Additional file 1 for

Effects of fungicides on aquatic fungi and bacteria: a comparison of morphological and molecular approaches from a microcosm experiment

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This document contains supporting information for the manuscript titled above and adds two text sections, 11 figures and 6 tables over 19 pages.

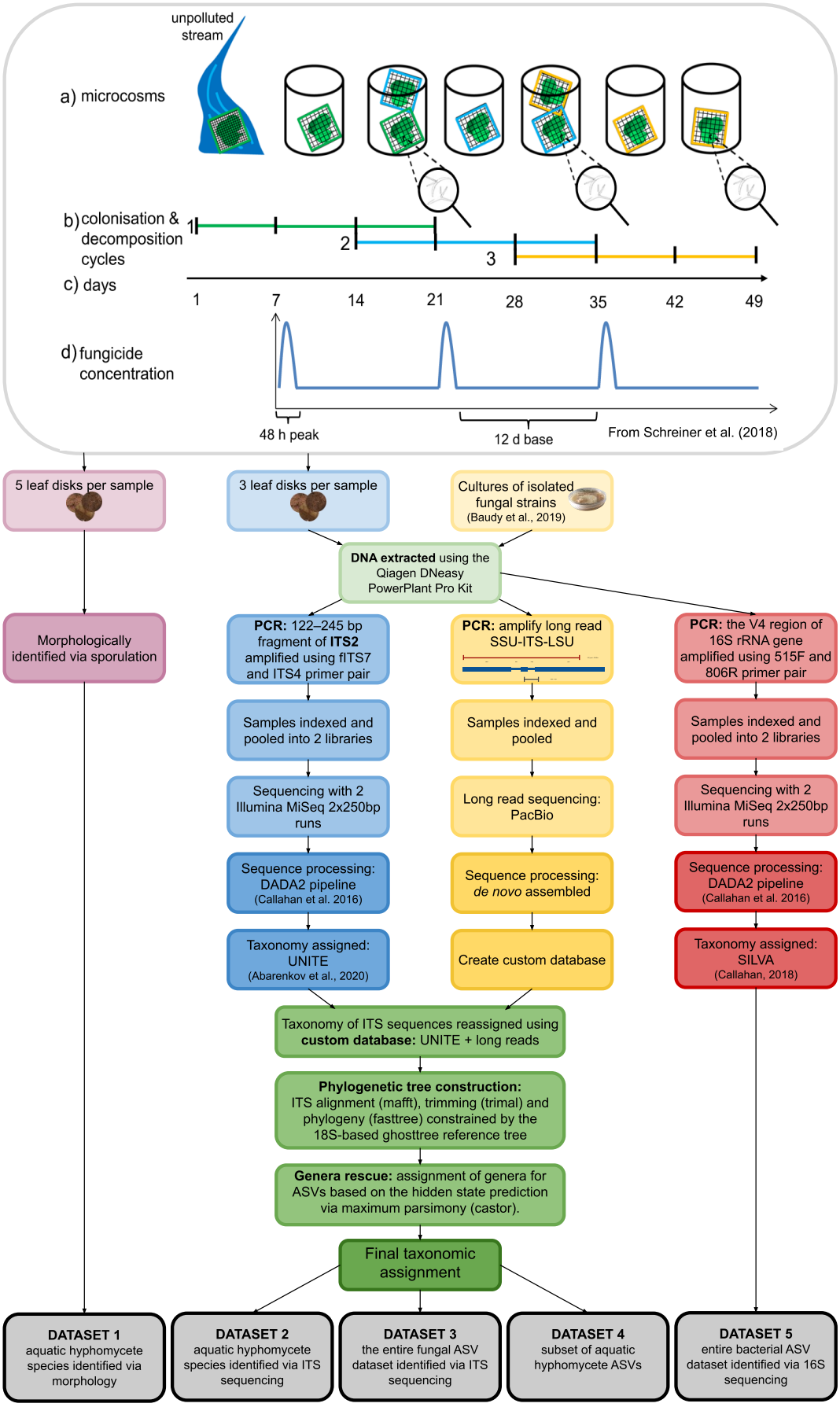

Fig. S1 Workflow resulting in the five community composition data sets. The uppermost box showing a schematic illustration of the experimental design was previously published in Schreiner et al. [1]. ASV: amplicon sequence variant.

Table S1 Fungal strains sequenced via long-read PacBio sequencing. The coverage refers to the subsection of the long-read sequence, which can be matched to the ITS barcode.

Species	Strain	Barcode	Sequence Length (bp)	Coverage (bp)
Cladosporium cladosporioides	DSM 104381	bc1001	4362	486
Tricladium cf. kelleri	DSM 104338	bc1002	4450	479
Alatospora acuminata	DSM 105546	bc1003	4372	480
Culicidospora sp.	DSM 105569	bc1004	5722	476
Lemonniera aquatica	DSM 104336	bc1005	4364	474
Tricladium splendens	DSM 104369	bc1006	4379	475
Filosporella annelidica	DSM 104337	bc1007	4377	484
Tetracladium marchalianum	DSM 104346	bc1008	4374	480
Helicosporium phragmitis	DSM 104385	bc1009	4407	478
Heliscella stellata	DSM 104357	bc1010	4438	373
Idriella lunata	DSM 104378	bc1012	4354	479
Anguillospora crassa	DSM 104363	bc1014	4378	474
Lemonniera terrestris	DSM 104343	bc1016	4364	477
Calloria sp.	DSM 105564	bc1017	4375	476
Anguillospora longissima	DSM 104384	bc1018	4864	471
Clavatospora longibrachiata	DSM 104364	bc1019	4512	351
Tumularia tuberculata	DSM 104368	bc1020	4346	478
Articulospora tetracladia	DSM 104345	bc1021	4366	477
Pseudoanguillospora stricta	DSM 104335	bc1022	4348	482
Flagellospora curvula	DSM 104334	bc1025	4374	464
Neonectria lugdunensis	DSM 104372	bc1026	4347	475

Table S2 Number of fungal amplicon sequence variants (ASVs) assigned to the different taxonomic ranks using the three methods of taxonomic assignment.

Assignment method	Kingdom	Phylum	Class	Order	Family	Genus	Species
1. UNITE	1882	1243	1161	1111	1018	907	635
2. UNITE + long reads	1882	1242	1153	1105	1011	896	640
3. UNITE + long reads + phylogenetic rescued	1882	1242	1153	1105	1025	939	640

UNITE database: Abarenkov et al. [2]

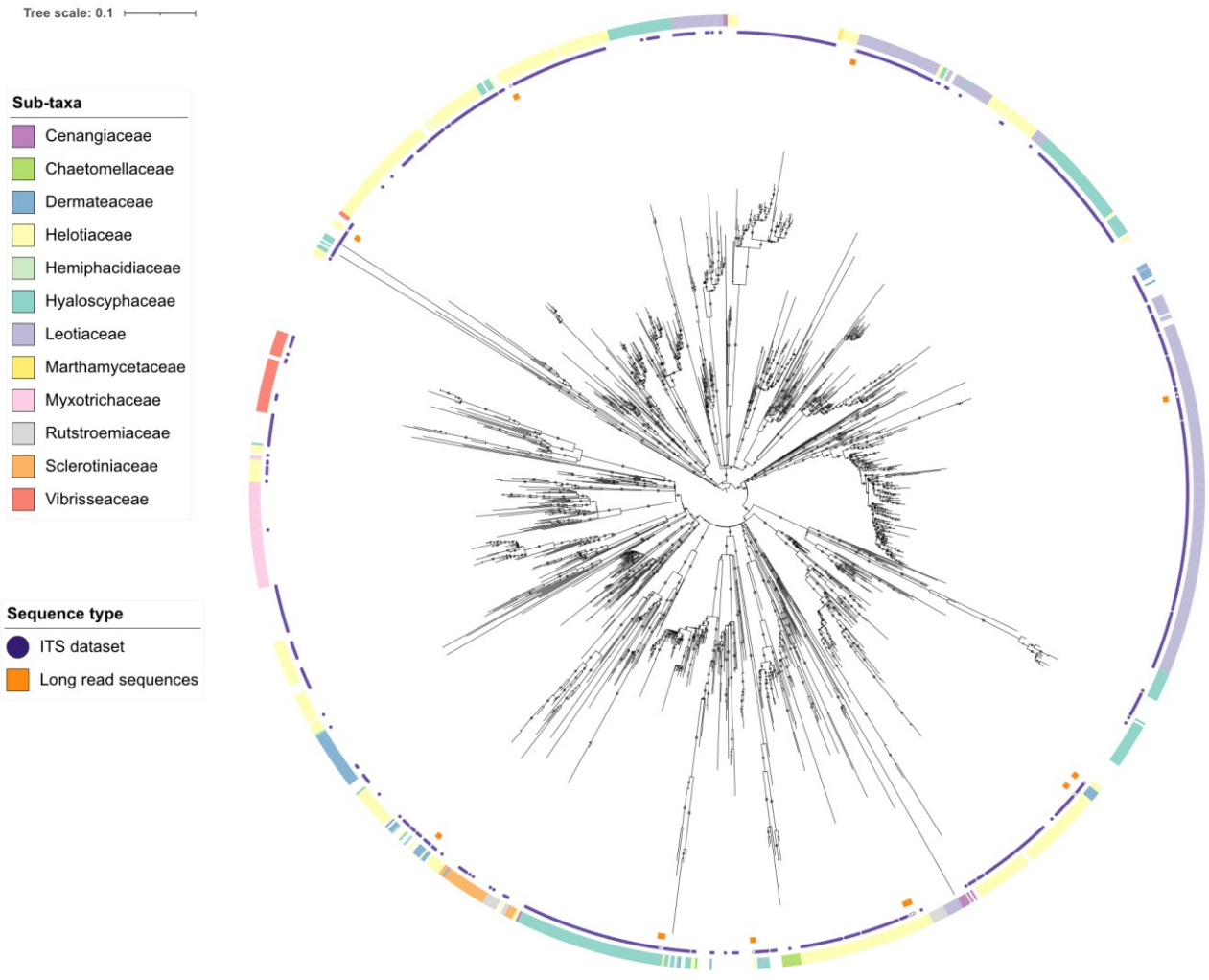

Fig. S2 Phylogenetic tree of the order **Helotiales** (fungi), constructed from sequences from the UNITE database [2], long read sequences of fungal cultures (indicated as orange bars) and ITS metabarcoding sequences from the experimental samples (indicated as purple dots). The outer colour strip indicates the families the sequences are assigned to (see legend on left-hand site).

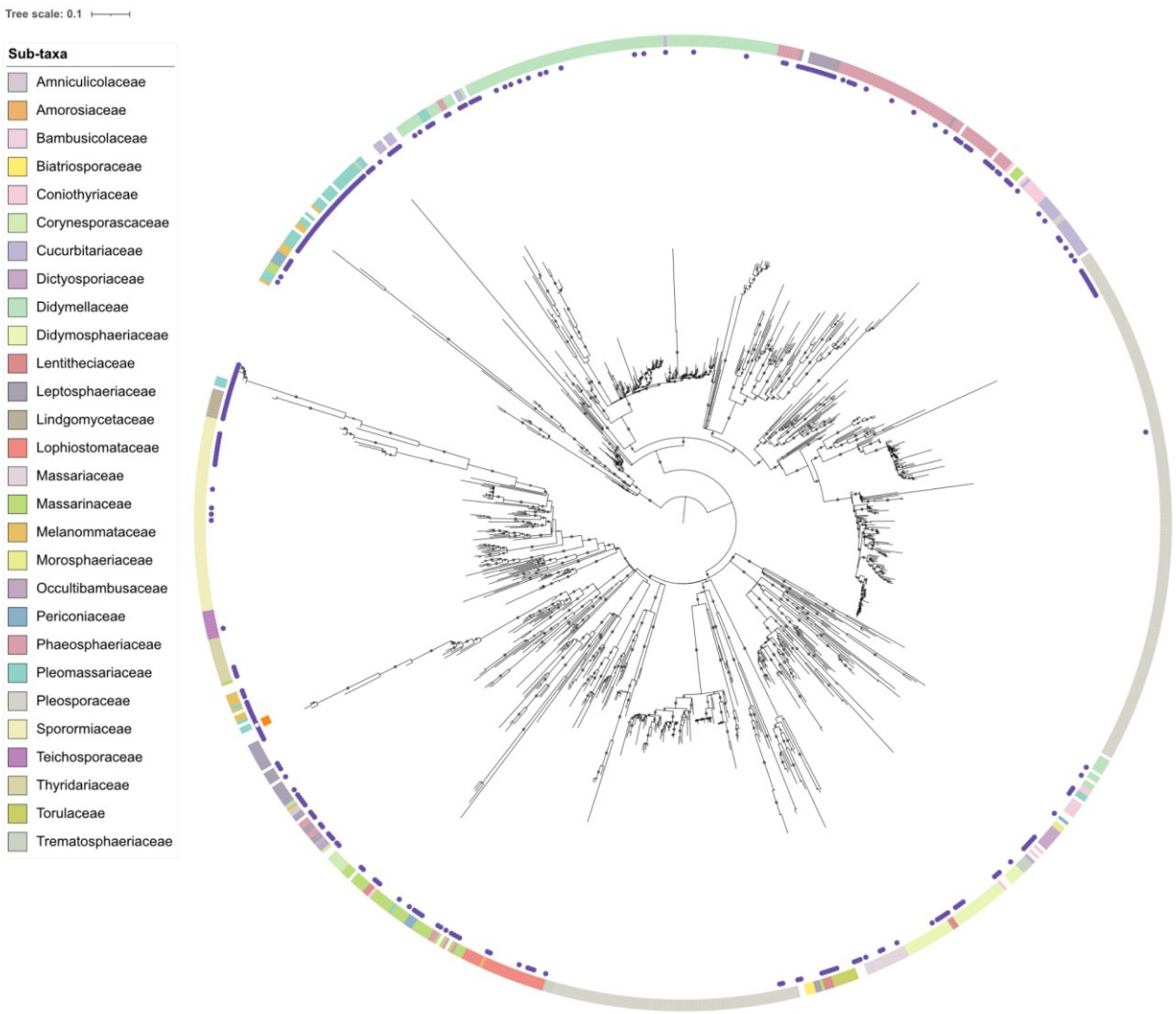

Fig. S3 Phylogenetic tree of the order **Pleosporales** (fungi), constructed from sequences from the UNITE database [2], long read sequences of fungal cultures (indicated as orange bars) and ITS metabarcoding sequences from the experimental samples (indicated as purple dots). The outer colour strip indicates the families the sequences are assigned to (see legend on left-hand site).

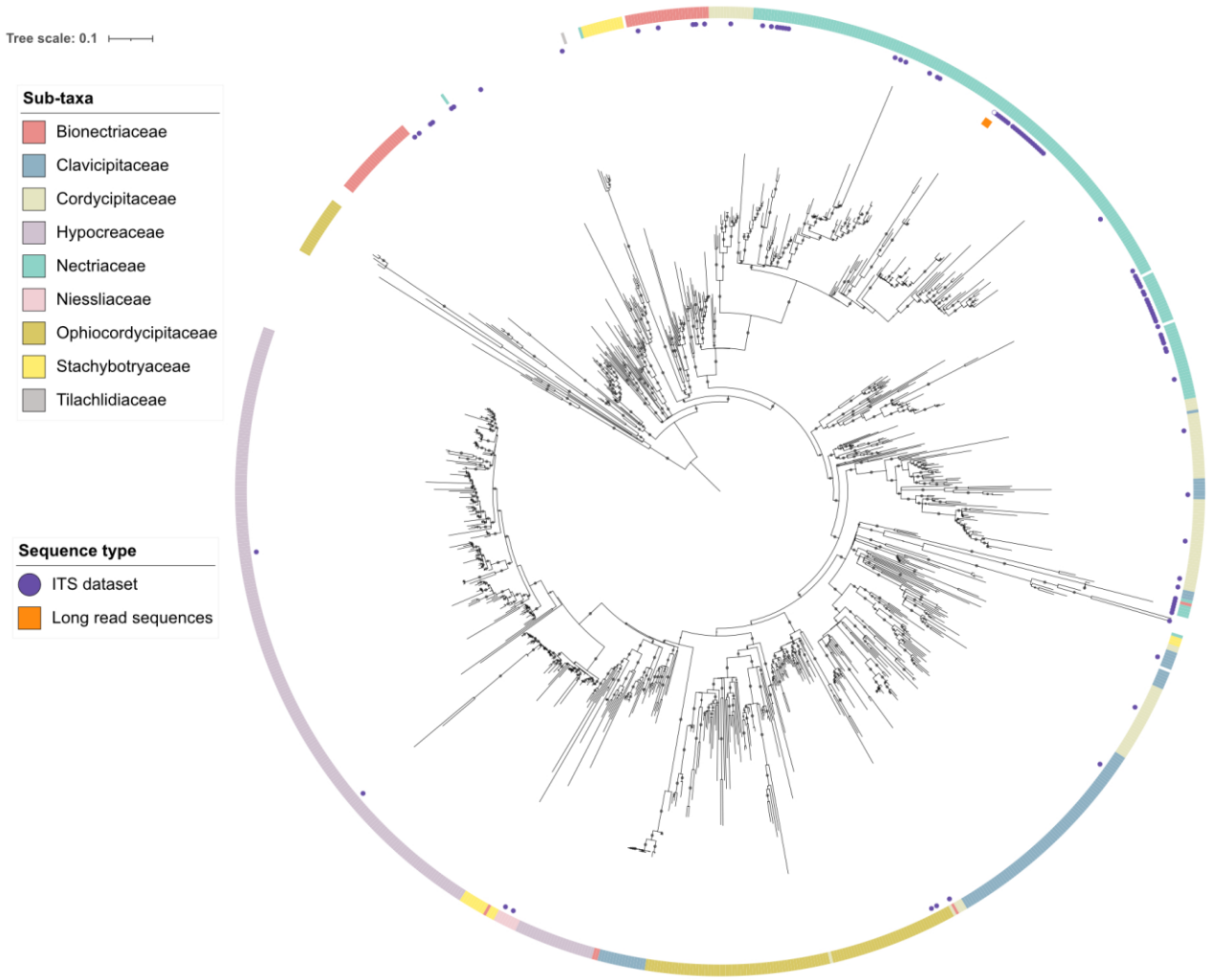

Fig. S4 Phylogenetic tree of the order **Hypocreales** (fungi), constructed from sequences from the UNITE database [2], long read sequences of fungal cultures (indicated as orange bars) and ITS metabarcoding sequences from the experimental samples (indicated as purple dots). The outer colour strip indicates the families the sequences are assigned to (see legend on left-hand site).

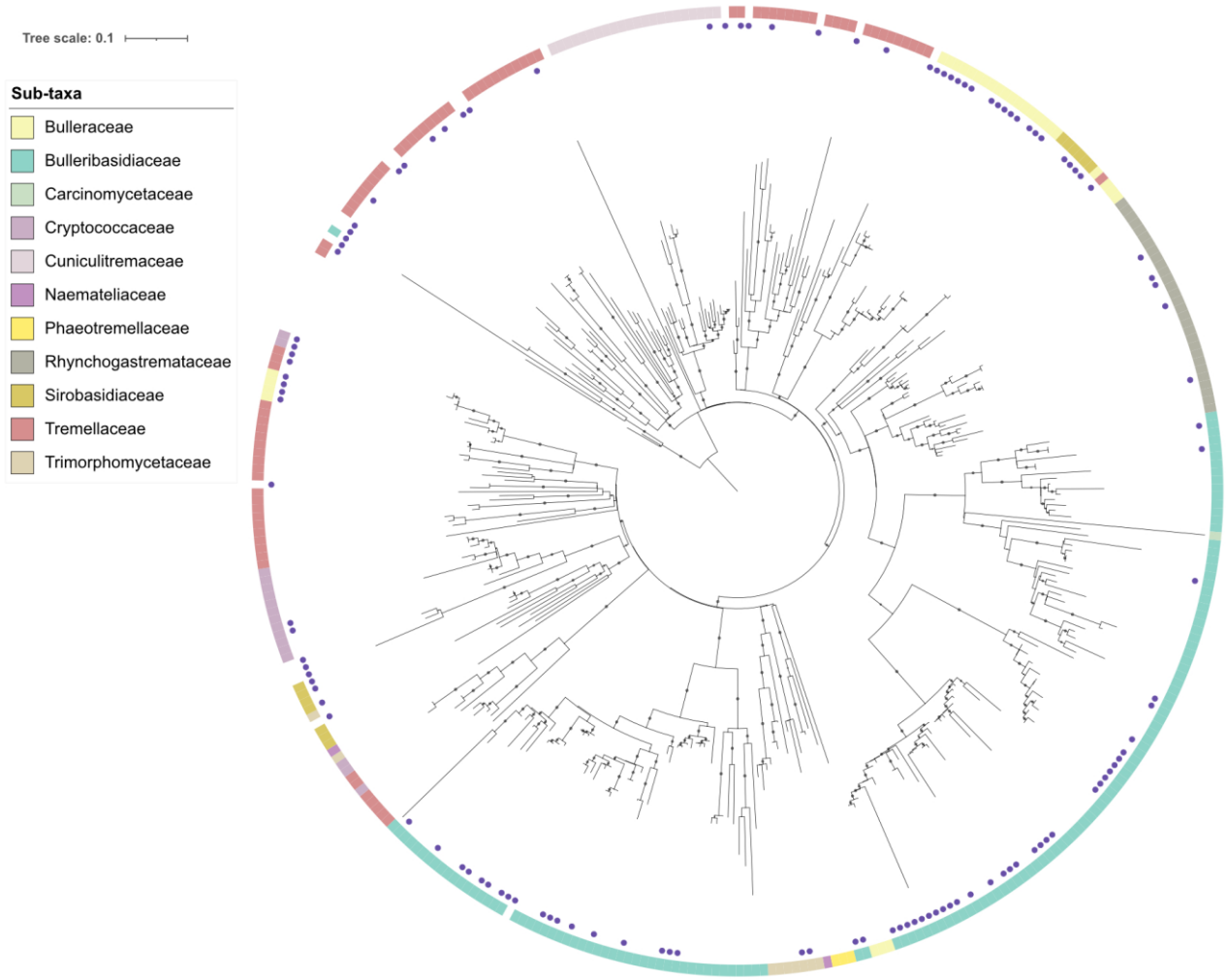

Fig. S5 Phylogenetic tree of the order **Tremellales** (fungi), constructed from sequences from the UNITE database [2] and ITS metabarcoding sequences from the experimental samples (indicated as purple dots). The outer colour strip indicates the families the sequences are assigned to (see legend on left-hand site).

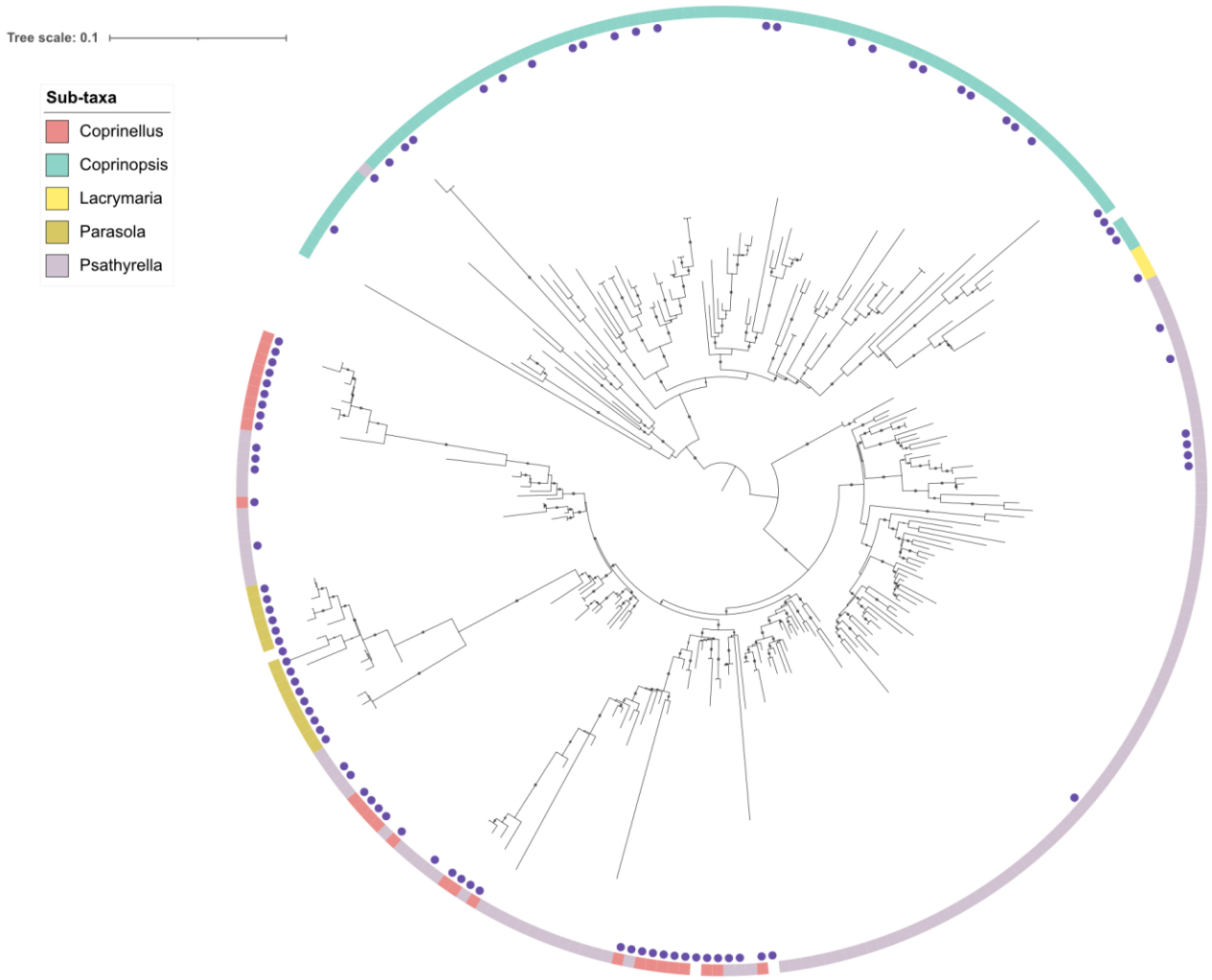

Fig. S6 Phylogenetic tree of the family **Psathyrellaceae** (fungi), constructed from sequences from the UNITE database [2] and ITS metabarcoding sequences from the experimental samples (indicated as purple dots). The outer colour strip indicates the families the sequences are assigned to (see legend on left-hand site).

Table S3 Results of the generalised linear models (GLM) testing the influence of explanatory variables on responses observed via the two identification methods and the five data sets generated. Bold *p* values indicate statistical significance. Data was missing for the morphologically identified hyphomycete community from cycle 1. LRT: likelihood-ratio test.

Response variable	Explanatory variable	df	LRT	p-value
Species richness: Morphologically-identified aquatic hyphomycete taxa (Cycle 2 and 3 only)	Treatment	1	1.78	0.183
	Cycle	1	0.50	0.481
	Treatment x Cycle	1	0.02	0.879
Species richness: ITS-identified aquatic hyphomycete taxa	Treatment	1	1.31	0.252
	Cycle	2	22.82	**< 0.001**
	Treatment x Cycle	2	0.843	0.656
ASV richness: ITS-identified aquatic hyphomycete taxa	Treatment	1	0.02	0.904
	Cycle	2	110.78	**< 0.001**
	Treatment x Cycle	2	7.29	**0.026**
ASV richness: Entire ITS data set	Treatment	1	4.00	**0.045**
	Cycle	2	317.57	**< 0.001**
	Treatment x Cycle	2	9.90	**0.007**
ASV richness: Bacteria 16S	Treatment	1	77.36	**< 0.001**
	Cycle	2	2173.30	**< 0.001**
	Treatment x Cycle	2	210.59	**< 0.001**

Table S4 Explained variance and *p* values of the Redundancy Discriminant Analysis (RDA) variables with Hellinger transformed data, tested by type III ANOVAs. Bold *p* values indicate statistical significance. Data was missing for the morphologically identified hyphomycete community from cycle 1.

Response variable	Explanatory variable	df	F	p-value	Explained variance (%)
Entire ITS data set (ASV level)	Treatment	1	0.76	0.581	2.6
	Cycle	2	11.32	**<0.001**	45.6
	Treatment x Cycle	2	0.89	0.502	3.7
ITS-identified aquatic hyphomycete taxa (ASV level)	Treatment	1	1.08	0.329	3.7
	Cycle	2	7.53	**<0.001**	35.8
	Treatment x Cycle	2	0.97	0.454	4.5
ITS-identified aquatic hyphomycete taxa (Species level)	Treatment	1	0.74	0.532	2.6
	Cycle	2	15.18	**<0.001**	52.9
	Treatment x Cycle	2	0.63	0.722	2.2
Morphologically-identified aquatic hyphomycete taxa (Species level, cyle 2 and 3 only)	Treatment	1	1.75	0.114	8.9
	Cycle	1	3.56	**<0.001**	16.5
	Treatment x Cycle	1	1.07	0.415	4.7
16S bacteria (ASV level)	Treatment	1	0.65	0.703	2.3
	Cycle	2	12.84	**<0.001**	48.7
	Treatment x Cycle	2	1.05	0.371	4.0

ASV: amplicon sequence variant

Table S5 ANOVA (type II) results testing the Influence of explanatory variables on the aquatic hyphomycete taxa richness observed via both identification methods, morphological and molecular.

Hyphomycete taxa	Method	Explanatory variable	df	F-value	p-value
Alatospora acuminata	sporulation				NS
	ITS relative abundance	Cycle (3<1,2)	2	27.5	<0.001
Articulospora tetracladia	sporulation				NS
	ITS relative abundance	Cycle (3>2>1)	2	43.0	<0.001
Flagellospora curvula	sporulation				NS
	ITS relative abundance				NS
Lemonniera aquatica	sporulation				NS
	ITS relative abundance				NS
Tetrachaetum elegans	sporulation	Cycle (3<2)	1	11.2	0.004
	ITS relative abundance	Cycle (3<1,2)	2	7.7	0.002
Tetracladium marchalianum	sporulation				NS
	ITS relative abundance	Cycle (3>1)	2	6.6	0.005

NS: not significant

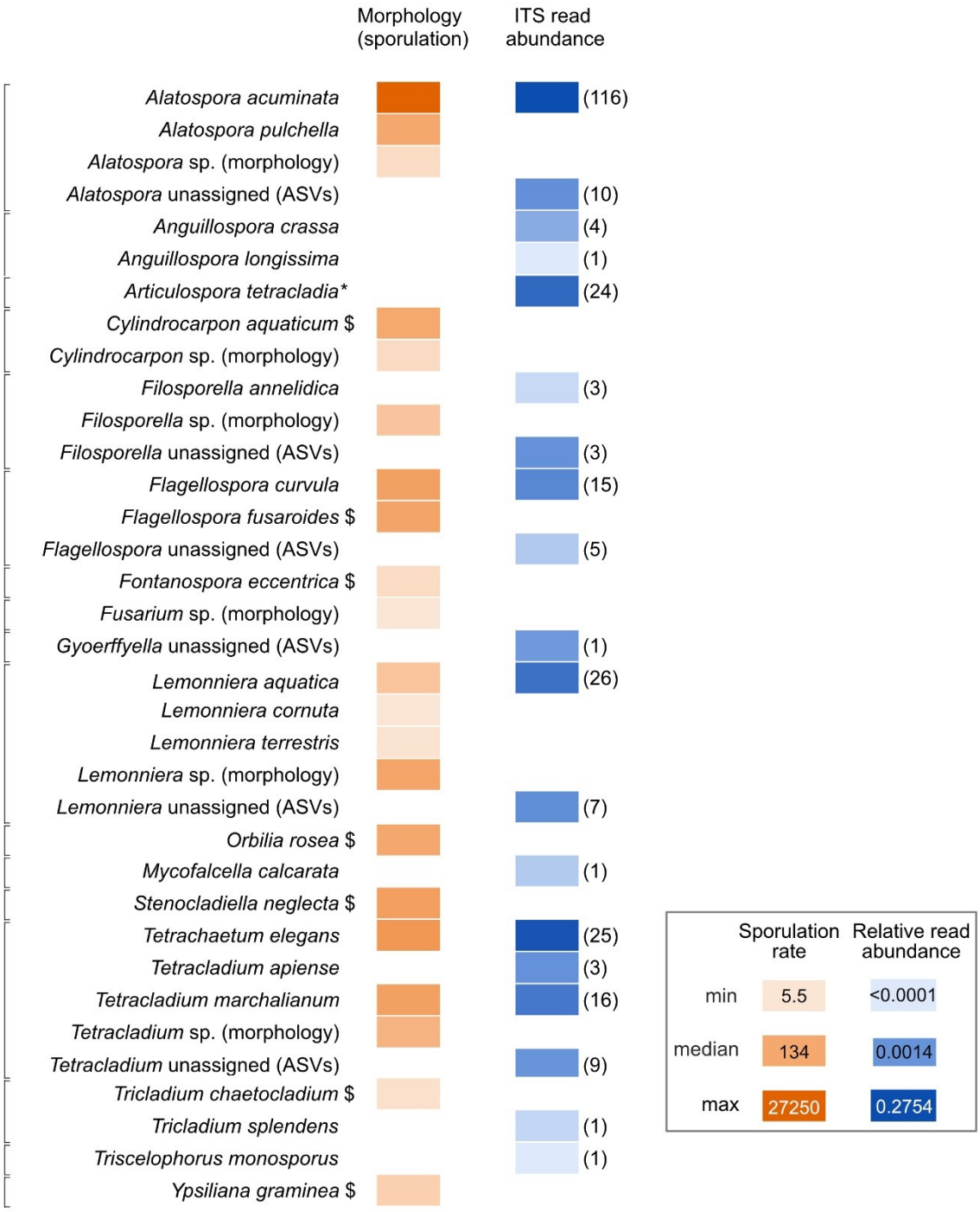

Fig. S7 Comparison between the aquatic hyphomycete community composition (over all cycles) identified morphologically based on morphologically and using ITS sequencing. Colour intensity indicates the relative sporulation rate (in conidia per g leaf dry mass) and relative read abundance, respectively (see legend). Numbers in parentheses (right of ITS read abundance) indicate the number of ASVs assigned to the species. **Articulospora tetracladia* (anamorph) recorded in the UNITE database as *Hymenoscyphus tetracladius* (teleomorph). $ indicates species identified morphologically for which there was no reference sequence in the database.

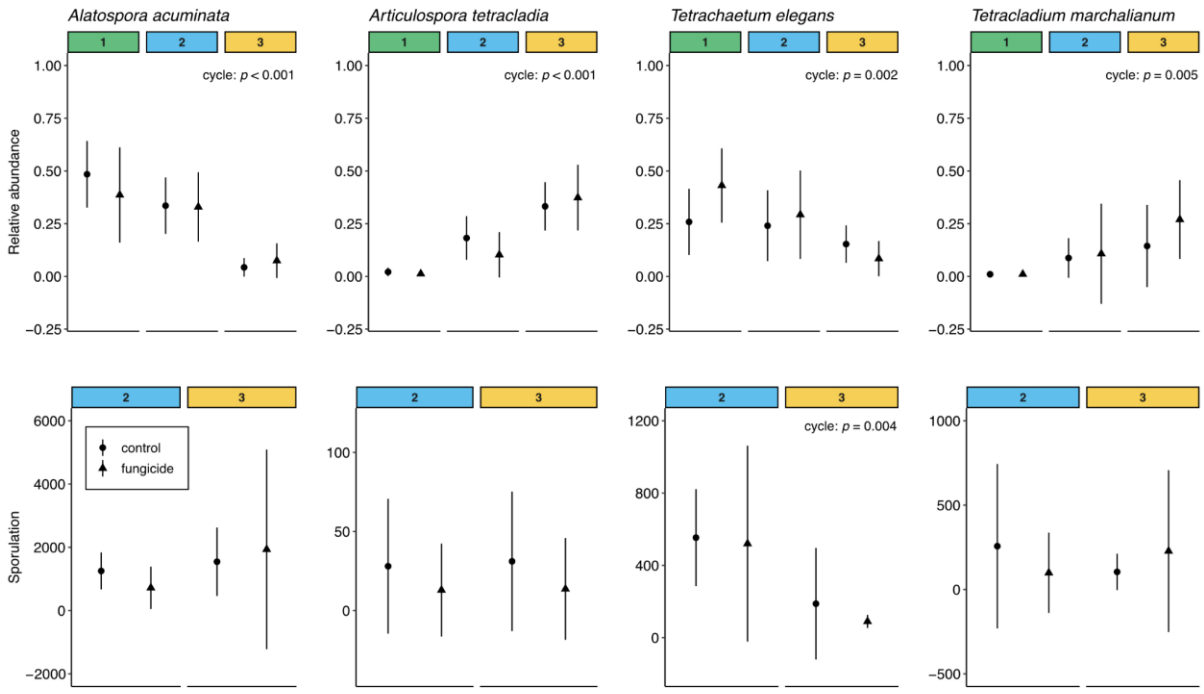

Fig. S8 Responses of four common aquatic hyphomycete taxa (that were identified via both morphological identification and metabarcoding and showed at least one significant response) to the different cycles (numbers on top) and treatments (circle: control; triangle: fungicide treatment). Mean relative abundance measured via sequencing is shown in the four upper plots and mean sporulation (morphological identification, in conidia per g leaf dry mass) is shown in the four lower plots. Error bars represent 95% confidence intervals. *P* values are provided for significant results based on the two-way ANOVAs (type II). Data was missing for the morphologically identified hyphomycete community from cycle 1.

Table S6 Differential analysis of fungal and bacterial amplicon sequence variants (ASVs) using DESeq2 [3]. Showing the log-fold change values of ASVs significantly different in treatment and controls for cycles 1, 2 and 3. Positive log fold change values means that ASV normalised read abundance increased in treatment compared to control, and negative log fold change value that ASVs read abundance decreased. Fungi marked with * are saprotrophs, with † are pathotrophs and with § are mixed (exhibit both trophic modes).

		Cycle 1		**Cycle 2**		**Cycle 3**	
Species	ASV ID	log2-fold change	*P*-value (adjusted)	log2-fold change	*P*-value (adjusted)	log2-fold change	*P*-value (adjusted)
Fungi:							
Alatospora acuminata	ASV93	-24.88	<0.001				
Alatospora acuminata	ASV130	-24.66	<0.001				
Alatospora acuminata	ASV170	-22.64	<0.001				
Alatospora acuminata	ASV119	12.22	<0.001				
Alatospora acuminata	ASV67			-26.34	<0.001		
Alatospora acuminata	ASV156			-25.93	<0.001		
Alatospora acuminata	ASV203			24.71	<0.001		
Alatospora acuminata	ASV188			-23.62	<0.001	24.82	<0.001
Alatospora acuminata	ASV43					25.11	<0.001
Alatospora acuminata	ASV37					25.65	<0.001
Alatospora acuminata	ASV26					26.01	<0.001
Alatospora acuminata	ASV50					26.52	<0.001
Alatospora acuminata	ASV22					26.68	<0.001
Alatospora acuminata	ASV32					26.76	<0.001
Alatospora acuminata	ASV33					26.77	<0.001
Alatospora acuminata	ASV14					27.04	<0.001
Alatospora acuminata	ASV17					27.32	<0.001
Alatospora acuminata	ASV12					28.22	<0.001
Ombrophila sp.§	ASV244	-23.10	<0.001				
Ascomycota	ASV208	-22.68	<0.001				
Filosporella annelidica	ASV518	-22.41	<0.001				
Tetrachaetum elegans	ASV73	26.13	<0.001				
Tetracladium sp.	ASV271	-22.84	<0.001				
Tetracladium sp.	ASV59			-23.18	<0.001		
Filosporella sp.	ASV74			-23.84	<0.001		
Erysiphe sp.†	ASV62			-22.63	<0.001		
Coprinellus disseminatus *	ASV498			22.31	<0.001		
Epicoccum sp.§	ASV109					7.76	0.008
Articulospora tetracladia	ASV204					11.92	0.001
Preussia dubia *	ASV549					20.95	<0.001
Bacteria:							
Rhizobacter sp.	ASV7	-2.72	0.006				
Enterobacteriaceae	ASV28	3.35	0.002				
Pseudomonas sp.	ASV114	21.67	<0.001				
Hydrogenophaga atypica	ASV1213	-7.83	0.005				
Chitinophagaceae	ASV1214	7.32	0.004				
Spirochaeta sp.	ASV347	-23.30	<0.001				
Burkholderiaceae	ASV450	-23.13	<0.001				
Klebsiella sp.	ASV494	8.96	0.001				
Rhizobacter sp.	ASV168			-22.67	<0.001		
Flavobacterium sp.	ASV613			22.70	<0.001		
Uliginosibacterium sp.	ASV181					-23.93	<0.001
Ancalomicrobium sp.	ASV486					-24.23	<0.001

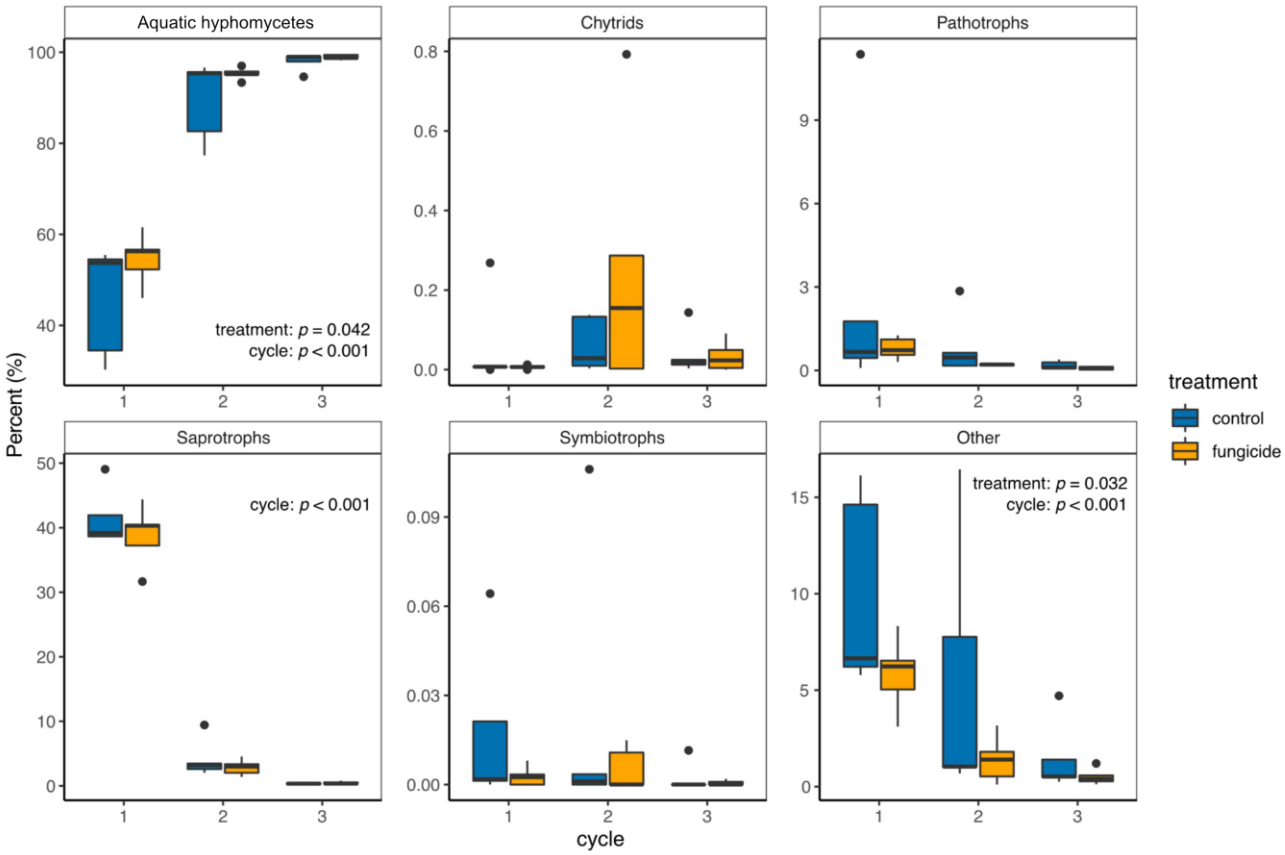

Fig. S9 Percentage of the different fungal groups (aquatic hyphomycetes and chytrids) and trophic modes (pathotrophs, saprotrophs, symbiotrophs and other unclassified fungi) in the different cycles and treatments. *P*-values are provided for significant results based on the two-way ANOVAs (type II).

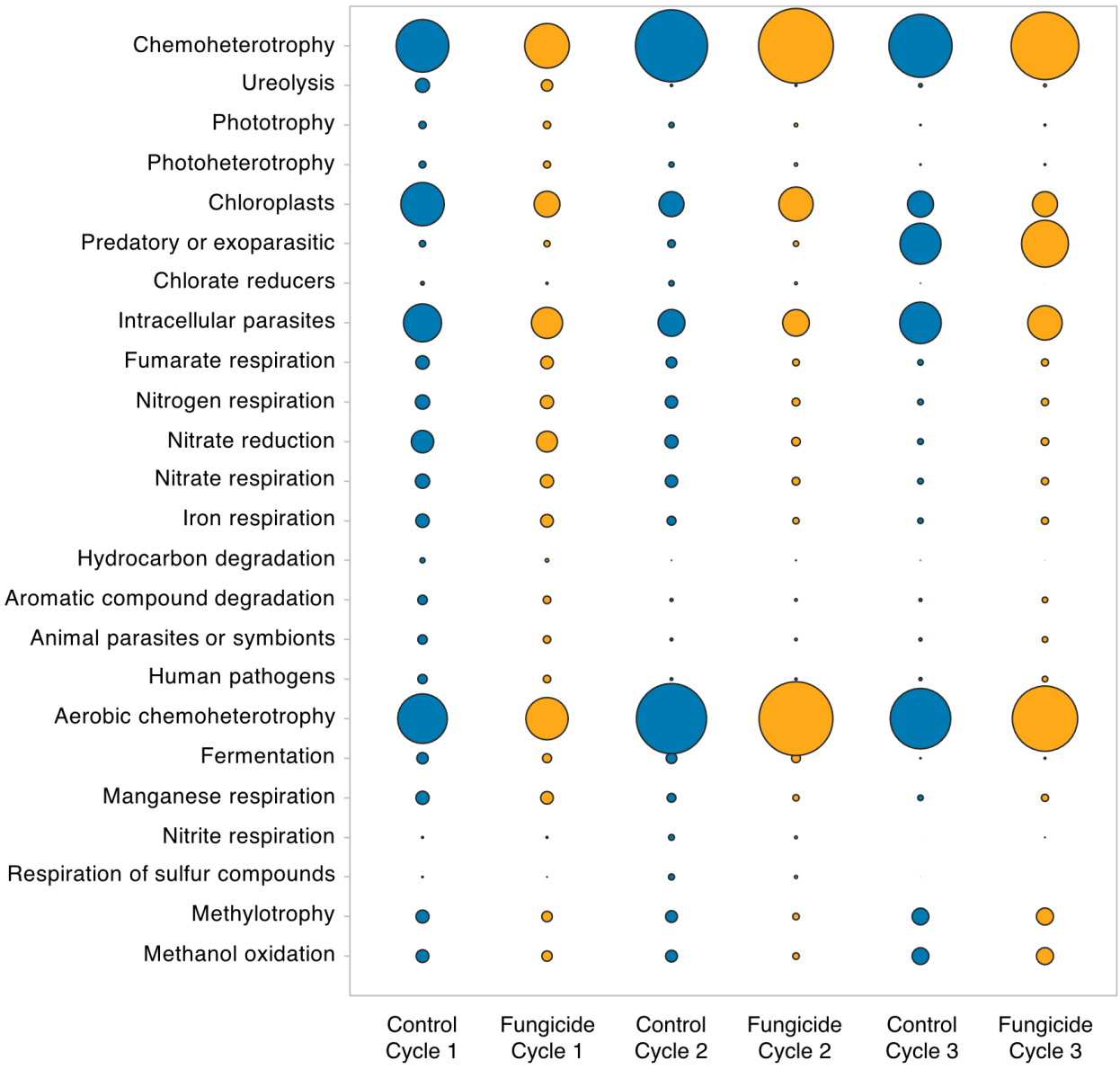

Fig. S10 Bubble plot showing the most common bacterial ecological functional groups. The size of the bubbles is referring to the mean read numbers per treatment and cycle, while the colour of the bubbles is referring to the treatment (control = blue, fungicide = yellow).

Text S1 – differences field and lab conditions:

Despite attempts to create laboratory conditions as close to natural conditions as possible, some significant differences remained. Among them are the temperature, which differed between the stream, where the colonisation was conducted, and the lab by 10 °C (8 °C in the stream in January during colonisation, 16 - 18 °C during the lab experiment) or the flow regime as the artificial current created by a magnetic stirrer differed from the longitudinal flow in the stream (Fig. S11).

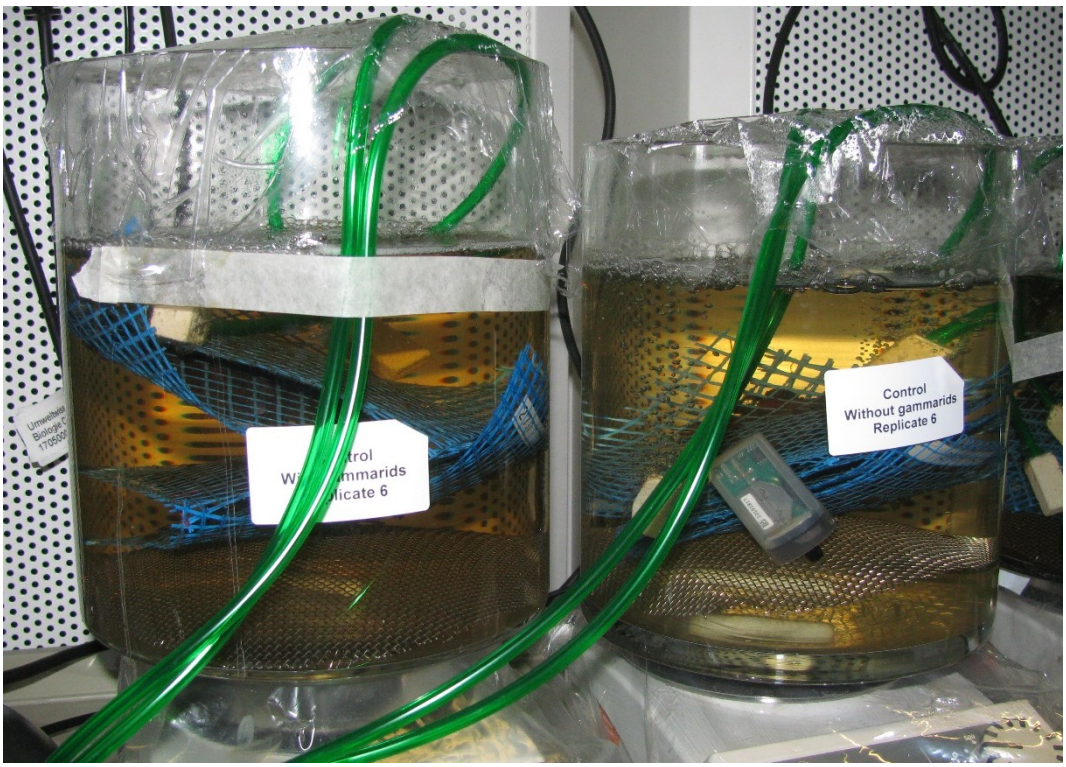

Fig. S11 Experimental setup of the microcosm experiment. Flow was created using magnetic stirrers. The magnetic stirrer bar was separated from the leaf bags using a stainless-steel mesh. Photo by Verena C. Schreiner.

Text S2 – response on the ASV level:

For *Alatospora acuminata*, we found no effect of the fungicide treatment at the species level for both morphological and molecular identification (Table S5, Fig. S8), but at the ASV level, we detected significant responses for 18 of 116 ASVs to the fungicide treatment (Table S6). Furthermore, the ASVs of *Alatospora acuminate* responded in different directions. We detected both positive and negative responses in cycles 1 and 2 to the fungicide exposure, but only positive responses in cycle 3 (Table S6). One ASV of *Alatospora acuminata* (ASV 188, Table S6) changed its response towards the fungicide treatment throughout the experiment. In cycle 2, the ASV responded negatively to the fungicide treatment, which changed to a positive response in cycle 3 (Table S6). This highlights that different genotypes of the sample species can show different responses to a stressor which might explain contrasting study results. In previous single species studies, unknown ASVs of *Alatospora acuminata* have been shown to exhibit a low sensitivity towards copper (an inorganic fungicide, Azevedo and Cássio [4]) and exhibited increasing DNA amounts with increasing concentrations of organic fungicides [5]. This contrasts the results of studies using whole communities which showed that fungicide exposure caused a reduction of the sporulation of *Alatospora acuminata* [6, 7] or even its elimination from the fungal community [8], while other studies found no effect on *Alatospora acuminata* [9, 10].

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