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BIODEGRADATION

Copper in Wood Preservatives Delayed Wood Decomposition and Shifted Soil Fungal but Not Bacterial Community Composition

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ABSTRACT Copper-based fungicides are routinely used for wood and plant protection, which can lead to an enrichment of copper-tolerant microbial communities in soil. To investigate the effect of such wood preservatives on the soil fungal and bacterial community compositions, five different vineyard and fruit-growing soil environments were evaluated using incubation studies over time. Pine sapwood specimens were impregnated with either water or different biocide treatment solutions containing a mixture of copper, triazoles, and quaternary ammonium compounds (CuTriQAC), a mixture of triazoles and quaternary ammonium compounds (TriQAC), or copper alone (Cu). Specimens were incubated in soil from each sample site for 8, 16, 24, and 32 weeks. The effects of preservative treatment on the modulus of elasticity (MOE) of the wood specimens and on the soil fungal as well as bacterial community composition at the soil-wood interface were assessed by quantitative PCR and amplicon sequencing of the fungal internal transcribed spacer (ITS) region and bacterial 16S rRNA gene. Specimens impregnated with CuTriQAC and Cu showed decreased MOE and reduced fungal and bacterial copy numbers over time compared to those impregnated with water and TriQAC. Fungal but not bacterial community composition was significantly affected by wood preservative treatment. The relative abundance of members of the family Trichocomaceae compared to other genera increased in the presence of the Cu and CuTriQAC treatments at three sites, suggesting these to be Cu-tolerant fungi. In conclusion, the copper-containing treatments resulted in marginally increased MOE, lowered microbial gene copy numbers compared to those in the TriQAC and water treatments, and thus enhanced wood protection against soil microbial wood degradation.

IMPORTANCE Copper-containing rather than TRIQAC formulations are efficient wood preservatives irrespective of the origin and composition of the soil microbial communities. However, some fungi appear to be naturally insensitive to copper and should be the focus of future wood preservative formulations to facilitate the life span of wooden construction in contact with soil while also minimizing the overall environmental impact.

KEYWORDS 16S rRNA gene, ITS region, amplicon sequencing, community composition, copper-based wood preservatives, soil incubation study

Modifications such as physical soil management and the use of fertilizers and pesticides influence the soil microbial community composition and function. The application of pesticides causes shifts in the composition of the soil microbial community as well as its activity through the inactivation of sensitive microbes and subsequent

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selection of more tolerant ones (1–3). Pesticides can thus directly and indirectly affect soil fertility and health as well as agro-ecosystem quality (4).

Copper is an essential trace element for living organisms but is toxic at elevated concentrations, and even sublethal concentrations affect microbial activities in many environments, including soil (5). While elemental copper is insoluble in water, copper oxides or copper sulfate are soluble at a low pH as Cu^{2+} as well as at a high pH as $Cu(OH)_2$ (6, 7). Copper persists by binding to organic matter in many soils for long periods and can accumulate (7). Copper is present in almost any pristine soil environment at concentrations of 2 to 276 mg kg^{-1} (8), and anthropogenic activities add much higher concentrations through fertilizer and fungicide applications. Soils in vineyards and fruit-growing environments are characterized by copper concentrations of greater than 1,500 mg kg^{-1} (9), probably as a result of the application of copper-containing plant protection products as well as leaching from wooden stakes and supports preserved with copper-based materials.

Small bioavailable quantities of copper in soil can influence microbial populations in terms of total biomass, diversity, and activity (10). The application of copper-containing fungicides (e.g., at rates of 16 to 48 kg copper ha^{-1}) over many years results in a microbial community that is characterized by high resistance to heavy metals (11).

Several directives and regulations have been issued within the European Union in recent years to regulate the use of chemicals, particularly biocides used in wood protection. As a consequence, only a limited number of biocides can be used for the preservation of wood (12), and most of these are based either solely on organic biocides or on copper-based systems. The latter are effective against the majority of wood-degrading microorganisms (5) and are used for wood that is intended to have contact with soil, such as the stakes and supports used for vines and fruit trees.

The effects of biocides used for wood protection on the soil microbial community as well as their microbial degradation are not well understood (5, 13). Moreover, the mechanisms by which such biocides inactivate the wood-degrading microbial community *in situ* are also poorly understood, making it difficult to assess how changes in formulation might affect biocide performance. Evidence for an impact of microbially diverse populations on wood degradation exists in the literature. Unsurprisingly, soil microbial communities with higher functional redundancies degraded preserved wood more efficiently than in laboratory-based experiments with only selected wood-decomposing fungi (5, 14).

Copper-containing wood preservatives have been shown to delay extensively the colonization of wooden field stakes by soil-inhabiting fungi (15). Although defined damage to preservative-treated wooden stakes is observed occasionally, it is unclear which components of the soil fungal and bacterial communities are associated with it. Therefore, it is necessary to elucidate the impact of preservatives on the effects on the composition of the soil microbial community.

This study investigated the degradation patterns of pine sapwood specimens that were impregnated with either water (H_2O) or different biocides containing either a mixture of copper, triazoles, and quaternary ammonium compounds (CuTriQAC), a mixture of triazoles and quaternary ammonium compounds (TriQAC), or copper alone (Cu) (Table 1). In addition, the impact of these treatments on the fungal and bacterial community compositions of soils derived from five sampling sites (central Germany [CG], northern France [NF], northern Germany [NG], Portugal [PO], and southern France [SF]) (Table 2) was studied. These soil environments were of interest because of prior exposure to copper-based fertilizers and biocides and consequently the presence of a microbial community anticipated to be adapted to copper-based preservatives.

RESULTS

Effect of wood preservatives on MOE. The decrease of the modulus of elasticity (MOE) was significantly affected by wood preservatives and incubation time (*F* value by two-sided analysis of variance [ANOVA] = 29.163; $P < 2.2 \times 10^{-16}$). The decrease of MOE was lower in the copper-containing wood preservative treatments (CuTriQAC and

TABLE 1 Wood preservative treatments used in this study

Wood preservative treatment (abbreviation)[a]	Uptake (kg m^{-3} wood)[b]		
	Copper	Triazole	Quaternary ammonium compounds
Copper triazole quaternary ammonium (CuTriQAC)	0.48 (0.0923)	0.0185 (0.0036)	0.24 (0.0462)
Triazole quaternary ammonium (TriQAC)		0.0185 (0.0036)	0.24 (0.0462)
Copper (Cu)	0.48 (0.0923)		
Water (H_2O)			

[a]Cu treatment was with the wood preservative Korasit CC (Kurt Obermeier GmbH & Co. KG, Bad Berleburg-Raumland, Germany), while the CuTriQAC and TriQAC formulations were not available as commercial wood preservatives at the time the study was conducted.
[b]The mass percentage relative to the weight of wood is denoted in parentheses.

Cu) than in the other treatments (H_2O and TriQAC) (Fig. 1). The decrease of MOE and mass loss of wood specimen were in line with each other (compare Fig. 1 with Fig. S1 in the supplemental material) and were independent of the sampling site (NG, CG, NF, SF, or PO) (Fig. 1).

Fungal and bacterial gene copy numbers at the soil-wood interface. The copy numbers of both the fungal internal transcribed spacer (ITS) region and the bacterial 16S rRNA gene were significantly lower in samples associated with copper-containing wood preservative treatments than in other treatments, irrespective of the sampling site (Fig. 2 and 3).

The copy numbers of the fungal ITS region as well as the bacterial 16S rRNA gene increased significantly over incubation time (for fungal operational taxonomic units [OTUs], F value by one-sided ANOVA = 8.0946 and $P = 3.285 \times 10^{-5}$; for bacterial OTUs, F value by one-sided ANOVA = 3.3922 and $P = 0.0183$). The fungal ITS region copy numbers were significantly different among sampling sites (F value by one-sided ANOVA = 6.8263; $P = 2.798 \times 10^{-5}$) and were ranked by numbers in the following order by sampling site: CG > NF > NG > PO > SF (Fig. 2). The bacterial 16S rRNA gene copy numbers decreased in the NG, CG, and PO sites in response to Cu and CuTriQAC treatments over time and were ranked by numbers in the following order by sampling site: CG > NG > NF > SF > PO (Fig. 3).

Soil fungal and bacterial community compositions. The fungal, but not bacterial, community composition was significantly affected by wood preservative treatment (for the fungal community, F ratio by the Monte Carlo test = 2.988 and $P = 0.0004$; for the bacterial community, F ratio by the Monte Carlo test = 1.461 and $P = 0.1785$) (Fig. 4)

TABLE 2 Soil parameters from five different sampling sites

Parameter	Value at site:				
	Northern Germany (NG)	Central Germany (CG)	Northern France (NF)	Southern France (SF)	Portugal (PO)
Land use type	Apple plantation	Prepared field site	Vineyard	Vineyard	Vineyard
pH	4.9	6.0	7.0	6.3	4.4
TOC content (mg/liter)	105	167	54	20	12
TN content (mg/liter)	27	13	4	3	3
C-to-N ratio	5.1	7.4	17.4	14.0	4.0
Al (mg/kg)	47,000	56,200	35,750	38,750	47,300
Cu (mg/kg)	60	18	119	51	25
Fe (mg/kg)	35,900	39,750	35,000	32,300	40,450
Mn (mg/kg)	827	1,220	1,040	393	387
Zn (mg/kg)	102	103	61	78	78
Rainfall (mm) (avg annual precipitation 2012–2014)	710	1,096	829	353	822
Maximum daily temp (°C) (avg 2012–2014)	13.5	8.8	17.2	17.3	18.8
Soil texture (%)					
Sand	8.8	10.6	9.2	26.1	14.0
Silt	70.2	51.2	58.0	7.6	45.9
Clay	2.0	1.2	1.2	0.2	1.0

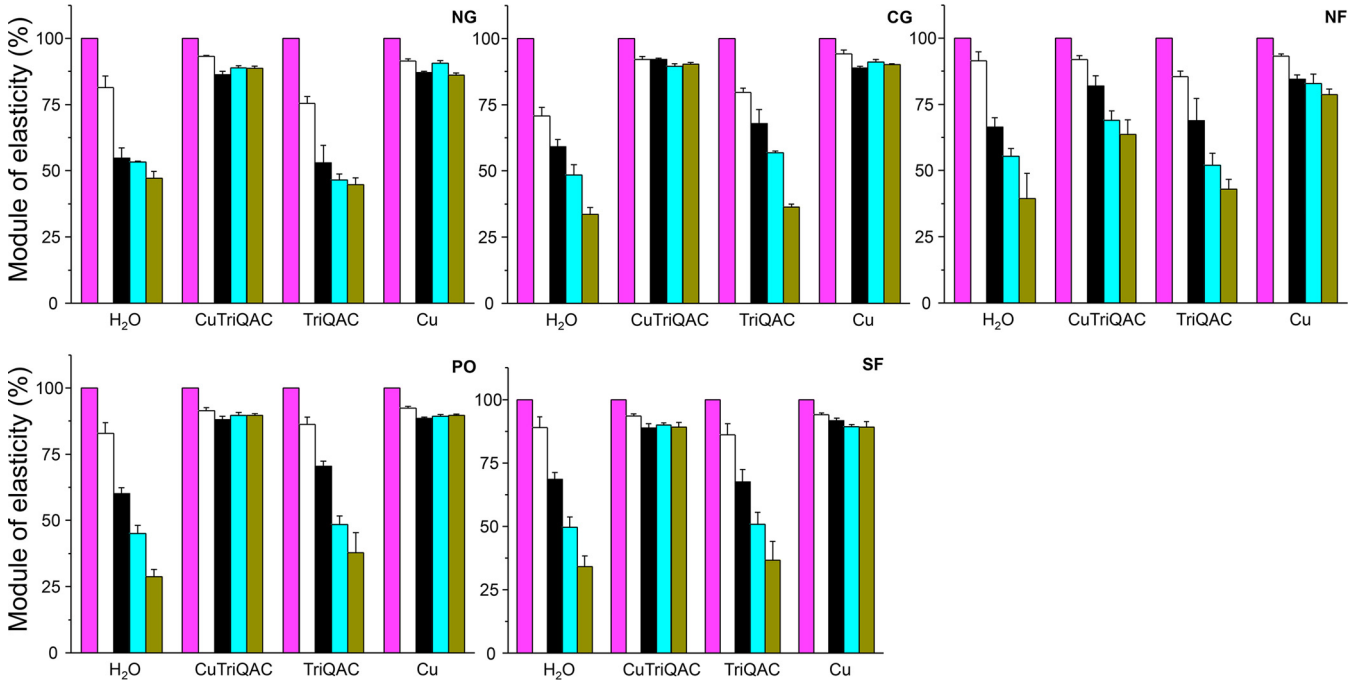

FIG 1 Percent reduction of the modulus of elasticity (MOE) of woods specimen exposed to soil derived from five sampling sites (NG, CG, NF, PO, and SF) (Table 2) and four wood preservative treatments (H_2O, CuTriQAC, TriQAC, and Cu) (Table 1) over time (pink, 0 weeks; white, 8 weeks; black, 16 weeks; cyan, 24 weeks; dark yellow, 32 weeks). Error bars indicate standard error ($n = 4$).

and differed significantly among sampling sites (for the fungal community, F ratio by the Monte Carlo test = 8.119 and $P = 0.0001$; for the bacterial community, F ratio by the Monte Carlo test = 1.631 and $P = 0.1316$) (Fig. 4). Moreover, the fungal and bacterial community compositions were both significantly affected by soil parameters as well as

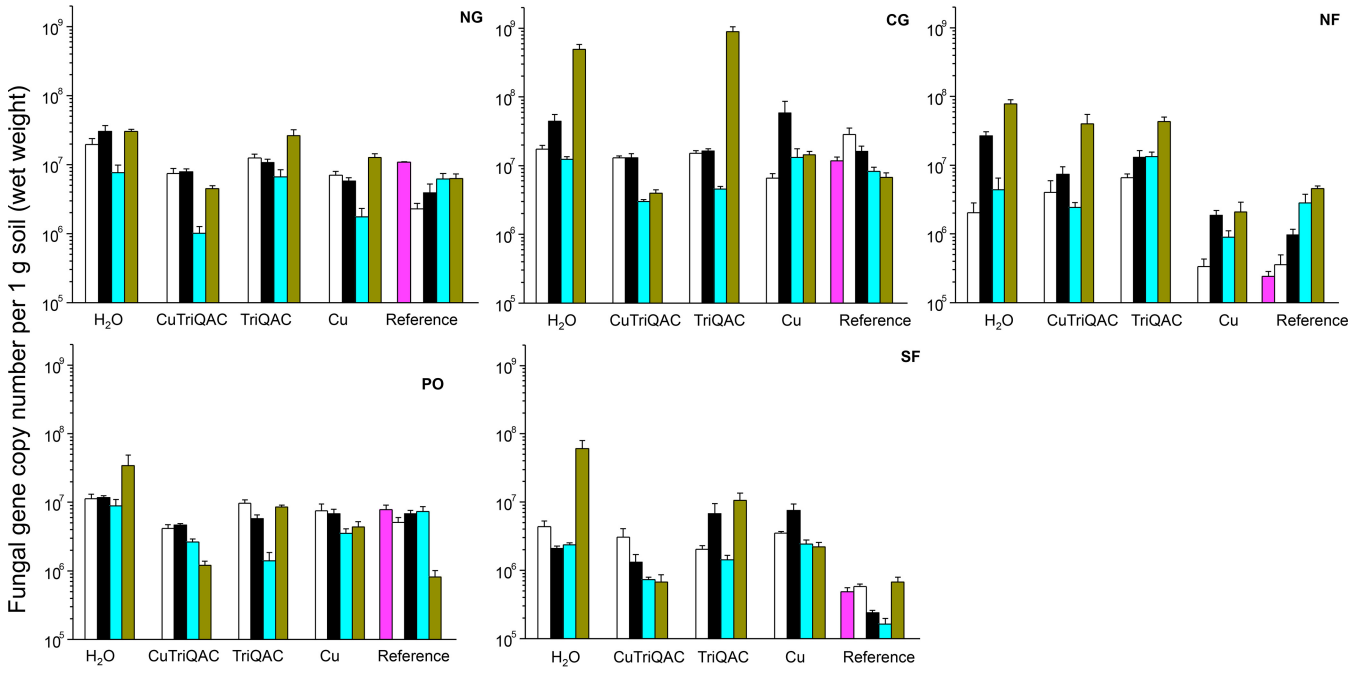

FIG 2 Fungal ITS region copy numbers of the soil-wood interface from five sampling sites (Table 2), four wood preservative treatments (Table 1), and soil without wood contact (reference) over time (pink, 0 weeks; white, 8 weeks; black, 16 weeks; cyan, 24 weeks; dark yellow, 32 weeks). Soil moisture was adjusted to 95% of water-holding capacity by adding double-distilled water to keep the moisture content constant during incubation. Error bars indicate standard error ($n = 4$).

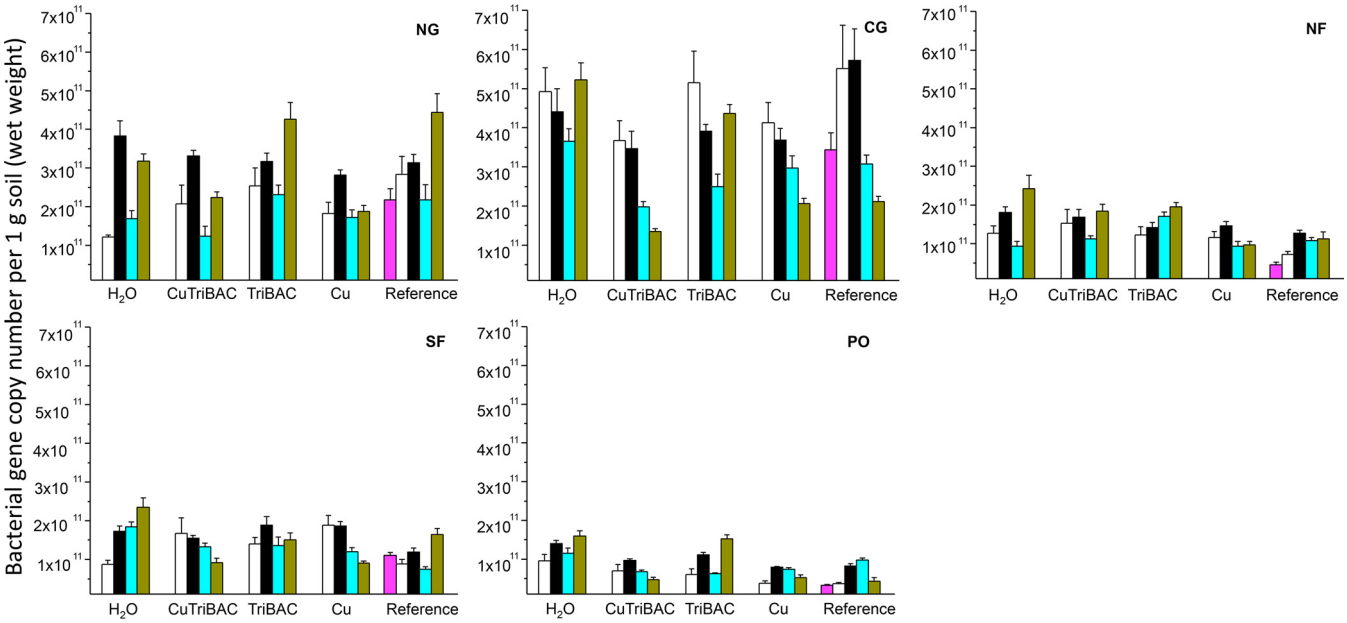

FIG 3 Bacterial 16S rRNA gene copy numbers at the soil-wood interface from five sampling sites (Table 2) and four wood preservative treatments (Table 1) over time (pink, 0 weeks; white, 8 weeks; black, 16 weeks; cyan, 24 weeks; dark yellow, 32 weeks). Soil moisture was adjusted to 95% of water-holding capacity by adding double-distilled water to keep the moisture content constant during incubation. Error bars indicate standard error ($n = 4$).

by incubation time (for fungal OTUs, F value by two-sided ANOVA = 6.8843 and $P = 5.354 \times 10^{-11}$; for bacterial OTUs, F value by two-sided ANOVA = 2.9925 and $P = 0.0006$) (see Table S1 in the supplemental material). The soil parameters and their respective fungal and bacterial community compositions are therefore highly site specific (Table S1). The fungal, but not bacterial, community composition shifted significantly over time (for the fungal community, F ratio by the Monte Carlo test = 1.657 and $P = 0.0342$; for the bacterial community, F ratio by the Monte Carlo test = 1.631 and $P = 0.1316$).

The relative abundance of amplicon sequence reads of OTUs potentially associated with wood degradation should be higher at the soil-wood interface than in soil only, which is true for the fungal OTUs affiliated with the genera *Conlarium*, *Pluteus*, *Pseudallescheria*, and *Scedosporium* (see Table S2 in the supplemental material). None of the bacterial OTUs had such high relative abundances at the soil-wood interface compared to in soil only (Table S2).

Furthermore, selected fungal taxa were highlighted if their relative abundance increased significantly over incubation time with Cu and/or CuTriQAC treatment (Table 3; see Fig. S2 in the supplemental material). The frequency of fungal OTUs (69 out of 80 OTUs) was lower with copper-containing preservative treatments than with other treatments (Fig. S2). However, some fungal OTUs demonstrated an increased relative abundance of amplicon sequence reads in copper-containing treatments independently of the sampling site (Table 3). A site-specific comparison of the copper-containing treatments revealed that each sampling site shared the same members of five genera (*Cryptococcus, Mortierella*, *Penicillium*, *Talaromyces*, and *Paecilomyces*) as well as unclassified members of the family Trichocomaceae, independent of incubation time. The relative abundance of unclassified Trichocomaceae (in SF) and *Penicillium* organisms (in NG, CG, and SF) increased in the presence of both copper-containing treatments (Cu and CuTriQAC).

Actinobacteria, *Alphaproteobacteria*, *Acidobacteria*, and *Chloroflexi* were the dominant bacterial phyla at every sampling site and incubation time (see Fig. S3 in the supplemental material). Approximately 50% of all bacterial OTUs were present in every treatment and at every sampling site (Fig. S2). The bacterial community composition was not affected by incubation time. However, a few bacterial OTUs increased over time

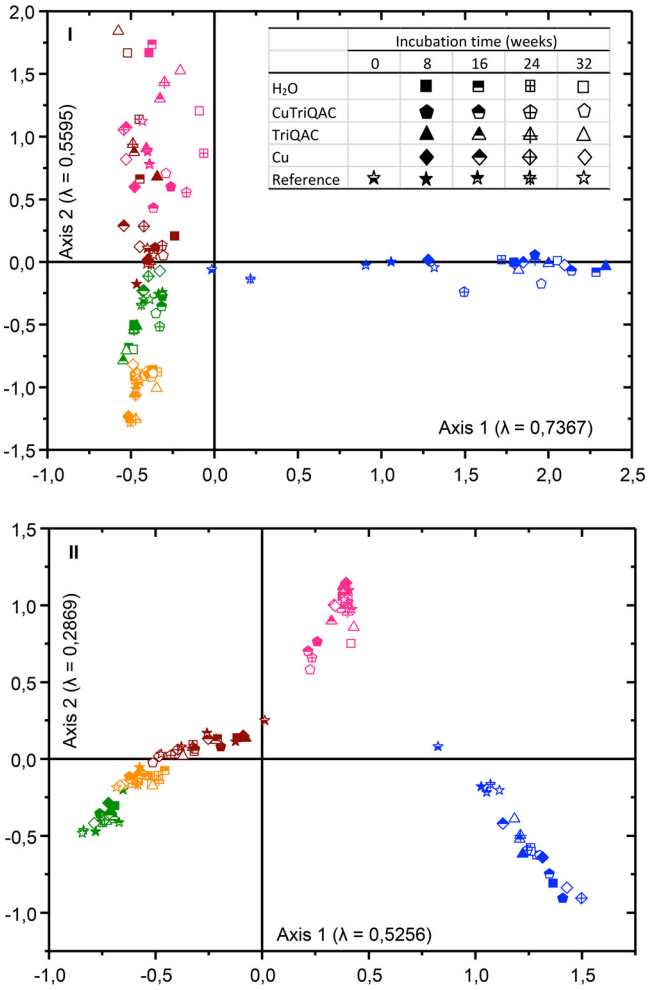

FIG 4 Correspondence analysis of fungal ITS region (I)-based and bacterial 16S rRNA gene (II)-based community compositions of soil derived from five sampling sites (green, NG; brown, CG; blue, NF; pink, SF; orange, PO) (Table 2) and four wood preservative treatments (H_2O, CuTriQAC, TriQAC, Cu, and reference) (Table 1) over time (0, 8, 16, 24, and 32 weeks). Eigenvalues are indicated for each graph. Correspondence analysis of the fungal ITS region and bacterial 16S rRNA gene-based community compositions for each sampling site can be found in Fig. S6 to S10 in the supplemental material.

in copper-containing treatments, for example, members of the genera *Sphingomonas, Bacillus*, and *Burkholderia*.

Fungal and bacterial diversity indices. The Shannon diversity index for the fungal community composition was unevenly distributed and differed between wood preservative treatments and sampling sites (see Fig. S4 in the supplemental material). In contrast, the bacterial community composition was more evenly distributed (see Fig. S5 in the supplemental material).

DISCUSSION

The sampling sites in this study differed in terms of location, climate conditions, and history of application of copper-containing biocides for plant and wood protection (long history [>50 years], NG, NF, SF, and PO; no history, CG). Wood specimens from all sampling sites were characterized by decreased MOE (Fig. 1) and increased mass loss (see Fig. S1 in the supplemental material), accompanied by decreased fungal ITS region and bacterial gene copy numbers (Fig. 2 and 3), where copper-containing wood preservatives (CuTriQAC and Cu) were applied. In addition, the fungal community composition was significantly shifted by wood preservative treatment, and both fungal and bacterial community compositions were significantly different between the sampling sites (Fig. 4; see Fig. S2, S11, and S12 in the supplemental material).

TABLE 3 Effect of wood preservative treatments and the soils derived from the five sampling sites on the relative abundances of fungal taxa[a]

Taxon	Wood specimen treatment	Sampling site	Relative abundance (%) at wk[b]:			
			8	16	24	32
Unclassified Chaetosphaeriaceae	H_2O	CG	8.5	29.8	50.1	81.9
	TriQAC		33.5	42.3	47.7	90.8
	TriQAC	SF	0.1	29.5	12.1	9.8
Geastrum	H_2O	SF	19.1	9.0	21.0	3.4
	CuTriQAC		13.5	3.3	10.8	7.0
	TriQAC		40.1	12.3	9.6	7.6
	Cu		20.4	12.6	19.8	10.9
Cryptococcus	CuTriQAC	NG	9.5	9.3	10.1	6.9
	Cu		19.0	15.5	12.4	9.7
	CuTriQAC	CG	12.8	13.1	8.4	8.0
	Cu		18.2	5.0	5.0	14.3
	CuTriQAC	PO	3.7	3.1	3.8	3.9
	Cu		4.4	6.2	5.4	6.5
Mortierella	CuTriQAC	NG	9.8	13.0	6.4	3.9
	Cu		8.6	11.0	7.7	2.9
	CuTriQAC	CG	10.6	10.6	11.6	13.0
	Cu		15.1	3.5	3.8	7.4
Fusarium	CuTriQAC	NG	3.8	4.8	7.2	16.3
Penicillium	Cu	NG	11.8	17.0	26.8	36.2
		CG	1.5	71.3	35.4	22.9
		SF	7.7	6.9	15.1	24.6
	CuTriQAC	SF	0.1	50.9	0.3	1.7
Unclassified Chaetomiaceae	CuTriQAC	CG	6.1	3.7	9.4	11.3
		NF	17.9	3.1	2.1	0.4
		SF	15.7	20.2	25.2	21.6
Chaetomium	CuTriQAC	NG	7.1	8.4	7.2	7.9
		NF	0.0	0.0	18.0	5.7
		PO	12.2	19.1	8.0	13.3
Pseudallescheria	CuTriQAC	NF	4.3	26.0	8.5	31.0
Scedosporium	H_2O	NF	27.1	43.8	38.0	52.4
	CuTriQAC		58.9	48.2	43.9	41.7
	TriQAC		72.1	49.6	56.3	54.5
	Cu		12.5	34.9	41.7	58.8

[a]For wood preservative treatments and soil parameters, see Tables 1 and 2, respectively.
[b]Relative abundances, based on the abundance of the sequence reads in relation to the entire amplicon sequence reads, with significant increases or decreases over time are shown.

The efficacy of copper-containing wood preservatives against wood-degrading fungi is exerted mainly by Cu in its soluble form (6, 7). This causes impaired cellulose decomposition and inhibited microbial cellulase activity (10). Therefore, application of copper-containing preservatives is highly effective against microbial wood degradation, but once released from the wood into the surrounding environment, they affect biological diversity and activity at some yet-unknown, but possibly significant, cost.

The copper content in the upper soil layer was between 18 and 110 mg kg^{-1} (Table 2), which can be sufficient to affect soil properties, plant cultivation, and harvest yields (9). The effects of copper-containing plant protection products and inputs from other copper-containing sources in agriculture, such as mineral and farm manure, compost, and sewage sludge, has been described extensively (16, 9). Shifts in microbial community composition and a decrease in biodiversity with an increasing copper content in copper-polluted locations can occur due to different sensitivities to copper (17). Zelles

et al. (18) pointed out that long-term usage of copper-containing fungicides in soils sustaining crops of hops can lead to an increased copper content, coupled with a change in the microbial community. Long-term application of copper-containing fungicides in fruit and vineyard growing environments also resulted in an increased copper content, which in turn could have a marked effect on the bacterial community composition (19).

The fungal ITS region and bacterial 16S rRNA gene copy numbers differed significantly among sampling sites, which was probably attributable to different soil properties (Table 2). Soil physical and chemical properties are pivotal parameters for fungal as well as bacterial community distributions across diverse environments (20, 21).

Fungal ITS region copy numbers initially increased with CuTriQAC treatment of NG and NF (Fig. 2). After 16 weeks of incubation, they decreased, and thereafter they increased again until the end of the incubation phase. These findings were accompanied by shifts in the fungal community composition (Fig. 4), suggesting that the fungal community members have undergone a selection and adaption process as previously postulated for copper-containing environments (22).

The presence of nonpreserved wood shifted the soil fungal community composition (see Table S2 in the supplemental material), and high abundances of the respective fungal genera have been found previously in environments containing wood without preservative treatment (23–26). These fungal community compositions were highly effective at degrading nonpreserved wood (Fig. 1 and S1), but only members of the genera *Pseudallescheria* and *Scedosporium* had a similar high abundance in the presence of wood treated with copper-containing preservatives. In addition, the relative abundance of members of the genera *Cryptococcus, Mortierella*, *Paecilomyces*, *Penicillium*, and *Talaromyces* as well as unclassified members of the family Trichocomaceae increased, compared to that of other fungal genera, in the presence of copper-containing treatments independent of the sampling site and incubation time (Table 3). Members of these genera are known to be metal tolerant (27–30), and some are tolerant to copper. Moreover, members of the genus *Talaromyces* are potent degraders of cellulose and demonstrate high tolerance to copper (29). In addition, *Paecilomyces* is known to metabolize the agricultural herbicide alachlor by employing cyanide hydratase (31), which can be a highly beneficial property with respect to the potential degradation of TRIQAC. Initial wood decay and either preservative modification or degradation likely are performed by copper-tolerant members of many fungal genera, including *Cryptococcus* and *Mortierella* (Table 3). Thereafter, presumably, the bioavailability of preservatives was lowered by copper precipitation, fungal translocation, and microbial transformation of preservative compounds. This should be analyzed in detail in future studies. Reduced bioavailability of preservatives may enable colonization by more preservative-sensitive members within the microbial community. A shift in microbial composition followed by increased relative abundances of unclassified Chaetomiaceae as well as members of the genera *Chaetomium* and *Fusarium* (Table 3) was indeed observed. Members of the genera *Chaetomium* and *Fusarium* are known to be copper tolerant (30). Furthermore, *Fusarium solani* has been described as one of the most powerful degraders of lignin in forest soils and possesses lignin-degrading enzymatic activities, such as laccase and lignin peroxidase (32).

In the absence of copper-containing treatments (i.e., H_2O and TriQAC), members of the fungal genus *Geastrum* and the family Tricholomataceae (both Agaricomycetes) and unclassified Chaetosphaeriaceae and *Scedosporium* (both Sordariomycetes) were present at high relative abundances (Table 3). This varied over time for *Geastrum*, which was present only in each treatment of SF. Some species of *Geastrum* are known to be saprotrophic (33), and they demonstrated increasing relative abundance over time in this study. Many wood-degrading fungi in our study are affiliated with the class Agaricomycetes, which are efficient degraders of recalcitrant biopolymers, such as lignin, and are known to secrete high-redox-potential peroxidases (34). Likewise, wood-degrading fungi of the family Tricholomataceae produce lignolytic enzymes such as manganese peroxidase and laccase (35). The relative abundance of unclassified

Chaetosphaeriaceae increased in CG with H_2O treatment and in CG and SF with TriQAC treatment over time. Chaetosphaeriaceae are capable of growing extensively on decomposed plant substrates and are distributed worldwide (36).

The bacterial community composition was not affected by the treatments (Fig. 4), which is in line with previous studies (37, 38). *Actinobacteria*, *Alphaproteobacteria*, *Acidobacteria*, and *Chloroflexi* (see Fig. S3 in the supplemental material) were the dominant bacterial phyla at every site and incubation time, and members of these phyla have been found previously in heavy-metal-contaminated soils (39, 40). *Bacillus* and *Burkholderia* were found to be dominant in heavy-metal-polluted soils and sediments (39, 40), which was in line with the findings in this study. Gremion et al. (41) showed that *Actinobacteria* prevailed within the bacterial community in contaminated soil. In addition, bacteria and fungi cooccur on dead wood less often by chance, and bacterial-fungal community interactions differ in wood decay between tree species (23). Therefore, more research is needed to understand bacterial-fungal community interactions and their contribution in the decay of both preserved and nonpreserved wood.

In summary, copper-containing wood preservatives were very powerful in delaying wood degradation and decreased the overall fungal and bacterial copy numbers at the soil-wood interface. Very heterogeneous fungal and bacterial community compositions were present at each sampling site, although they caused similar wood degradation rates. The design of innovative wood preservatives requires more finely tuned organic biocide compositions as counterparts to copper compounds to allow the development of wood preservatives with a lower copper content than used in this study. Further laboratory-based studies, in combination with upcoming field-based studies, will provide a basis for such new formulations, which aim to maximize the service life of wooden construction as well as to minimize the environmental impact.

MATERIALS AND METHODS

Study sites and soil sampling. Soil samples were collected in October 2012 from five sampling sites across Europe, differing in climate and land use conditions. Soils were collected from three different vineyards (NF, SF, and PO) and one fruit orchard (NG), each with a history of copper-based treatments, and from a field site to which saw dust and cuttings have been added but without prior biocide treatment (CG) (Table 2). The upper litter was removed from each sampling site, and then soil from the A horizon was collected from randomly allocated locations at each sampling site. The soil was then processed through a sieve with a 4-mm mesh and transported at 4°C to the laboratory.

Soil properties. Each soil parameter was determined in three independent replicates. The pH was determined in a 1:5 (wt/vol) suspension of soil-tap water. Each soil-water slurry was mixed for 60 min to create a homogeneous suspension of soil particles, and then the pH in the supernatant was determined with a glass electrode (WTW Sentix 41; Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim, Germany). Conductivity was measured according to DIN ISO 11265 by mixing 20 g air-dried soil with 100 ml tap water for 30 min at 20°C. The electrical conductivity was determined at 25°C with a conductivity meter (WTW microprocessor conductivity meter LF537; Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim, Germany). The determination of the carbonate content was performed as described by Müller and Gastner (42). Grain density was determined as described previously (43). Soil type was determined according to DIN 18123 by the grain size composition of the mineral soil material. The coarse-grained components (>2 mm) were removed by sieving through a 2-mm sieve. Fine soil (<2 mm) was characterized by the relative proportions of clay, silt, and sand, which were determined using oven-dried soils (105°C). Elements were released from 100 ml of a 2:1 (wt/vol) suspension of soil-tap water by addition of 0.5 ml 70% (wt/vol) nitric acid. Thereafter, Al, Cu, Fe, Mn, and Zn were determined by inductively coupled plasma atomic emission spectroscopy according to DIN EN ISO 11885. The samples were atomized, and aerosols were transported via helium (5.0; Westfalen AG, Münster, Germany) into inductively coupled plasma. The characteristic emission lines were disassembled with a photometer, and the line intensities were measured. The total organic content (TOC) was determined according to DIN 18128 by drying a soil sample overnight at 105°C and thereafter heating it for two hours at 550°C in a muffle furnace (Heraeus Instruments, Hanau, Germany). TOC was calculated as the difference between the initial weight of the soil sample and that of the cooled residues. Dissolved organic carbon (DOC) and total nitrogen (TN) were determined in a 2:1 (wt/vol) suspension of tap water-soil and measured with an automatic analyzer (TOC-VCPH; Shimadzu, Kyoto, Japan) according to the manufacturer's instructions.

Preservative treatment of wood specimens. Pine (*Pinus sylvestris* L.) sapwood specimens (100 mm [longitudinal] by10 mm by 5 mm) were kiln dried (103°C) for several days, cooled over a desiccant (silica gel; VWR International, Darmstadt, Germany), precisely weighed under kiln-dried conditions, and then stored in a desiccator prior to treatment. Each specimen was impregnated either with water (H_2O) as a reference or with CuTriQAC, TriQAC, or Cu (Table 1). A prevacuum of 10^4 Pa (30 min) and a pressure of

9×10^5 to 1×10^6 Pa (90 min) were used for the impregnation process. To accelerate microbial wood degradation in a laboratory-based study, the minimum copper concentration 0.48 kg m^{-3} wood (0.0923% [wt/wt]) was applied to pass the experimentally similar DIN V ENV 807:2001 (44). After drying and climatizing at 20°C and 65% relative humidity (RH), the impregnated specimens were immediately weighed to enable the calculation of any loss in weight after soil burial. Wood specimens from each treatment were then aged artificially according to DIN EN 84:1997 (45). Demineralized water was made by reverse osmosis, electrodeionization, and UV sterilization (Lenntech, Delfgauw, The Netherlands) according to the manufacturer's instructions until a conductivity of 0.1 μS was reached. Vacuum was applied to specimens from each treatment prior to immersion in demineralized water for two hours, followed by leaching in demineralized water for 14 days (20°C, 65% RH) and drying (20°C, 65% RH) for an additional 14 days.

Experimental setup and soil sampling. Soil from each of the five sampling sites was adjusted to 95% of its water-holding capacity. Samples of each soil (8 kg) were then placed in individual lidded boxes. Treated wood specimens were inserted into each soil according to DIN V ENV 807:2001 (44) to determine the wood degradation potential of each soil type (14). Sixteen replicate specimens of each treatment were placed in the lidded boxes along with eight controls (nonimpregnated wood specimens). Thirty-six wood specimens (eight replicate specimens of each treatment and four controls) were placed into each box (four rows with nine specimens, inserted vertically). In total, 360 wood specimens (five soil sampling sites [CG, NG, NF, PO, and SF] [Table 2] $\times$ four treatments [CuTriQAC, TriQAC, Cu, and H_2O] [Table 1] $\times$ four incubation times [8, 16, 24, and 32 weeks of incubation] $\times$ four replicate measurements, plus 40 controls) were incubated at 27°C $\pm$ 2°C and 70% $\pm$ 5% relative humidity (RH) in the dark. The soil moisture was adjusted to 95% of water-holding capacity by adding double-distilled water to maintain the moisture content during incubation constant. At each incubation interval, replicate (4 per treatment) wood specimens were removed from the boxes to measure MOE and weight loss. In addition, the soil at the interface of each wood specimen was sampled and analyzed by nucleic acid extraction and quantitative PCR (qPCR). A corer with a rectangular cross-section (inner dimensions, 17 mm by 8 mm) was used to take one soil sample immediately adjacent to, and surrounding, each specimen prior to its removal (see Fig. S13 in the supplemental material). Replicate soil samples ($n = 10$) that had no contact with wood were also collected for each soil and at each sampling time. All samples were cooled on ice before storage at -20°C for subsequent nucleic acid extraction.

MOE and weight loss of wood specimens. Prior to the incubation studies, the modulus of elasticity (MOE) of samples of wood was measured to record its initial value. For these and the samples taken during the study, the specimens were dried overnight in a climatic chamber and then placed in lidded boxes. The weight of each specimen was measured. The specimens were then immersed in demineralized water overnight at room temperature to ensure that the wood moisture content was above the fiber saturation point so that moisture content did not influence elasticity (46).

The wood specimens were then wiped with a soft cloth and weighed to determine the current moisture content, and the MOE was measured using the Tira test 2805 universal testing machine (Tira GmbH, Schalkau, Germany) as described earlier (47). After testing, the wood specimens were oven dried for 18 to 24 h at 103°C $\pm$ 2°C, cooled to room temperature in a desiccator, and then weighed.

Extraction of nucleic acids, qPCR, and amplicon sequencing analysis. Total microbial community DNA was isolated from subsamples (250 mg) taken from the interface of each wood specimen and the associated soil by employing the PowerSoil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative PCR (qPCR) was applied to determine the copy number of sequences from the fungal internal transcribed spacer (ITS) DNA regions with the primer set fITS7 (48) and ITS4 (49). Fungal ITS qPCR was performed in 20-μl reaction mixtures containing a 10-fold dilution of soil DNA in 1 μl of DNA template (approximately 10 to 30 ng), 0.3 μM each primer, 1$\times$ TaqMaster PCR enhancer (5-Prime, Hamburg, Germany), 1$\times$ iTaq universal SYBR green supermix (Bio-Rad, Munich, Germany), and nuclease-free water (Carl Roth GmbH, Karlsruhe, Germany). Reaction conditions involved an initial 2-min denaturation at 96°C, followed by 50 cycles of 15 s of denaturation at 96°C, annealing at 53.4°C for 30 s, and elongation at 72°C for 60 s. The final elongation step was at 72°C for 6 min. In addition, qPCR was performed to enumerate total bacterial populations by using the 16S rRNA gene as described by Zaprasis et al. (50) with primer set Eub341F and Eub534R (50). 16S rRNA gene qPCR was performed in 25-μl reaction mixtures containing a 10-fold dilution of soil DNA in 1 μl of DNA template, 0.3 μM each primer, 1$\times$ TaqMaster PCR enhancer (5-Prime), 1$\times$ iTaq universal SYBR green supermix (Bio-Rad), and nuclease-free water (Carl Roth GmbH). Reaction conditions involved an initial 8-min denaturation at 95°C, followed by 45 cycles of 40 s of denaturation at 95°C, annealing at 55°C for 30 s, and elongation at 72°C for 15 s. The final elongation step was at 72°C for 5 min. The gene copy number was calculated by comparing PCR cycle threshold values (C_T) to a standard curve of triplicate 10-fold dilutions of genomic DNA (gDNA) extracted from a known concentration of *Escherichia coli* K-12 (DSM 423) and *Aspergillus terreus* (DSM 1958) by employing the PowerSoil DNA isolation kit, as appropriate. The gDNA concentrations per PCR of *E. coli* and *A. terreus* standard ranged from 5×10^{12} to 5×10^3 and from 5×10^{11} to 5×10^3 gene copies, respectively. The copy numbers of the 16S rRNA gene and ITS region on the genomes of *E. coli* and *A. terreus* were seven and one, respectively, as found in NCBI nucleotide sequence databases. All reactions were performed in triplicate in 96-well plates using the CFX96 real-time system (Bio-Rad), and nuclease-free master mix blanks were run as negative controls. Multiple dilutions were run simultaneously to check for inhibitors in qPCR assays. Based on these results, 10-fold-diluted DNA extracts were best suited for qPCR analyses. The C_T and efficiency (E) were calculated by the Bio-Rad software CFX manager version 3.1.

To analyze the taxonomic compositions of the soil fungal and bacterial communities, the DNA of the four replicate soil samples in treatments H_2O, CuTriQAC, TriQAC, and Cu (Table 1), as well as the reference soil samples, were pooled in order to represent one sample per treatment. In total, 105 pooled samples were chosen for the amplification and subsequent amplicon sequencing (MiSeq V3; Illumina, San Diego, CA, USA) of fungal ITS region and bacterial 16S rRNA gene sequences. The primer set for the amplification of ITS region sequences was fITS7 (48) and ITS4 (51), while the primer set for 16S rRNA gene amplifications was 341F-785R (52). PCR, including tagging and adapter ligation, was carried out by LGC Genomics GmbH, Berlin, Germany. In addition, cluster generation and 300-bp paired-end sequencing on an Illumina MiSeq V3 system were performed by LGC Genomics GmbH. Raw data were demultiplexed and quality filtered, and an analysis of microbial communities was performed using Mothur 1.35.1 (53), CD-HIT 4.6.1 (54), and QIIME 1.9.0. (55). ITS region and 16S rRNA gene-based sequences were preprocessed and operational taxonomic units (OTUs) picked from amplicons with Mothur 1.35.1 (53). Sequences were removed if they contained ambiguous bases with homopolymer stretches of more than 8 bases or with an average Phred quality score of below 35. An alignment was performed against the 16S Mothur-Silva SEED r119 reference alignment. Short alignments were filtered regarding truncated or unspecific PCR products. Sequence subsampling to 40,000 sequences per sample was included, as well as sequencing error reduction by preclustering in Mothur. Chimeras were eliminated with the uchime algorithm (56). Taxonomical classification of the 16S rRNA gene-based sequences using the Silva reference classification and removal of sequences from other domains of life were conducted based on 97% identity level. Taxonomical classification of ITS region sequences to OTUs was performed against the UNITE version 6 reference database based on a 97% identity level.

After sequence processing, 0.64×10^6 and 2.85×10^6 sequences were obtained from the fungal ITS region and 16S rRNA genes, respectively. Sequences were clustered into 489 (fungal ITS region) and 867 (bacterial 16S rRNA gene) OTUs. In total, seven fungal and 36 bacterial phyla and candidate divisions were identified from soils of the five sampling sites; five fungal and 15 bacterial phyla contained a relative abundance of >1% OTUs per sample (see Fig. S3 in the supplemental material).

Statistics. The distribution characteristics of the data sets were determined using the Shapiro-Wilk test as described by Armougom et al. (57). Two-factor analysis of variance analysis (ANOVA) was performed with the ANOVA (58) function from R used on the linear model function lm to determine the statistical significance of any differences observed in the MOE, weight loss measurements, and fungal as well as bacterial gene copy numbers ($P \leq 0.05$). Diversity indices were calculated using Canoco 4.5 (Microcomputer Power, Ithaca, NY, USA). Explorative statistical analyses were performed with correspondence analysis (CA), and relative abundances of OTU composition were calculated as described by Noll et al. (59). CA was performed with relative abundances of ≥2% of fungal ITS region sequence and bacterial 16S rRNA genes using Canoco 4.5 and visualized using Origin 6.1 (OriginLab Corporation, Northampton, MA, USA). To test the effect of environmental variables (sampling site, incubation time, specimen treatment, fungal and bacterial gene copy number, mass loss, soil pH, soil content of TOC, TN, Cu, Al, Mn, Fe, and Zn) on the fungal and bacterial community compositions, canonical correspondence analyses (CCA) was employed as described elsewhere (59). To obtain tests of independent effects, the influence of each environmental variable (e.g., soil pH) was tested after adjusting for the effect of other environmental variables (e.g., sampling site, incubation time, specimen treatment, fungal and bacterial gene copy number, mass loss, soil, soil content of TOC, TN, Cu, Al, Mn, Fe, and Zn) and vice versa, and that of the interaction, after adjusting for each environmental variable, by specifying the effects to be eliminated as covariables in CCA. The effect of each environmental variable and their interaction on the sum of all canonical eigenvalues was tested by Monte Carlo permutation tests available in Canoco using 9,999 replicate runs to assess significance on the trace values as explained earlier (60). Venn diagrams of relative abundances of fungal and bacterial OTUs in R that were present at every sampling site and at each sampling time were constructed.

Accession number(s). The fungal ITS region and 16S rRNA gene sequences were deposited in the NCBI nucleotide sequence databases under accession no. PRJNA315846.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02391-18.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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REFERENCES

1. Gianfreda L, Rao MA. 2008. Interactions between xenobiotics and microbial and enzymatic soil activity. Crit Rev Environ Sci Technol 38:269–310. https://doi.org/10.1080/10643380701413526.
2. Liu Y-J, Liu S-J, Drake HL, Horn MA. 2011. *Alphaproteobacteria* dominate active 2-methyl-4-chlorophenoxyacetic acid herbicide degraders in agricultural soil and drilosphere. Environ Microbiol 13:991–1009. https://doi.org/10.1111/j.1462-2920.2010.02405.x.
3. Dallinger A, Horn MA. 2014. Agricultural soil and drilosphere as reservoirs of new and unusual assimilators of 2,4-dichlorophenol carbon. Environ Microbiol 16:84–100. https://doi.org/10.1111/1462-2920.12209.
4. Verma JP, Jaiswal DK, Sagar R. 2014. Pesticide relevance and their microbial degradation: a-state-of-art. Rev Environ Sci Biotechnol 13:429–466. https://doi.org/10.1007/s11157-014-9341-7.
5. Jakobs-Schönwandt D, Mathies H, Abraham W-R, Pritzkow W, Stephan I, Noll M. 2010. Biodegradation of a biocide (Cu-*N*-cyclohexyldiazenium dioxide) component of a wood preservative by a defined soil bacterial community. Appl Environ Microbiol 76:8076–8083. https://doi.org/10.1128/AEM.01092-10.
6. McCallan SEA. 1949. The nature of the fungicidal action of copper and sulfur. Bot Rev 15:629–643. https://doi.org/10.1007/BF02861716.
7. Flemming CA, Trevors JT. 1989. Copper toxicity and chemistry in the environment. A review. Water Air Soil Pollut 44:143–158. https://doi.org/10.1007/BF00228784.
8. Kratz S, Haneklaus S, Schnug E. 2009. Kupfergehalte in Acker- und Grünlandböden und das Verhältnis dieser Gehalte zu den durch Pflanzenschutz ausgebrachten Kupfermengen. J Kulturpflanzen 61:112–116.
9. Strumpf T, Stendel U, Vetter C. 2009. Gesamtgehalte von Kupfer in Böden des Kernobstanbaus, Weinbergen und Hopfenanlagen. J Kulturpflanzen 61:117–125.
10. Malkomes H-P. 2010. Einfluss Kupfer-haltiger anthropogener Einträge auf Bodenmikroorganimen—eine Übersicht. II. Mikrobielle Aktivitäten. J Kulturpflanzen 62:429–443.
11. Ranjard L, Nowak V, Echairi A, Faloya V, Chaussod R. 2008. The dynamics of soil bacterial community structure in response to yearly repeated agricultural copper treatments. Res Microbiol 159:251–254. https://doi.org/10.1016/j.resmic.2008.02.004.
12. European Commission. 2012. Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Off J Eur Union 55:1–123. https://doi.org/10.3000/19770677.L_2012.167.eng.
13. Lo C-C. 2010. Effect of pesticides on soil microbial community. J Environ Sci Health B 45:348–359. https://doi.org/10.1080/03601231003799804.
14. Edlund M-L, Nilsson T. 1999. Performance of copper and non-copper based wood preservatives in terrestrial microcosms. Holzforschung 53:369–375. https://doi.org/10.1515/HF.1999.061.
15. Kirker GT, Prewitt ML, Schultz TP, Diehl SV. 2012. Community analysis of preservative-treated southern pine (Pinus spp.) using terminal restriction fragment length polymorphism (T-RFLP) analysis. 1. Fungal field study. Holzforschung 66:529–535. https://doi.org/10.1515/HF.2011.171.
16. Klepper O, Traas TP, Schouten AJ, Korthals GW, de Zwart D. 1999. Estimating the effect on soil organisms of exceeding no-observed effect concentrations (NOECs) of persistent toxicants. Ecotoxicology 8:9–21. https://doi.org/10.1023/A:1008845226732.
17. Pedersen MB, Axelsen JA, Strandberg B, Jensen J, Attrill MJ. 1999. The impact of a copper gradient on a microarthropod field community. Ecotoxicology 8:467–483. https://doi.org/10.1023/A:1008964021344.
18. Zelles L, Bai QY, Ma RX, Rackwitz R, Winter K, Beese F. 1994. Microbial biomass, metabolic activity and nutritional status determined from fatty acid patterns and poly-hydroxybutyrate in agriculturally-managed soils. Soil Biol Biochem 26:439–446. https://doi.org/10.1016/0038-0717(94)90175-9.
19. Viti C, Quaranta D, Philippis R, Corti G, Agnelli A, Cuniglio R, Giovannetti L. 2008. Characterizing cultivable soil microbial communities from copper fungicide-amended olive orchard and vineyard soils. World J Microbiol Biotechnol 24:309–318. https://doi.org/10.1007/s11274-007-9472-x.
20. Whitaker RJ. 2009. Evolution: spatial scaling of microbial interactions. Curr Biol 19:954–956. https://doi.org/10.1016/j.cub.2009.09.035.
21. Li J, Ma Y-B, Hu H-W, Wang J-T, Liu Y-R, He J-Z. 2015. Field-based evidence for consistent responses of bacterial communities to copper contamination in two contrasting agricultural soils. Front Microbiol 6:31. https://doi.org/10.3389/fmicb.2015.00031.
22. Parry KE, Wood RKS. 1958. The adaptation of fungi to fungicides. Adaptation to copper and mercury salts. Ann Appl Biology 46:446–456. https://doi.org/10.1111/j.1744-7348.1958.tb02225.x.
23. Hoppe B, Kahl T, Karasch P, Wubet T, Bauhus J, Buscot F, Krüger D, Desvaux M. 2014. Network analysis reveals ecological links between N-fixing bacteria and wood-decaying fungi. PLoS One 9:e88141. https://doi.org/10.1371/journal.pone.0088141.
24. Liu F, Hu D-M, Cai L. 2012. *Conlarium duplumascospora* gen. et. sp. nov. and *Jobellisia guangdongensis* sp. nov. from freshwater habitats in China. Mycologia 104:1178–1186. https://doi.org/10.3852/11-379.
25. Kaltseis J, Rainer J, De Hoog GS, Kaltseis J, Rainer J, De Hoog GS. 2009. Ecology of *Pseudallescheria* and *Scedosporium* species in human-dominated and natural environments and their distribution in clinical samples. Med Mycol 47:398–405. https://doi.org/10.1080/13693780802585317.
26. Novinscak A, DeCoste NJ, Surette C, Filion M. 2009. Characterization of bacterial and fungal communities in composted biosolids over a 2 year period using denaturing gradient gel electrophoresis. Can J Microbiol 55:375–387. https://doi.org/10.1139/W08-152.
27. Ezzouhri L, Castro E, Moya M, Espinola F, Lairini K. 2009. Heavy metal tolerance of filamentous fungi isolated from polluted sites in Tangier, Morocco. Afr J Microbiol Res 3:35–48.
28. Zafar S, Aqil F, Ahmad I. 2007. Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil. Bioresour Technol 98:2557–2561. https://doi.org/10.1016/j.biortech.2006.09.051.
29. Romero MC, H Reinoso E, Urrutia MI, Moreno Kiernan A. 2006. Biosorption of heavy metals by *Talaromyces helicus*. A trained fungus for copper and biphenyl detoxification. Electron J Biotechnol 9:0. https://doi.org/10.2225/vol9-issue3-fulltext-11.
30. Pečiulytė D, Dirginčiutė-Volodkienė V. 2012. Effect of zinc and copper on cultivable populations of soil fungi with special reference to entomopathogenic fungi. Ekologija 58. https://doi.org/10.6001/ekologija.v58i2.2524.
31. Szewczyk R, Soboń A, Słaba M, Długoński J. 2015. Mechanism study of alachlor biodegradation by *Paecilomyces marquandii* with proteomic and metabolomic methods. J Hazard Mater 291:52–64. https://doi.org/10.1016/j.jhazmat.2015.02.063.
32. Lozovaya VV, Lygin AV, Zernova OV, Li S, Widholm JM, Hartman GL. 2006. Lignin degradation by *Fusarium solani* f. sp. *glycines*. Plant Dis 90:77–82. https://doi.org/10.1094/PD-90-0077.
33. Hibbett DS, Bauer R, Binder M, Giachini AJ, Hosaka K, Justo A, Larsson E, Larsson KH, Lawrey JD, Miettinen O, Nagy LG, Nilsson RH, Weiss M, Thorn RG. 2014. Agaricomycetes, p 373–429. *In* McLaughlin DJ, Spatafora JW (ed), Systematics and evolution. The Mycota VII, part A, 2nd ed. Springer-Verlag, Berlin, Germany.
34. Liers C, Pecyna MJ, Kellner H, Worrich A, Zorn H, Steffen KT, Hofrichter M, Ullrich R. 2013. Substrate oxidation by dye-decolorizing peroxidases (DyPs) from wood- and litter-degrading agaricomycetes compared to other fungal and plant heme-peroxidases. Appl Microbiol Biotechnol 97:5839–5849. https://doi.org/10.1007/s00253-012-4521-2.
35. Kuhad RC, Singh A. 2007. Lignocellulose biotechnology: future prospects. IK International Pvt Ltd, New Delhi, India.
36. Fernández FA, Huhndorf SM. 2005. New species of *Chaetosphaeria*, *Melanopsammella* and *Tainosphaeria* gen. nov. from the Americas. Fungal Divers 18:15–57.
37. Altimira F, Yáñez C, Bravo G, González M, Rojas LA, Seeger M. 2012. Characterization of copper-resistant bacteria and bacterial communities from copper-polluted agricultural soils of central Chile. BMC Microbiol 12:193. https://doi.org/10.1186/1471-2180-12-193.
38. Deng H, Li X-F, Cheng W-D, Zhu Y-G. 2009. Resistance and resilience of Cu-polluted soil after Cu perturbation, tested by a wide range of soil microbial parameters. FEMS Microbiol Ecol 70:137–148. https://doi.org/10.1111/j.1574-6941.2009.00741.x.
39. Kozdrój J, van Elsas JD. 2001. Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches. J Microbiol Methods 43:197–212. https://doi.org/10.1016/S0167-7012(00)00197-4.
40. Zhang H-B, Yang M-X, Shi W, Zheng Y, Sha T, Zhao Z-W. 2007. Bacterial diversity in mine tailings compared by cultivation and cultivation-independent methods and their resistance to lead and cadmium. Microb Ecol 54:705–712. https://doi.org/10.1007/s00248-007-9229-y.

41. Gremion F, Chatzinotas A, Harms H. 2003. Comparative 16S rDNA and 16S rRNA sequence analysis indicates that *Actinobacteria* might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. Environ Microbiol 5:896–907. https://doi.org/10.1046/j.1462-2920.2003.00484.x.
42. Müller G, Gastner M. 1971. The 'Karbonat-Bombe', a simple device for the determination of carbonate content in sediment, soils, and other materials. Neues Jahrbuch Mineralogie-Monatshefte 10:466–469.
43. Chang CS. 1988. Measuring density and porosity of grain kernels using a gas pycnometer. Cereal Chem 65:13–15.
44. Kartal SN, Brischke C, Rapp AO, Imamura Y. 2006. Biological effectiveness of didecyl dimethyl ammonium tetrafluoroborate (DBF) against basidiomycetes following preconditioning in soil bed tests. Wood Sci Technol 40:63–71. https://doi.org/10.1007/s00226-005-0048-3.
45. Thaler N, Lesar B, Humar M. 2013. Performance of copper-ethanolamine-impregnated Scots pine wood during exposure to terrestial microorganisms. Bioresources 8:3299–3308.
46. Zilch K, Diederichs CJ, Katzenbach R, Beckmann KJ (ed). 2013. Konstruktiver Ingenieurbau und Hochbau. Springer Vieweg, Berlin, Germany.
47. Naumann A, Seefeldt H, Stephan I, Braun U, Noll M. 2012. Material resistance of flame retarded wood-plastic composites against fire and fungal decay. Polym Degrad Stab 97:1189–1196. https://doi.org/10.1016/j.polymdegradstab.2012.03.031.
48. Ihrmark K, Bödeker ITM, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, Strid Y, Stenlid J, Brandström-Durling M, Clemmensen KE, Lindahl BD. 2012. New primers to amplify the fungal ITS2 region—evaluation by 454-sequencing of artificial and natural communities. FEMS Microbiol Ecol 82:666–677. https://doi.org/10.1111/j.1574-6941.2012.01437.x.
49. White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. *In* Innis MA (ed), PCR Protocols. Elsevier, New York, NY.
50. Zaprasis A, Liu Y-J, Liu S-J, Drake HL, Horn MA. 2010. Abundance of novel and diverse *tfdA*-like genes, encoding putative phenoxyalkanoic acid herbicide-degrading dioxygenases, in soil. Appl Environ Microbiol 76:119–128. https://doi.org/10.1128/AEM.01727-09.
51. Toju H, Tanabe AS, Yamamoto S, Sato H. 2012. High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. PLoS One 7:e40863. https://doi.org/10.1371/journal.pone.0040863.
52. Stewardson AJ, Gaïa N, François P, Malhotra-Kumar S, Delémont C, Martinez de Tejada B, Schrenzel J, Harbarth S, Lazarevic V. 2015. Collateral damage from oral ciprofloxacin versus nitrofurantoin in outpatients with urinary tract infections: a culture-free analysis of gut microbiota. Clin Microbiol Infect 21:344.e1–311. https://doi.org/10.1016/j.cmi.2014.11.016.
53. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541. https://doi.org/10.1128/AEM.01541-09.
54. Fu L, Niu B, Zhu Z, Wu S, Li W. 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28:3150–3152. https://doi.org/10.1093/bioinformatics/bts565.
55. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336. https://doi.org/10.1038/nmeth.f.303.
56. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R, Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200. https://doi.org/10.1093/bioinformatics/btr381.
57. Armougom F, Henry M, Vialettes B, Raccah D, Raoult D, Ratner AJ. 2009. Monitoring bacterial community of human gut microbiota reveals an increase in *Lactobacillus* in obese patients and *methanogens* in anorexic patients. PLoS One 4:e7125. https://doi.org/10.1371/journal.pone.0007125.
58. R Core Team. 2015. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.
59. Noll M, Matthies D, Frenzel P, Derakshani M, Liesack W. 2005. Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. Environ Microbiol 7:382–395. https://doi.org/10.1111/j.1462-2920.2005.00700.x.
60. Verdonschot PFM, Ter Braak CJF. 1994. An experimental manipulation of oligochaete communities in mesocosms treated with chlorpyrifos or nutrient additions. Multivariate analyses with Monte Carlo permutation tests. Hydrobiologia 278:251–266. https://doi.org/10.1007/BF00142333.