollut. 158, 737-748.

Rong, Q., Sridhar, K.R., Bärlocher, F., 1995. Food selection in three leaf-shredding stream invertebrates. Hydrobiologia 316, 173e181.

Solé, M., Fetzer, I., Wennrich, R., Sridhar, K.R., Harms, H., Krauss, G., 2008. Aquatic hyphomycete communities as potential bioindicators for assessing anthropogenic stress. Sci. Total Environ. 389, 557-565.

Sridhar, K.R., Bärlocher, F., Krauss, G.-J., Krauss, G., 2005. Response of aquatic hyphomycete communities to changes in heavy metal exposure. Int. Rev. Hydrobiol. 90, 21-32.

Sridhar, K.R., Krauss, G., Bärlocher, F., Wennrich, R., Krauss, G.J., 2000. Fungal diversity in heavy metal polluted waters in central Germany. Fungal Divers. 5, e129.

Steinberg, C.E.W., 2012. Stress Ecology: Environmental Stress as Ecological Driving Force and Key Player in Evolution. Springer, Dordrecht, Netherlands.

Stenersen, J., 2004. Chemical Pesticides Mode of Action and Toxicology. CRC Press, Brussels.

Suberkropp, K., Arsuffi, T.L., Anderson, J.P., 1983. Comparison of degradative ability, enzymatic activity, and palatability of aquatic hyphomycetes grown on leaf litter. Appl. Environ. Microbiol. 46, 237-244.

Tsui, C.K.M., Baschien, C., Goh, T.-K., 2016. Biology and ecology of freshwater fungi. In: Li, D.-W. (Ed.), Biology of Microfungi. Springer International Publishing, Cham, pp. 285-313.

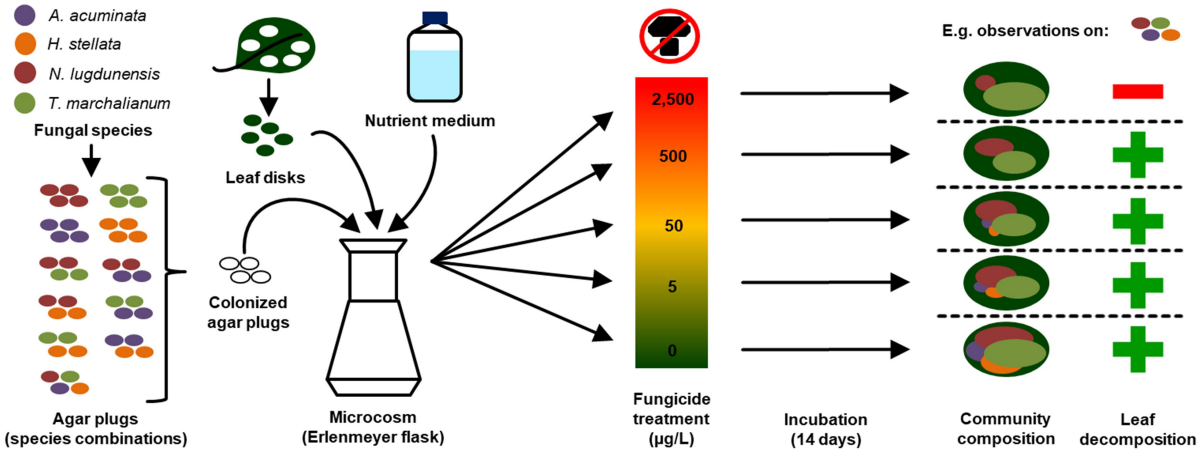
Vörösmarty, C.J., McIntyre, P.B., Gessner, M.O., Dudgeon, D., Prusevich, A., Green, P., Glidden, S., Bunn, S.E., Sullivan, C.A., Liermann, C.R., Davies, P.M., 2010. Global threats to human water security and river biodiversity. Nature 467, 555-561.

Zubrod, J.P., Bundschuh, M., Arts, G., Brühl, C.A., Imfeld, G., Knäbel, A., Payraudeau, S., Rasmussen, J.J., Rohr, J., Scharmüller, A., Smalling, K., Stehle, S., Schulz, R., Schäfer, R.B., 2019. Fungicides: an overlooked pesticide class? Environ. Sci. Technol. 53, 3347-3365.

Zubrod, J.P., Englert, D., Feckler, A., Koksharova, N., Konschak, M., Bundschuh, R., Schnetzer, N., Englert, K., Schulz, R., Bundschuh, M., 2015a. Does the current fungicide risk assessment provide sufficient protection for key drivers in aquatic ecosystem functioning? Environ. Sci. Technol. 49, 1173-1181.

Zubrod, J.P., Englert, D., Wolfram, J., Wallace, D., Schnetzer, N., Baudy, P., Konschak, M., Schulz, R., Bundschuh, M., 2015b. Waterborne toxicity and diet-related effects of fungicides in the key leaf shredder *Gammarus fossarum* (Crustacea: Amphipoda). Aquat. Toxicol. 169, 105-112.

## GRAPHICAL ABSTRACT



## SUPPLEMENTARY DATA

DETAILS ON FUNGAL STRAINS

Table S1. Information on fungal strain identity, GenBank accession numbers, functional characteristics and genetic divergence

| Species | Strain specification | ITS sequence (GenBank accession number) | Growth rate in fungicide-free monocultures (µg ergosterol / g leaf dry mass / day) | Mean specific leaf mass loss (% leaf mass loss / µg DNA / g leaf dry mass)* | Mean specific ergosterol concentration (µg ergosterol / g leaf dry mass / µg DNA / g leaf dry mass)# | Genetic divergence (% base substitutions) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *Alatospora acuminata* | *Heliscella stellata* | *Neonectria lugdunensis* | *Tetracladium marchalianum* |
| *Alatospora acuminata* | DSM 104360 | MH930815 | 23.30 | 0.145 | 11.562 | 0 | - | - | - |
| *Heliscella stellata* | DSM 104386 | MH930820 | 6.11 | -0.651 | 12.406 | 39 | 0 | - | - |
| *Neonectria lugdunensis* | DSM 104361 | MH930822 | 39.11 | 3.334 | 74.203 | 22 | 41 | 0 | - |
| *Tetracladium marchalianum* | DSM 104373 | MH930823 | 51.71 | 0.266 | 10.170 | 18 | 37 | 25 | 0 |

Abbreviations: ITS = internal transcribed spacer; DNA = deoxyribonucleic acid

* Calculated from fungicide-free monocultures as: $F_{lml} = \frac{\sum_{i=1}^{n} L_i \,/\, c_{DNA\,i}}{n}$, where $L_i$ and $c_{DNA\,i}$ are the leaf mass loss and DNA concentration in replicate *i*, respectively, and *n* is the number of replicates of a monoculture treatment

\# Calculated from fungicide-free monocultures as: $F_{erg} = \frac{\sum_{i=1}^{n} E_i \,/\, c_{DNA\,i}}{n}$, where $E_i$ and $c_{DNA\,i}$ are the ergosterol concentration and DNA concentration in replicate *i*, respectively, and *n* is the number of replicates of a monoculture treatment

ASSESSMENT OF BACTERIAL CONTAMINATION

To check for contamination with bacteria, which potentially may have interfered with fungal activity, 24 randomly chosen microcosms were analyzed for bacterial densities following the method described in Buesing (2005). Briefly, bacteria were detached from the leaf sample, filtered onto membrane filters, and nucleic acids were stained with SYBR Green II (Molecular Probes, USA). Using a camera-mounted epifluorescence microscope (Axioscope.A1, Carl Zeiss AG, Germany), 1000-fold magnified microscopic fields were digitally captured and quantified for bacterial cells by image analysis software (AxioVision 4.8.2; Carl Zeiss, Germany). The detailed procedure is described in Zubrod et al. (2011).

Among the analyzed microcosms, 20 were free of bacterial contamination (i.e., 83%). In the remaining four microcosms (replicates of *A. acuminata-N. lugdunensis*: 50 µg/L, *A. acuminata-N. lugdunensis*: 2,500 µg/L, *H. stellata-T. marchalianum*: 50 µg/L, and the quaternary culture: 50 µg/L), cell densities between $0.5*10^6$ and $12.9*10^6$ cells per mg leaf dry weight ($\overline{x}=6.9*10^6$) indicated slight bacterial contamination (cf. Zubrod et al., 2017), while other parameters did not show any signs of bacterial influence.

## ASSOCIATIONS BETWEEN LEAF MASS LOSS AND ENZYME ACTIVITIES

Using analysis of covariance (ANCOVA) on ranks, it was assessed whether cumulated enzyme activities and fungicide treatment or their interaction affected leaf mass loss. As absolute enzyme activities do not necessarily reflect the relative importance of an enzyme compared to others (German et al., 2011), individual activities were rank-transformed before replicate-wise summation. The ANCOVA output shown in Table S5 reveals a strong linear relationship between leaf mass loss and cumulative enzyme activities ($F$=124.287; $p$<0.001). While fungicide treatment has a slight but statistically significant impact on leaf mass loss ($F$=4.080; $p$=0.003), its impact on the relationship between leaf mass loss and enzyme activities is considered negligible, as indicated by the non-significant interaction term ($F$=2.142; $p$=0.076).

Table S2. Results of the ANCOVA assessing the effects of the sum of rank-transformed enzyme activities and fungicide treatment as well as their interaction on rank-transformed leaf mass loss (for interpretation of the output, see text above).

| **Source of variation** | ***D.f.*** | ***SS*** | ***MS*** | ***F*** | ***p*** |
|---|---|---|---|---|---|
| Enzyme activities | 1 | 520061 | 520061 | 124.287 | **<0.001** |
| Fungicide treatment | 4 | 68281 | 17070 | 4.080 | **0.003** |
| Enzyme activities × fungicide treatment | 4 | 35846 | 8961 | 2.142 | 0.076 |
| Residuals | 265 | 1108859 | 4184 | | |

## EFFECTS ON ENZYME ACTIVITY PROFILES

Table S3. Results of the three-way PERMANOVA assessing the effects of diversity, fungicide treatment, species combination (nested within diversity) as well as their interactions on enzyme activity profiles.

| **Source of variation** | ***D.f.*** | ***SS*** | ***MS*** | ***F*** | ***$R^2$*** | ***p*** |
|---|---|---|---|---|---|---|
| Diversity | 2 | 8.227 | 4.1133 | 8.7086 | 0.03911 | **0.001** |
| Fungicide treatment | 4 | 24.820 | 6.2049 | 13.1369 | 0.11799 | **0.001** |
| Species combination (Diversity) | 8 | 43.170 | 5.3963 | 11.4249 | 0.20523 | **0.001** |
| Diversity × fungicide treatment | 8 | 4.995 | 0.6244 | 1.3219 | 0.02375 | 0.096 |
| Species combination (Diversity) × fungicide treatment | 32 | 25.223 | 0.7882 | 1.6688 | 0.11991 | **0.001** |
| Residuals | 220 | 103.912 | 0.4723 | | 0.49400 | |

ASSOCIATIONS BETWEEN NET DIVERSITY EFFECTS AND FUNGAL GROWTH RATE OR GENETIC DIVERGENCE

Average growth rates were calculated as the arithmetic mean from growth rates (Table S1) of combinations of two species and the combination of all four fungi. Genetic divergence between combinations of two and four species was calculated based on the internal transcribed spacer (ITS) region of the ribosomal DNA operon (i.e., the most frequently used barcoding region for fungi; Schoch et al., 2012). Therefore, ITS sequences of the four fungi (Table S1) were trimmed to a common section (i.e., partial ITS1, 5.8S, and ITS2). The trimmed sequences were aligned and paired distances as well as overall mean distance of all four fungi were calculated, using the software MEGA X (Kumar et al., 2018) with default settings (Maximum Composite Likelihood substitution model). The mean overall mean distance was 30% and pairwise distances are shown in Table S1.

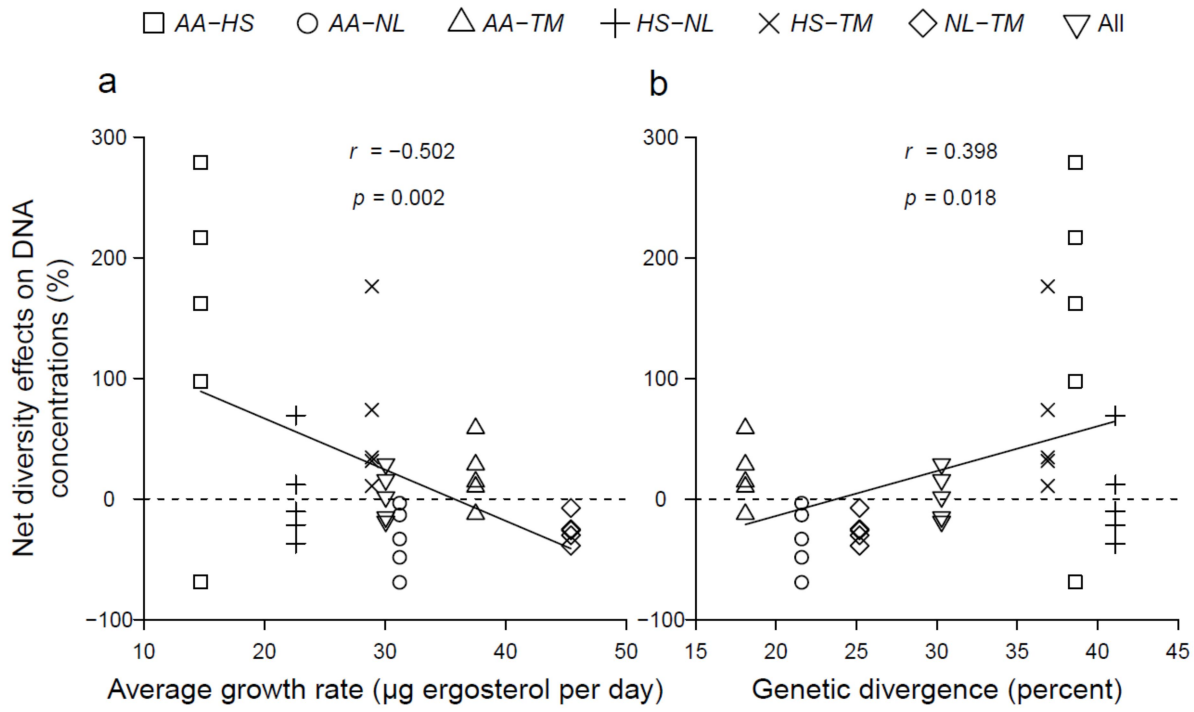



Figure S1. Correlations between net diversity effects on DNA concentrations and average growth rates (a) or genetic divergence (b) of fungal mixed cultures (fungicide-free control) with Pearson's correlation coefficients (r) and p-values. The regression lines were added to visualize the relationships between the variables. (*AA*: *A. acuminata*, *HS*: *H. stellata*, *NL*: *N. lugdunensis*, and *TM*: *T. marchalianum*).

REFERENCES

Buesing, N., 2005. Bacterial counts and biomass determination by epifluorescence microscopy., in: Graça, M.A.S., Bärlocher, F., Gessner, M.O. (Eds.), Methods to study litter decomposition: a practical guide. Springer-Verlag, Dordrecht, Netherlands, pp. 203–208.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35, 1547–1549.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America 109, 6241–6246.

Zubrod, J.P., Bundschuh, M., Feckler, A., Englert, D., Schulz, R., 2011. Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. Environmental Toxicology and Chemistry 30, 2718–2724.

Zubrod, J.P., Englert, D., Wolfram, J., Rosenfeldt, R.R., Feckler, A., Bundschuh, R., Seitz, F., Konschak, M., Baudy, P., Luderwald, S., Fink, P., Lorke, A., Schulz, R., Bundschuh, M., 2017. Long-term effects of fungicides on leaf-associated microorganisms and shredder populations-an artificial stream study. Environmental Toxicology and Chemistry.

**APPENDIX A.5**

## CURRICULUM VITAE


| | |
|---|---|
| Name: | Patrick Baudy-Groh (né Baudy) |
| Born: | December 11, 1988 in Landau, Rhineland-Palatinate, Germany |
| Address: | Trifelsring 11<br>76857 Albersweiler, Rhineland-Palatinate<br>Germany |


### EDUCATION

| | |
|---|---|
| Since 04/2016 | Scientist at the Institute for Environmental Sciences, University of Koblenz-Landau, Campus Landau, Germany |
| 03/2016 | Diploma Environmental Sciences (thesis title: „Does long-term fungicide exposure affect the reproductive performance of leaf-shredders? A partial life-cycle study using *Hyalella azteca*", published in *Environmental Pollution*) |
| 10/2008 - 03/2016 | Studies in Environmental Sciences at the University of Koblenz-Landau, Campus Landau, Germany |
| 05/2008 | Final secondary school (Abitur) at the Pamina Gymnasium, Herxheim, Germany |

**PUBLICATION LIST**

PEER-REVIEWED ARTICLES

2021:

**Baudy, P.**, Zubrod, J.P., Konschak, M., Röder, N., Nguyen, T.H., Schreiner, V.C., Baschien, C., Schulz, R., Bundschuh, M., 2021. Environmentally relevant fungicide levels modify fungal community composition and interactions but not functioning. Environmental Pollution 285, 117234.

**Baudy, P.**, Zubrod, J.P., Konschak, M., Kolbenschlag, S., Pollitt, A., Baschien, C., Schulz, R., Bundschuh, M., 2021. Fungal-fungal and fungal-bacterial interactions in aquatic decomposer communities: bacteria promote fungal diversity. Ecology 102, e03471.

Konschak, M., Zubrod, J.P., Duque Acosta, T.S., Bouchez, A., Kroll, A., Feckler, A., Röder, N., **Baudy, P.**, Schulz, R., Bundschuh, M., 2021. Herbicide-Induced Shifts in the Periphyton Community Composition Indirectly Affect Feeding Activity and Physiology of the Gastropod Grazer *Physella acuta*. Environmental Science & Technology 55, 14699-14709

Konschak, M., Zubrod, J.P., **Baudy, P.**, Fink, P., Pietz, S., Duque Acosta, T.S., Bakanov, N., Schulz, R., Bundschuh, M., 2021. Mixture effects of a fungicide and an antibiotic: assessment and prediction using a decomposer-detritivore system. Aquatic Toxicology 232, 105762.

Konschak, M., Zubrod, J.P., **Baudy, P.**, Fink, P., Kenngott, K.G.J., Englert, D., Röder, N., Ogbeide, C., Schulz, R., Bundschuh, M., 2021. Chronic effects of the strobilurin fungicide azoxystrobin in the leaf shredder *Gammarus fossarum* (Crustacea; Amphipoda) via two effect pathways. Ecotoxicology and Environmental Safety 209, 111848.

Bundschuh, M., Zubrod, J.P., Konschak, M., **Baudy, P.**, Frombold, B., Schulz, R., 2021. Photoactive titanium dioxide nanoparticles modify heterotrophic microbial functioning. Environmental Science and Pollution Research, 1-9

Bundschuh, M., Zubrod, J.P., Wernicke, T., Konschak, M., Werner, L., Brühl, C.A., **Baudy, P.**, Schulz, R., 2021. Bottom-up effects of fungicides on tadpoles of the European common frog (*Rana temporaria*). Ecology and Evolution, 00:1-13.

2020:

**Baudy, P.**, Konschak, M.., Sakpal, H., Baschien, C., Schulz, R., Bundschuh, M., Zubrod, J.P., 2020. The fungicide tebuconazole confounds concentrations of molecular biomarkers estimating fungal biomass. Bulletin of Environmental Contamination and Toxicology 105, 620-625.

Konschak, M., Zubrod, J.P., **Baudy, P.**, Fink, P., Kenngott, K., Lüderwald, S., Englert, K., Jusi, C., Schulz, R., Bundschuh, M., 2020. The importance of diet-related effects of the antibiotic ciprofloxacin on the leaf-shredding invertebrate *Gammarus fossarum* (Crustacea; Amphipoda). Aquatic Toxicology 222, 105461.

2019:

**Baudy, P.**, Zubrod, J.P., Röder, N., Baschien, C., Feckler, A., Schulz, R., Bundschuh, M., 2019. A glance into the black box: Novel species-specific quantitative real-time PCR assays to disentangle aquatic hyphomycete community composition. Fungal Ecology 42, 100858.

Konschak, M., Zubrod, J.P., **Baudy, P.**, Englert, D., Herrmann, B., Schulz, R., Bundschuh, M., 2019. Waterborne and diet-related effects of inorganic and organic fungicides on the in-sect leaf shredder *Chaetopteryx villosa* (Trichoptera). Aquatic Toxicology 206, 33-42.

2018:

Newton, K., Zubrod, J.P., Englert, D., Lüderwald, S., Schell ,T., **Baudy, P.**, Konschak, M., Feckler, A., Schulz, R., Bundschuh, M., 2018. The evil within? Systemic fungicide application in trees enhances litter quality for an aquatic decomposer-detritivore system. Environmental Pollution 241, 549-556.

2017:

**Baudy, P.**, Zubrod, J.P., Konschak, M., Weil, M., Schulz, R.; Bundschuh, M., 2017. Does long-term fungicide exposure affect the reproductive performance of leaf-shredders? A partial life-cycle study using *Hyalella azteca*. Environmental Pollution 222, 458-464.

Feckler, A., Schrimpf, A., Bundschuh, M., Bärlocher, F., **Baudy, P.**, Cornut, J., Schulz, R., 2017. Quantitative real-time PCR as a promising tool for the

detection and quantification of leaf-associated fungal species – a proof of principle using *Alatospora pulchella* PLoS ONE. 12, e0174634.

Wendland, S., **Baudy, P.**, Fusser, M. S., Entling, M.H., Schirmel, J., 2017. Contrasting predation pressure of different carabids on slugs and their eggs. BioControl 62, 749-756.

Zubrod, J.P., Englert, D., Wolfram, J., Rosenfeldt, R.R., Feckler, A., Bundschuh, R., Seitz, F., Konschak, M., **Baudy, P.**, Lüderwald, S., Fink, P., Lorke, A., Schulz, R., Bundschuh, M., 2017. Long-term effects of fungicides on leaf-associated microorganisms and shredder populations – an artificial stream study. Environmental Toxicology and Chemistry 36, 2178-2189.

2015:

Zubrod, J.P., Englert, D., Wolfram, J., Wallace, D., Schnetzer, N., **Baudy, P.**, Konschak, M., Schulz, R., Bundschuh, M., 2015. Waterborne toxicity and diet-related effects of fungicides in the key leaf shredder *Gammarus fossarum* (Crustacea: Amphipoda). Aquatic Toxicology 169, 105-112.

2014:

Zubrod, J.P., **Baudy, P.**, Schulz, R., Bundschuh, M., 2014. Effects of current-use fungicides and their mixtures on the feeding and survival of the key shredder *Gammarus fossarum*. Aquatic Toxicology 150, 133-143.

ORAL PRESENTATIONS

2019:

Konschak, M., Zubrod, J.P., **Baudy, P.**, Fink, P., Kenngott, K., Lüderwald, S., Englert, D., Jusi, C., Schulz, R., Bundschuh, M., 2019. Effekte des Antibiotikums Ciprofloxacin auf den Zerkleinerer *Gammarus fossarum* – eine Untersuchung zweier Expositionspfade. SETAC GLB 24th Annual Meeting 2019, Landau, Rhineland-Palatinate, Germany.

2018:

Zubrod, J.P., Wernicke, T., Konschak, M., **Baudy, P**., Brühl, C., Schulz, R., Bundschuh, M., 2018. Altered development of the grass frog (*Rana temporaria*) as a consequence of fungicide induced modifications of food

quality. SETAC North America 39th Annual Meeting 2018, Sacramento, California, USA.

2017:

**Baudy, P.**, Zubrod, J.P., Röder, N., Konschak, M., Baschien, C., Schulz, R., Bundschuh, M., 2017. Beeinflussen Fungizide die funktionelle Leistungsfähigkeit laubzersetzender aquatischer Pilzgemeinschaften. SETAC GLB 22nd Annual Meeting, Neustadt, Rhineland-Palatinate, Germany.

**Baudy, P.**, Zubrod, J.P., Röder, N., Konschak, M., Baschien, C., Schulz, R., Bundschuh, M., 2017. Do anthropogenic stressors impact the functional performance of leaf-degrading fungi? ExotoxicoMic 1st International Conference on Microbial Ecotoxicology 2017, Lyon, France.

**Baudy, P.**, Zubrod, J.P., Konschak, M., Weil, M., Schulz, R., Bundschuh, M., 2017. Chronic exposure to field-relevant fungicide concentrations impairs reproduction in the amphipod leaf-shredder *Hyalella azteca*. SETAC Europe 27th Annual Meeting 2017, Brussels, Belgium.

**Baudy, P.**, Zubrod, J.P., Konschak, M., Weil, M., Schulz, R., Bundschuh, M., 2017. Chronic fungicide exposure impairs the reproductive performance of the amphipod leaf-shredder *Hyalella azteca*. Sixth Young Environmental Scientists Meeting 2017, Stockholm, Sweden.

Konschak, M., Zubrod, J.P., **Baudy, P.**, Herrmann, B., Englert, D., Schulz, R., Bundschuh, M., 2017. Are waterborne and diet-related fungicide effects on amphipod leaf-shredders transferable to their insect counterparts? Sixth Young Environmental Scientists Meeting 2017, Stockholm, Sweden.

Zubrod, J.P., Wernicke, T., Konschak, M., **Baudy, P.**, Brühl, C., Schulz, R., Bundschuh, M., 2017. Fungizid-induzierte Veränderungen der Detritus-Qualität beeinflussen die Nahrungsverwertung und Entwicklung des Grasfroschs (*Rana temporaria*). SETAC GLB 22nd Annual Meeting 2017, Neustadt, Rhineland-Palatinate, Germany.

2016:

Konschak, M., Zubrod, J.P., **Baudy, P.**, Herrmann, B., Englert, D., Schulz, R., Bundschuh, M., 2016. Sind wasserexpositionsbezogene und nahrungsbedingte Fungizideffekte unabhängig von der Shredderspezies? SETAC GLB 21st Annual Meeting 2016, Tübingen, Germany.

POSTER PRESENTATIONS

2018:

**Baudy, P.**, Zubrod, J.P., Fahlman, J., Brodin, T., Klaminder, J., Bundschuh, M., 2018. Impact of the antihistamine fexofenadine on the structure and functioning of leaf-associated microbial communities. SETAC Europe 28th Annual Meeting 2018, Rome, Italy.

Konschak, M., Zubrod, J.P., **Baudy, P.**, Lüderwald, S., Jusi, C., Englert, K., Schulz, R., Bundschuh, M., 2018. Direct and indirect effects of antibiotics in the leaf-shredding macroinvertebrate *Gammarus fossarum*. SETAC Europe 28th Annual Meeting 2018, Rome, Italy.

Newton, K., Zubrod, J.P., Englert, D., Schell, T., **Baudy, P.**, Konschak, M., Feckler, A., Schulz, R., Bundschuh, M., 2018. Leaf litter originating from trees treated with systemic fungicides – a new exposure pathway for aquatic decomposer detritivore systems. SETAC Europe 28th Annual Meeting 2018, Rome, Italy.

Röder, N., **Baudy, P.**, Zubrod, J.P., Baschien, C., Schulz, R., Bundschuh, M., 2018. Does fungicide exposure alter interspecific relationships of aquatic fungi during leaf decomposition? A case study using species-specific qPCR assays. SETAC Europe 28th Annual Meeting 2018, Rome, Italy.

2017:

**Baudy, P.**, Zubrod, J.P., Schulz, R., Bundschuh, M., 2017. Development of a standardized aquatic hyphomycete laboratory assay for fungicide risk assessment. ExotoxicoMic 1st International Conference on Microbial Ecotoxicology 2017, Lyon, France.

Konschak, M., Zubrod, J.P., **Baudy, P.**, Englert, D., Lüderwald, S., Jusi, C., Ogbeide, C., Schulz, R., Bundschuh, M., 2017. Antimicrobials modify decomposer detritivore interactions – the role of substances' target microorganisms. SETAC Europe 27th Annual Meeting 2017, Brussels, Belgium.

2016:

**Baudy, P.**, Zubrod, J.P., Konschak, M., Weil, M., Schulz, R., Bundschuh, M., 2016. Does long-term fungicide exposure affect the reproductive performance of leaf-shredders? SETAC Europe 26th Annual Meeting 2016, Nantes, France.

Konschak, M., Zubrod, J.P., **Baudy, P.**, Englert, D., Schulz, R., Bundschuh, M., 2016. Are waterborne and diet-related effects of fungicides transferable among leaf-shredders? SETAC Europe 26th Annual Meeting 2016, Nantes, France.

2013:

Zubrod, J.P., **Baudy, P.**, Schulz, R., Bundschuh, M., 2013. Effects of organic and inorganic current-use fungicides and their mixtures on the feeding of a key shredder species. SETAC North America 34th Annual Meeting 2013, Nashville, Tennessee, USA.

Zubrod, J.P., Koksharova, N., **Baudy, P.**, Konschak, M., Englert, K., Schulz, R., Bundschuh, M., 2013. (In)Direct effects of (in)organic fungicides on an aquatic decomposer-detritivore-system. Third Young Environmental Scientists Meeting 2013, Krakow, Poland.