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ORIGINAL PAPER

Fungal communities do not recover after removing invasive *Alliaria petiolata* (garlic mustard)

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Abstract The negative impacts of non-native invasive plants on native plants has prompted intensive eradication efforts, but whether eradication can restore soil microbial communities that are also sensitive to invasion is generally not considered. Some invasive plants, like *Alliaria petiolata* (garlic mustard), specifically alter soils in ways that promote the invasion process. Garlic mustard disrupts mycorrhizas, increases fungal pathogen loads, and elevates soil nutrient availability and soil pH; thus, the fungal community and soil property responses to garlic mustard eradication may be key to restoring ecosystem function in invaded forests. We conducted a garlic mustard eradication experiment at eight temperate, deciduous forests. 1 and 3 years after initiating annual garlic mustard removal (hand pulling), we collected soil samples and characterized fungal community structure using DNA metabarcoding alongside a suite of edaphic properties. We found that fungal richness, the number of shared fungal species, fungal biomass, and the relative abundance of fungal guilds became less similar to invaded plots by year three of eradication and more similar to uninvaded reference plots. However, fungal community composition did not resemble uninvaded communities by the third year of eradication and remained comparable to invaded communities. Soil chemical and physical properties also remained similar to invaded conditions. Overall soil abiotic–biotic restoration was not observed after 3 years of garlic mustard removal. Garlic mustard eradications may therefore not achieve management goals until soil physical, chemical, and biological properties become more similar to uninvaded forested areas or at least more dissimilar to invaded conditions that can promote invasion.

Keywords *Alliaria petiolata* · Fungi · Garlic mustard · Invasive species · Mycorrhizal fungi · Mycorrhizal symbiosis · Restoration

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Introduction

Non-native invasive species pose significant threats to biodiversity (Wilcove et al. 1998) and ecosystem function (Ehrenfeld 2003) and are often intensively managed (Pimental 2007; Corbin and D'antonio 2012). A central goal of managing biotic invasions is to bring community composition and function back to

a set of desired reference conditions (Török et al. 2018). Most often this is back to an uninvaded state (Bradshaw 1996; Stanturf et al. 2014). In practice, ecosystems may fully recover to an uninvaded state (restoration) or they may partially recover (rehabilitate), remain in an invaded state (no response), or turnover to form a third, novel state (replacement; Bradshaw 1996). While there is evidence that management can rehabilitate or even restore native plant communities (Rejmánek and Pitcairn 2002; Stinson et al. 2007; Simberloff 2009; Pyšek and Richardson 2010), few studies have addressed soil microbial responses to invasive plant management (Reid et al. 2009; Lankau et al. 2014). Microbes have been widely considered functionally redundant (Martiny et al. 2017; Louca et al. 2018) and so species membership has not been afforded the same attention as plants and animals. There are several benefits to considering microbial restoration in concert with plants and animals, especially fungal restoration (Heilmann-Clausen et al. 2015). Since fungi form mycorrhizas, cause disease as plant pathogens, and mediate nutrient cycling processes as decomposers, fungal restoration could have particular importance for native plant recovery (e.g. Stinson et al. 2006).

Invasive plants can strongly influence fungal communities. Some invasive plants produce allelochemicals that directly suppress fungi (Rodgers et al. 2008). Others are non-mycorrhizal and can shift the dominant fungal trophic guild from mycorrhizal to saprotrophic (Pringle et al. 2009; Anthony et al. 2017). Some invasive plants also produce far more biomass with greater chemical complexity than native species litter, and this can reorganize saprotrophic communities (Mincheva et al. 2014) and alter soil C levels (Tamura and Tharayil 2014). Despite well-documented changes in the fungal community in response to invasive species (Lankau 2011; Lankau et al. 2014; Mincheva et al. 2014; Anthony et al. 2017), little work has addressed fungal recovery after invasive plant management. Those that have find only certain fungal species recover following eradication (Grove et al. 2012; Lankau et al. 2014). Plant species known to disperse efficiently can recover more quickly than dispersal limited species (Kiehl et al. 2010), but plants most likely to succeed in areas undergoing restoration are also those adjusted to the physical conditions of the eradicated area (Prach and Pyšek 2001). Fungi have diverse dispersal capacities, and while many are efficient dispersers (Webster and Weber 2007; Davison et al. 2015), others experience varying degrees of dispersal limitation (Peay et al. 2012; Glassman et al. 2017). While dispersal is an important aspect of community recovery, fungal re-establishment may further vary depending on local edaphic conditions. The outcome of eradication may be especially variable across fungal guilds since saprotrophic, mycorrhizal, and pathotrophic fungi assemble differentially in relation to soil properties (Tedersoo et al. 2014). In this study, we tracked fungal communities and soil physical and chemical properties following *Alliaria petiolata* (garlic mustard) eradication and compared them to reference conditions in nearby uninvaded areas of the same forests. We worked at eight forests across southern New England where garlic mustard is well established in the forest understory (Rodgers et al. 2008) and which allowed us to overcome the limitations of studying recovery at a single site and make broader inferences about the recovery of soil fungi in response to eradication (*sensu* Stinson et al. 2007; Lankau et al. 2014).

Garlic mustard is a non-native, invasive forb in North America with well-known impacts on fungal communities and soil properties (Rodgers et al. 2008). We previously found that garlic mustard invasion is associated with depleted mycorrhizal fungal biomass and a shift towards increased dominance by saprotrophic and pathogenic fungi known to influence biogeochemical cycles and native plant health (see Anthony et al. 2017). Garlic mustard invasion can also accelerate decomposition and nutrient cycling (Rodgers et al. 2008), decrease soil C concentrations (Anthony et al. 2017), and increase soil pH (Anderson and Kelley 1995; Rodgers et al. 2008; Anthony et al. 2017). Garlic mustard has invaded 37 states in the USA and is found throughout southern Canada (USDA NRCS National Plant Data Team 2018). Given the extensive introduced range of garlic mustard, complete eradication is not realistic, but garlic mustard removal initiatives are common (e.g. The Stewardship Network Garlic Mustard Challenge). At the moment, land managers are removing garlic mustard from the landscape without knowing if this promotes soil restoration, or whether restoration is consistent across geographic scales. We have already found that eradicating *A. petiolata* can increase native plant diversity and tree seedling abundances (Stinson et al. 2007) and reduce non-native earthworm

abundances (Stinson et al. 2018), forming communities that mirror those without a history of invasion. The objectives of this study were therefore to characterize fungal communities and soil properties from invaded and post one- and three-year eradication areas in comparison to adjacent uninvaded reference areas in multiple forested locations.

Methods

Sites and study design

This work was conducted at eight temperate, deciduous forests in southern New England, USA, a detailed description of which can be found in Anthony et al. (2017). Briefly, the overstory at all sites is of mixed composition with maple (*Acer saccharum, A. rubrum*), oak (*Quercus rubra*), ash (*Fraxinus americana*), and white pine (*Pinus strobus*) canopy trees. Tree seedlings, Canada mayflower (*Mianthemum candense*), trout lily (*Erythronium americanum*), and jack-in-the-pulpit (*Arisaema triphyllum*) are the most abundant understory vegetation. Within each site, we established replicate 3 × 3 m plots in June 2013 to be able to compare uninvaded, invaded, and 'to-be' eradicated plots. All invaded plots had garlic mustard at densities > 20 plants m^2 at the time of establishment. Each plot was separated by at least 10 m, with replicate uninvaded, invaded, and eradicated plots paired based on similar native understory vegetation, slope, aspect, and relief. Despite pairing plots across invasion statuses in order to account for site level heterogeneity, we cannot discern whether invaded and 'to-be' eradicated plots were previously similar to reference uninvaded plots prior to being invaded. We began removing all garlic mustard by hand pulling from the eradicated plots in May, 2014. We maintained eradication thereafter on an annual basis. In total, there were three replicate uninvaded, invaded, and eradicated plots at each forest (8 sites × 3 invasion statuses × 3 replicates = 72 plots).

Soil sampling, processing, and analyses

We collected soil samples from each plot one (June 2015) and three (June 2017) years after starting the eradications. The organic horizon was sampled by removing three intact 10 × 10 cm of the forest floor to the depth of the mineral soil surface (~ 3–5 cm). Mineral soil was collected to a depth of 5 cm beneath each organic horizon sample using a 5 cm diameter sledge-hammer soil corer. Samples from each plot were homogenized by depth for a total of 144 samples and stored in a 4 °C cooler in the field until being processed within 24 h.

All soil was passed through a 2 mm sieve to remove rocks, roots, and coarse organic debris > 2 mm. A soil subsample (~ 5 g) was immediately frozen at − 80 °C for molecular analysis and another (~ 10 g) at − 20 °C for microbial biomass assessment via phospholipid fatty acid (PLFA) analysis. Within 48 h of sampling, we measured gravimetric soil moisture after drying at 60 °C for 48 h. Bulk density was estimated as the mass of dry soil after correcting for the mass of rocks divided by the volume of collected soil. Soil pH was determined using air-dried soil and distilled water (1 g: 10 mL). Total organic C and N was measured on air-dried, finely ground soil using dry combustion on a Perkin Elmer 2400 Series II CHN elemental analyzer (Waltham, MA). The remaining soil (10 g) was extracted for inorganic N using 2M KCl (40 mL) and analyzed for ammonium and nitrate using a colorimetric microplate assay (Braman and Hendrix 2002).

Microbial biomass was estimated on freeze-dried soil (Freezone 6, Labconco, Kansas City, MO) using PLFA analysis. Briefly, lipids were extracted from soil (1 g) using phosphate buffer, chloroform, and methanol (0.8:1:2; v:v:v). The polar lipids were isolated using silicic acid chromatography and then methylated using 0.2 M methanolic potassium hydroxide (1 mL) and incubating at 60 °C for 30 min to form fatty acid methyl esters (FAMES). The FAMES were dried and reconstituted in hexane for quantification on a Varian CP-3800 gas chromatograph equipped with a flame ionization detector (Palo Alto, CA). We compared FAME peaks against a standard library of FAMES specific to fungi (18:2ω6, 9c, 18:1ω9c) (Matreya, LLC, Pleasant Gap, PA). A standard control biomarker (c19:0) was used to convert peak area concentrations into nmol PLFA g^{-1} dry soil.

Fungal community structure (richness and community composition) was characterized using ITS2 metabarcoding on the Illumina MiSeq platform. DNA was extracted from soil (0.25 g) using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). The ITS2 region was amplified using

the fungal specific primer pair fITS7 (Ihrmark et al. 2012) and ITS4 (White et al. 1990). PCR primers contained the Illumina adaptor sequence, an 8 bp pad sequence, a 2 bp linker sequence, and were dual indexed to include two unique 8 bp sequences (see custom PCR primer constructs, Supplementary Table 1). PCR reactions were performed in triplicate for each sample in 25 μL reactions with the following reagents: PCR Grade H_2O (13 μL), PCR master mix (10 μL; Phusion® High Fidelity Master Mix, New England Biolabs, Ipswitch, MA), 10 μM fITS7 (0.5 μL), 10 μM ITS4 (0.5 μL), and template DNA (1 μL). Thermocycler conditions followed that of Anthony et al. (2017). PCR products were cleaned using the AxyPrep MAG PCR Clean-up kit (Corning, Tewksbury, MA). Final PCR products were inspected on an agarose gel and DNA concentration was measured by fluorometry on a Qubit® 3.0 Fluorometer (Life Technologies, Grand Island, NY). Equimolar libraries of the samples were split on two separate Illumina MiSeq runs (2 × 250 bp chemistry) for each sampling year for a total of four MiSeq runs at the Center for Genomics and Bioinformatics at Indiana State University (Bloomington, IN). Raw sequences were deposited in the NCBI Sequence Read Archive under bioproject number PRJNA504442.

Sequences were passed through a series of quality control measures. First, we removed low quality bases and reads (Phred score < 2), short reads (< 100 bp), and Illumina adapter and PCR primer sequences using Trimmomatic (v0.26-0.32; Bolger et al. 2014). We then merged forward and reverse reads at a 20-50 bp overlap allowing 5% mismatch using the join_paired_ends.py function in QIIME and the fastq_mergepairs function in USEARCH (v11; Edgar 2010) for the one and three-year samples, respectively. Next, we isolated the ITS2 fragment from each sequence using the ITSx software (v1.011; Bengtsson-Palme et al. 2013). We used the USEARCH (v8 and v11; Edgar 2010) pipeline to create OTU tables. We dereplicated the sequences and excluded sequences < 150 bp (derep_fulllength). Then we sorted the sequences and removed singletons (sortbysize), and calculated sequence similarity at a 97%

Table 1 Table of soil variables

	Soil pH	Ammonium (μg g^{-1} dry soil)	Nitrate (μg g^{-1} dry soil)	Soil C (g C m^{-2})	Soil C:N ratio	Fungal biomass (nmol PLFA g^{-1} dry soil)
1 year						
Organic						
Uninvaded	4.9 (0.17)a	21.6 (3.5)a	66.0 (18.1)a	18.08 (1.78)a	13.1 (0.85)a	66 (7)ab
Invaded	5.5 (0.13)b	17.3 (2.4)a	143.5 (40.3)b	17.55 (1.83)a	13.57 (0.83)a	56 (5)b
Eradicated	5.3 (0.14)b	22.5 (5.2)a	185.9 (35.7)b	17.21 (1.45)a	13.97 (0.27)a	79 (7)a
Mineral						
Uninvaded	5.0 (0.13)a	21.4 (3.5)a	56.7 (10.5)a	18.7 (1.21)a	11.66 (1.14)a	42 (6)a
Invaded	5.2 (0.11)b	13.2 (1.9)b	58.7 (15.8)a	16.85 (1.81)a	12.11 (0.77)a	35 (4)a
Eradicated	5.1 (0.1)ab	19.1 (3.1)ab	42.7 (12.8)a	18.84 (2.39)a	12.48 (0.8)a	41 (4)a
3 years						
Organic						
Uninvaded	4.3 (0.17)a	8.3 (1)a	0.3 (0.1)a	20.94 (2.33)a	15.09 (0.51)a	68 (6)a
Invaded	4.7 (0.16)b	13.0 (3)ab	0.6 (0.1)b	17.37 (2.02)b	14.18 (0.27)a	68 (7)a
Eradicated	4.4 (0.17)ab	10.9 (1)b	0.7 (0.1)b	18.98 (2.82)b	14.17 (0.38)a	73 (6)a
Mineral						
Uninvaded	4.2 (0.16)a	5.9 (0.5)a	0.3 (0.1)a	21.87 (2.45)a	14.66 (0.51)a	33 (2)a
Invaded	4.6 (0.12)b	8.5 (1.07)b	0.3 (0.1)a	16.48 (2.06)a	12.85 (0.42)b	33 (3)a
Eradicated	4.4 (0.11)ab	7.5 (1.07)ab	0.3 (0.1)a	17.94 (1.37)a	13.32 (0.41)b	36 (4)a

Values represent the mean, with standard errors shown in parentheses. Values with different lowercase letters are significantly different across invasions statuses in the organic horizon and mineral soil, respectively

cutoff while removing chimeras (cluster_otus). We assigned taxonomy to OTUs using the UNITE reference database (version 7; January 2016 release) and the utax reference database (January 2017 release) using the assign_taxonomy.py function in QIIME and the Sintax approach in USEARCH for the one- and three-year analyses, respectively. OTUs without a match to fungi were blasted against the entire NCBI *nr* database, and OTUs assigned to non-fungal organisms were removed from subsequent analyses. Genus-level taxonomic assignments were compared to the FUNGuild database to make functional guild annotations (Nguyen et al. 2016) and all 'probable' or above probable matches were included for subsequent analyses. We grouped fungi by trophic mode (saprotrophic, pathotrophic) and whether they were ectomycorrhizal fungi (EMF). Arbuscular mycorrhizal fungi were not included in functional guild analyses because the primers we used poorly target the Glomeromycotina. A table of sequence retention after quality control steps, taxonomic annotation, and guild annotations is found in the supplementary materials (Supplementary Table 2).

Statistical analyses

All statistical analyses were conducted in R 3.0.2 (R Development Core Team 2008), with significance across all tests set at $P \leq 0.05$. Linear mixed effects models were used to look for significance of univariate response variables (i.e. fungal richness, biomass, soil properties) across sites, invasion statuses, and site × invasion statuses using the lme function within the nlme package (Pinheiro et al. 2007). We created beyond optimal models that parameterized for autocorrelation and unequal variance across predictor variables. We used *t*-tests with heteroscedastic variance to make multiple comparisons. All of the edaphic properties were also analyzed together using Euclidean distance and principle correspondence analysis (PCoA) with the pcoa function in the ape package (Paradis et al. 2004). Fungal community analyses were run after randomly rarifying the OTU table 1000 times at the lowest sequencing depth of 4000 and 1025 sequences per sampling unit using the rrarefy function for the one- and three-year samples, respectively. Species (OTU) richness was calculated using the specnumber function within the vegan package (Oksanen et al. 2013). The number of shared OTUs (species overlap) among invasion statuses was calculated using the ChaoShared function within the SpadeR package (Chao et al. 2016). Multivariate analyses of fungal community composition were run using resemblance-based permutation methods. Permutation ANOVA (PERMANOVA; Anderson 2001) and heterogeneity of multivariate dispersion (PERMDISP; Anderson et al. 2006) were run using the functions adonis and betadisper in the vegan package. Heterogeneity of multivariate dispersion is a measure of beta diversity. Communities are divergent if community composition is variable across space (high beta diversity) and convergent if composition is similar across space (low beta diversity; Anderson et al. 2006). Distance-based analyses for fungi were performed on Bray–Curtis dissimilarity matrices calculated from OTU relative abundances. Significance of permutation methods was determined after 1000 permutations. Different visualization approaches were used depending on whether one versus two dimensions we were being analyzed. Non-metric multidimensional scaling (NMDS) was used to visually display fungal community composition using the metaMDS function (vegan) since NMDS does not use eigenvalues (*sensu* PCoA) and is most appropriate for visualizing the highest amount of variation in communities using two dimensions. We used PCoA axes to account for variation in fungal community composition based on a single axis since PCoA decomposes variation to many individual axes (unlike NMDS). To compare abiotic–biotic soil recovery pathways, we analyzed co-variation between the first PCoA axis (most explanatory) of all soil properties (Euclidean distance) and the first PCoA axis of fungal community composition (Bray–Curtis).

Results

Soil physical and chemical properties and fungal biomass

Soil pH and inorganic N contents were elevated in association with invasion and remained higher in the eradicated compared to uninvaded reference plots (Table 1). By year three, soil pH in eradicated plots was in between that of the uninvaded and invaded plots. Soil C stocks and C:N ratio tended to be lower in invaded and eradicated plots compared to uninvaded

reference plots, but this was only significant in year three soils in the organic and mineral soils, respectively. Overall, fungal biomass varied across invasion statuses in year one ($F_{(2,94)}$; $P = 0.01$) but not in year three ($F_{(2,91)}$, $P = 0.61$; Supplementary Table 3). Specifically, the eradicated plots had higher fungal biomass than the invaded plots in year one (*t* test; $P = 0.005$), but this disappeared by year three. There were otherwise no differences in fungal biomass.

Fungal richness, evenness, species overlap, and community composition

Fungal richness was elevated in association with invasion ($F_{(2,90)}$= 8.5, $P = 0.004$) and remained elevated in the eradicated plots one year after eradication (Supplementary Table 4). The eradicated and invaded plots also shared 484 OTUs while eradicated and uninvaded reference plots only shared 246 OTUs (Supplementary Fig. 1). By the third year of eradication, species membership became less similar to invaded plots. Fungal richness was no longer elevated in the eradicated plots compared to uninvaded plots (*t* test; $P = 0.32$; Supplementary Table 4), and eradicated and invaded communities only shared 217 OTUs while approximately the same number of OTUs (240) were shared with uninvaded plots as in year one.

In contrast to the number of shared OTUs, community composition differed between uninvaded reference plots and the invaded and eradicated plots in both years (PERMANOVA: Table 2; Fig. 1), while eradicated and invaded communities were not different from each other in either year. Specifically, variation in community composition (beta diversity) was lower in both the invaded and eradicated plots compared to uninvaded reference plots (PERMDISP: Table 2).

Guild relative abundances

EMF relative abundance was lower while saprotrophic and pathotrophic fungal relative abundances were higher in the invaded compared to uninvaded plots (Fig. 2). After one year of eradication, the relative abundances of EMF, saprotrophic, and pathotrophic groups were similar between eradicated and uninvaded reference plots. After 3 years of eradication, there were no differences between uninvaded, invaded, and eradicated plots in terms of guild relative abundances. In contrast, the taxonomic composition of functional guilds varied across invasion statuses in year one and in year three. In year one, there were reduced relative abundances of some EMF taxa (Russulales, Sebacinales, Cantharellales) and increased relative abundances of some saprotrophs

Table 2 Table of statistical values showing the influence of site, invasion, soil horizon, and site × invasion on fungal community composition (PERMANOVA) and variation in heterogeneity of community composition (PERMDISP)

Analyses were performed on Bray–Curtis dissimilarities, and significance was determined after 1000 permutations. Dashes indicate where a statistical output was not generated. Unv., Erad., and Inv. is short for uninvaded, eradicated, and invaded, respectively

Values in bold are significant

	1 year			3 years		
	F value	R^2	*P* value	*F* value	R^2	*P* value
PERMANOVA						
Site	4.95	0.19	**0.001**	2.21	0.14	**0.001**
Invasion	2.398	0.03	**0.001**	1.**52**	0.02	**0.001**
Unv. × Erad.	1.92	0.02	**0.001**	1.55	0.02	**0.002**
Unv. × Inv. Erad	1.94	0.02	**0.001**	1.63	0.02	**0.001**
Erad. × Inv.	1.07	0.01	0.27	1.05	0.01	0.33
Horizon	2.11	0.01	**0.001**	1.78	0.01	**0.001**
Site × Invasion	1.62	0.03	**0.03**	0.01	0.12	**0.001**
PERMDISP						
Site	2.94	–	**0.007**	31.3	–	**< 0.0001**
Invasion	9.61	–	**0.0001**	6.65	–	**0.002**
Unv. × Erad.	7.27	–	**0.008**	12.76	–	**0.001**
Unv. × Inv. Erad	17.58	–	**< 0.0001**	9.69	–	**0.004**
Erad. × Inv.	3.49	–	0.07	0.11	–	0.74
Horizon	0.26	–	0.61	8.29	–	**0.005**
Site × Invasion	1.37	–	0.14	12.5	–	**< 0.0001**

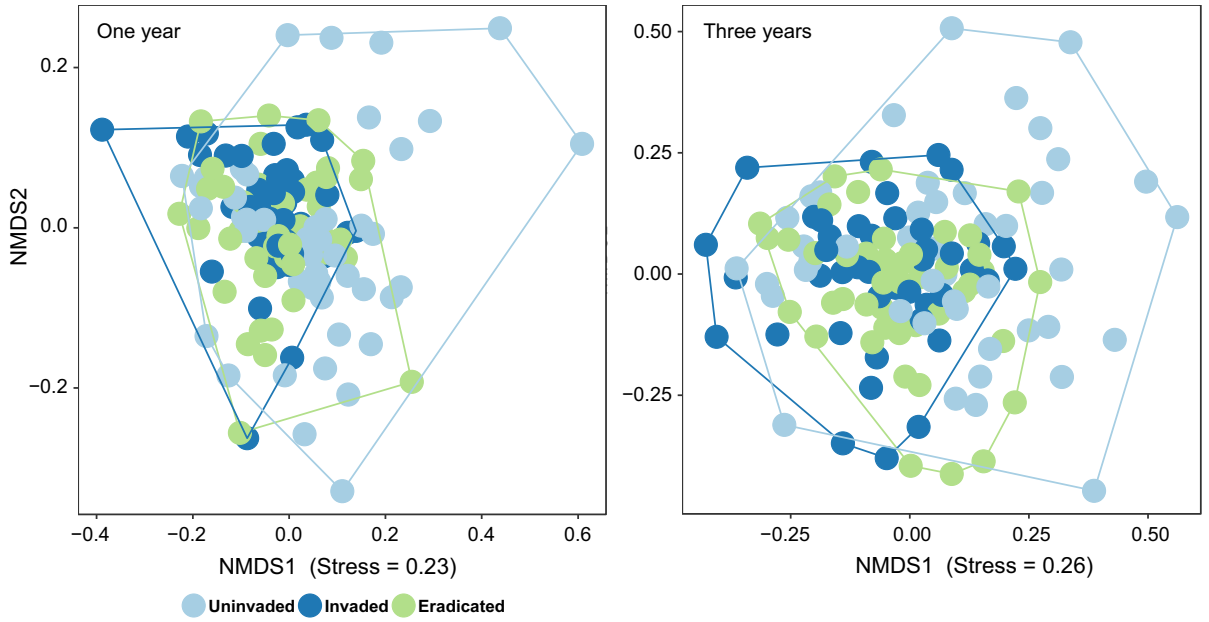

Fig. 1 Fungal community composition 1 and 3 years after garlic mustard eradication. Points represent the Bray–Curtis dissimilarity across sites, with the range in dissimilarity across invasion statuses shown using polygons

(Mortierellomycetes, Sordariomycetes, Wallemiomycetes) and pathotrophs (Polyporales, Urocystidales, Olpidiales) in the invaded plots compared to uninvaded plots (Table 3). The relative abundance of certain EMF (Russulales and Sebacinales) increased to levels between uninvaded and invaded plots in the eradicated plots while other EMF had higher relative abundances in the eradicated plots compared to reference uninvaded plots (Agaricales, Pezizales). Some of the same saprotrophs (Wallemiomycetes, Basidiobolales) and pathotrophs (Polyporales, Urocystidales, Olpidiales) had similarly high relative abundances in eradicated plots as in invaded ones, in addition to different saprotrophs (Basidiobolales) and pathotrophs (Spizellomycetales). In the year three sampling, taxonomic composition still varied among all three invasion statuses (Table 3). Notably, there was still higher relative abundances of pathotrophic Urocystidales and Olpidiales in the invaded and eradicated plots compared to uninvaded plots.

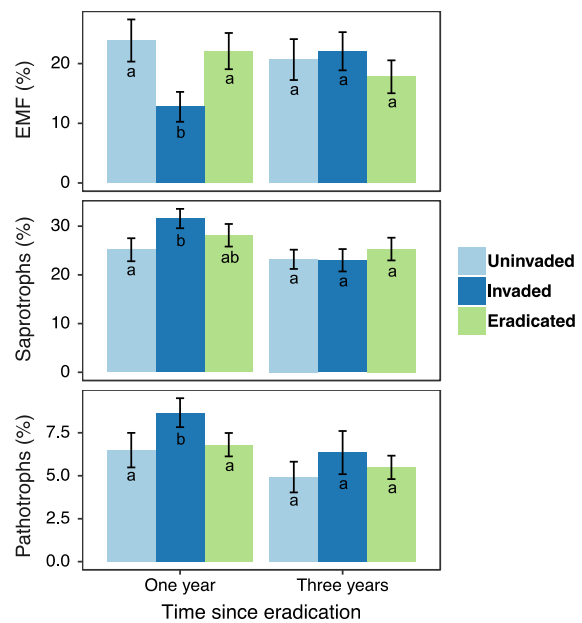

Fig. 2 The relative abundance of fungal functional guilds 1 and 3 years after garlic mustard eradication. Bars represent the mean and error bars are the standard error. Different lowercase letters are significantly different

Relationships between environmental variables and the fungal community

Shifts in the fungal community were correlated to soil properties and may have prevented overall abiotic–biotic soil system recovery. First, variation in fungal community composition was restricted (*a.k.a.* converged) in association with invasion and eradication

Table 3 The relative abundance of ectomycorrhizal (EMF; orders), saprotrophic (classes), and pathotrophic (orders) taxonomic relative abundances that were significantly altered by invasion status one and three year after eradication

	Uninvaded	Invaded	Eradicated
Year one			
EMF			
Russulales	0.147 (0.027)a	0.064 (0.016)b	0.105 (0.024)ab
Agaricales	0.036 (0.01)ab	0.02 (0.008)a	0.055 (0.015)b
Sebacinales	0.013 (0.007)a	< 0.001b	0.002 (0.001)ab
Cantharellales	0.01 (0.004)a	0.001 (0)b	0.016 (0.009)a
Pezizales	0.001 (0)a	0.009 (0.002)b	0.005 (0.002)b
Saprotrophs			
Mortierellomycetes	0.144 (0.014)a	0.225 (0.015)b	0.174 (0.013)a
Sordariomycetes	0.005 (0.001)a	0.01 (0.002)b	0.008 (0.002)ab
Wallemiomycetes	0.003 (0.001)a	0.011 (0.002)b	0.006 (0.001)c
Kickxellales	0.001 (0)a	< 0.001b	0.001 (0)ab
Basidiobolales	< 0.001a	< 0.002ab	< 0.002b
Pathotrophs			
Polyporales	0.004 (0.001)a	0.009 (0.001)b	0.006 (0.001)c
Spizellomycetales	0.001 (0.001)a	0.001 (0.001)ab	< 0.001b
Urocystidales	< 0.001a	0.006 (0.003)b	0.002 (0.001)b
Olpidiales	Absent	< 0.001b	< 0.001c
Year three			
EMF			
Agaricales	0.0293 (0.0092)a	0.065 (0.0162)b	0.0445 (0.0101)ab
Mytilinidiales	0.0059 (0.002)a	0.0036 (0.0021)ab	0.002 (0.0011)b
Pezizales	0.0011 (0.0005)a	0.0044 (0.0011)b	0.0063 (0.0024)ab
Gomphales	Absent	0.0018 (0.0014)	Absent
Saprotrophs			
Agaricomycetes	0.0949 (0.0161)a	0.0696 (0.0127)b	0.1162 (0.0213)a
Mortierellomycetes	0.0457 (0.0053)a	0.0714 (0.0079)b	0.0673 (0.0058)b
Geoglossomycetes	0.0145 (0.0039)a	0.0028 (0.0012)b	0.0038 (0.0016)b
Umbelopsidomycetes	0.0023 (0.0007)a	0.0059 (0.0018)b	0.0056 (0.0014)b
Tremellomycetes	0.0013 (0.0005)a	0.0014 (0.0003)a	0.002 (0.0005)a
Geminibasidiomycetes	0.0011 (0.0004)a	0.0027 (0.0007)b	0.0032 (0.0013)ab
Pathotrophs			
Rhizophydiales	0.002 (0.0004)a	0.0057 (0.0016)b	0.0046 (0.0017)ab
Spizellomycetales	0.0013 (0.0008)ab	0.0001 (0.0001)a	0.0005 (0.0002)b
Thelebolales	0.0005 (0.0002)a	0.0005 (0.0002)a	0.0013 (0.0004)b
Urocystidales	0.0004 (0.0002)a	0.0045 (0.0024)b	0.0009 (0.0006)ab
Xylariales	0.0004 (0.0002)a	0.0003 (0.0002)ab	0.0001 (0.0001)b
Venturiales	0.0001 (0)a	0.0005 (0.0002)b	0.0016 (0.0011)ab
Eurotiales	Absent	0.0005 (0.0003)b	0.0003 (0.0002)b
Olpidiales	Absent	0.0001 (0)a	0.0002 (0.0002)a

Guild annotations were made at the genus and species level and then summarized at the class and order levels. Values are the mean and error bars are the standard error. Values with different lowercase letters within a lineage are significantly different

(Fig. 1), and the degree of community convergence (i.e. decrease in fungal beta diversity) was positively correlated to soil pH (Fig. 3; $R^2 = 0.48$, $P < 0.0001$). Overall, when we examined variation in fungal community composition (biotic) relative to all abiotic soil variables, soil systems in the eradicated treatments were not different from invaded soil systems (Fig. 4). This indicates that eradication did not restore soil

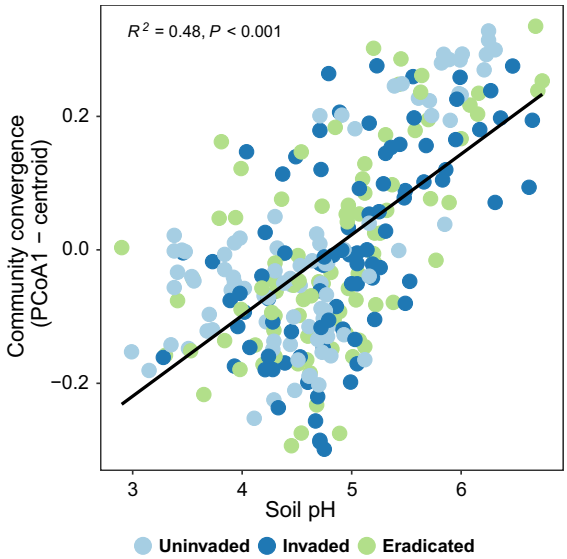

Fig. 3 Community convergence in relation to soil pH across invasion statuses. Positive values indicate that communities are converging towards a common composition (decreasing beta diversity) while negative values indicate that communities are diverging away from a common composition (increasing beta diversity)

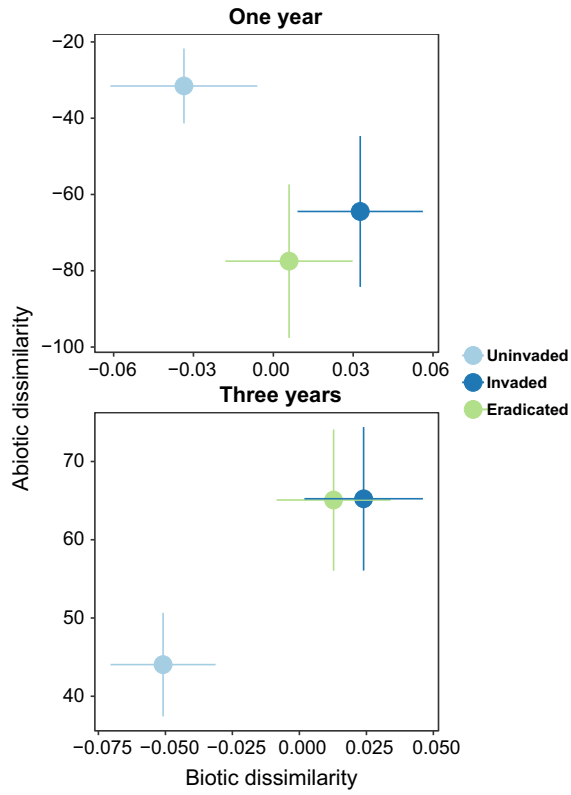

Fig. 4 Overall dissimilarity in the fungal (biotic) and edaphic (abiotic) components of soils one and three years after eradication in the reference uninvaded, invaded, and eradicated plots. Points represent the centroid of fungal community composition (Bray–Curtis) in principle correspondence analysis (axis 1) and edaphic property composition (Euclidean) in principle correspondence analysis (axis 1) and error bars are the standard deviation

systems back to an uninvaded state, despite partial rehabilitation in fungal richness, community evenness, species-overlap, and total fungal guild relative abundances.

Discussion

Extensive research and conservation initiatives have sought to understand and control non-native invasive species (Simberloff 2009). Despite this attention, invasion ecology has largely overlooked what happens in soils after an invasive plant has been removed (Lankau et al. 2014), even though mycorrhizas and pathogens (Bennett et al. 2017) and soil fertility (Meekins and McCarthy 2000) all effect plant regeneration and growth. If soil physical, chemical, and biological conditions remain similar in eradicated compared to invaded areas, then sites undergoing restoration may be prone to re-invasion and require additional management in the future (Ries et al. 2004). To that end, our most important findings were that fungal richness, species overlap, and functional guild relative abundances partially recovered to reference conditions after 3 years of garlic mustard removal, while fungal community composition and soil chemical and physical attributes remained fundamentally different in eradicated relative to uninvaded reference plots. Since fungi differentiating uninvaded and eradicated plots have different ecological strategies, the functioning of areas undergoing eradication has likely not recovered to an uninvaded state. Further, soil pH, inorganic N concentrations, and soil C stocks remained altered in the eradicated plots, likely prohibiting fungal community restoration via environmental filtering (Kivlin et al. 2014). Although we hypothesize that some ecosystem functions in the eradicated plots have been restored due to increased total ectomycorrhizal and reduced total pathotrophic fungal abundances, overall soil abiotic–biotic

recovery to uninvaded reference conditions was not responsive to three years of annual garlic mustard removal.

Soil properties did not recover

Garlic mustard invasion has well-known impacts on soil properties (Rodgers et al. 2008; Anthony et al. 2017), but whether soil properties respond to garlic mustard eradication is unclear. By the third year of eradication in our study, eradicated plots had higher ammonium and nitrate contents and reduced organic soil C stocks and mineral soil C:N ratios compared to uninvaded plots. Soil pH decreased to be in between uninvaded and invaded levels, but it was still elevated following eradication. It is worth noting that differences in soil properties across invasion statuses were similar across years despite some fluctuations in actual soil property values. In particular, first year samples had higher soil pH and nitrate contents than we observed in our baseline analysis (Anthony et al. 2017) and compared to year three (Table 1). We think this may have to do with a drought in 2015 (mean annual precipitation: 102 cm) compared to our baseline data collected in 2013 (116 cm) and data collected in 2017 (115 cm) (Northeast RCC 2018). Despite fluctuations in actual edaphic values, soil pH and nitrate contents were still elevated in the invaded and eradicated plots in both years. 3 years of garlic mustard removal therefore did not restore soil chemical and physical properties back to reference uninvaded levels.

Restoration of fungal richness but not community composition

Fungal richness was elevated in the invaded plots prior to eradication (Anthony et al. 2017) but decreased to uninvaded levels after three years of eradication. Our results are consistent with another study showing that arbuscular mycorrhizal fungal richness was restored to uninvaded levels after six years of garlic mustard eradication (Lankau et al. 2014). This provides strong evidence that garlic mustard invasions are responsible for changes in fungal richness, either directly, as has been suggested before (Lankau 2011; Anthony et al. 2017) or indirectly through changes in plant communities (Stinson et al. 2007) and earthworm densities (Stinson et al. 2018), but we cannot discern what the composition of fungal communities was like in the invaded and eradicated plots prior to invasion. Restoration of fungal richness is only indicative of potential fungal establishment, however, since it does not consider abundance (i.e. community composition). If fungi are like plants, then fungi must colonize and increase abundances dramatically to become viable community members (Williamson and Fitter 1996; Tilman 2004). Community composition, measured as Bray–Curtis dissimilarity, measures species membership alongside relative abundances. It remained altered in the eradicated plots, indicating that soil fungal communities have not been fully restored to uninvaded reference plots. There are therefore other elements of garlic mustard invasion beside the presence of garlic mustard that influence fungal establishment and persistence (i.e. legacy effects, soil properties).

We previously found that garlic mustard invasion was associated with lower regional beta diversity since fungal communities in invaded plots converged towards a common composition (Anthony et al. 2017), and we found in our current study that fungal beta diversity remained lower in the eradication treatments. Communities can converge towards a common community composition when environmental variation is low (Caruso et al. 2012). Since soil properties in garlic mustard invaded soils are more homogenous than uninvaded soils (Anthony et al. 2017), and there was little restoration of soil properties in the eradicated plots, we suggest that community homogenization is related to low environmental variation. In particular, soil pH remained elevated across the eradicated plots compared to the uninvaded plots. We also found that the degree of convergence in community composition was positively correlated to soil pH (Fig. 3). Elevated soil pH in the eradicated and invaded plots likely contributed to homogeneity of fungal communities. Since soil properties have well known environmental filtering effects on fungi (Kivlin et al. 2014; Glassman et al. 2017), there will likely be legacy effects of invasion on fungal community composition as long as soil properties remain altered. However, it is important to acknowledge the correlative nature of our study since we do not know what the soil conditions in invaded and eradicated plots were prior to invasion.

Fungal guild relative abundance but not taxonomic membership and relative abundance were restored

Invasive plants leave a signature in the microbial community (legacy effect) even after their removal (Elgersma et al. 2011; Grove et al. 2012), but relatively little is known about which fungal groups or individual taxa are responsible for these legacy effects (Lankau et al. 2014). We found that garlic mustard invasion decreased EMF relative abundance but increased that of saprotrophs and pathotrophs, mirroring our earlier results comparing uninvaded and invaded communities (Anthony et al. 2017). Eradication appears to return total guild relative abundances back to uninvaded reference levels within the first year of eradication, with an increase in EMF and decreases in saprotrophic and pathotrophic relative abundances. There was generally no impact of invasion or eradication on fungal biomass, with one exception. There was higher fungal biomass in the eradicated plots after the first year compared to invaded plots. Since EMF increased in relative abundance in the eradicated compared to invaded plots, we hypothesize that the temporal increase in fungal biomass was due to initial recolonization by EMF. There were EMF taxa that increased in relative abundance in the eradicated compared to invaded plots in the first year that were not significantly more abundant by the third year (Agaricales and Cantharellales). Of highest relative abundance were EMF Agaricales, including *Amanita, Gliophorus, Hebeloma, Hymenogaster, and Inocybe* (Supplementary Table 5). Differences in fungal traits, including growth rates and dispersal mode, may make certain taxa especially effective at initial recolonization (Twieg et al. 2007; Moeller et al. 2014). For example, *Amanita* produces larger mushrooms than most EMF and has high spore production (Bässler et al. 2015), while members of the *Hebeloma*, *Hymenogaster*, and *Inocybe* genera have ornamented spores that may promote dispersal by animals (Güler and Türkoglu 2015; Halbwachs et al. 2015).

At the third year of eradication, fungal biomass was similar to uninvaded reference levels and was not different from invaded plots. The relative abundance of EMF Agaricales was still higher in the eradicated plots, but this was no longer significant. Rather, the EMF community was depleted in Mytilinidiales (*Cenococcum*) and Gomphales (*Ramaria*). The loss of *Cenococcum* is especially concerning because this taxon was previously identified as an indicator taxon of uninvaded soils and is sensitive to garlic mustard invasion (Anthony et al. 2017). *Cenococcum* is also resistant to abiotic stressors and may be especially important during times of drought (Fernandez and Koide 2013). Ectomycorrhizas without *Cenococcum* may be particularly vulnerable to climatic stressors, especially in garlic mustard invaded forests. In addition to the EMF that were not present in the eradicated plots, there were also those that recovered back to reference uninvaded levels by year three. The most abundant EMF lineage, the Russulales, comprised of *Russula* and *Lactarius,* were one such group. *Russula* and *Lactarius* are known to have ligninolytic decomposing abilities (Looney et al. 2018), and they are often found between decomposing leaves and root tips, so they are especially beneficial symbionts for nutrient acquisition (Agerer 2001). Since EMF Russulales are also the dominant EMF across the study sites, recovery of these fungi should benefit ectomycorrhizal tree seedlings that are vulnerable to garlic mustard invasions (Stinson et al. 2007; Castellano and Gorchov 2012). Overall, we found that EMF have been partially rehabilitated since some taxa have recovered while others remain in an altered state.

Saprotrophic fungi also exhibited partial recovery to garlic mustard eradication with sustained alteration to some taxa in relation to reference uninvaded plots. We have previously found that garlic mustard invasion associated with increased relative abundance of Mortierellomycetes (Anthony et al. 2017) and they were still at higher relative abundances in the eradicated plots in our current study. At year three, there were reduced relative abundances of Geoglossomycetes (*Geoglossum*, *Glutinoglossum*, *Trichoglossum*) but increased relative abundance of Umbelopsidomycetes (*Umbelopsis*) in the eradicated plots compared to uninvaded plots. It is notable that none of the saprotrophic fungi that had different relative abundances in the eradicated plots were Basidiomycota. Saprotrophic Agaricomycetes (Basidiomycota), which include the strongest lignocellulose decomposers (Floudas et al. 2012), were comparable between uninvaded and eradicated plots. We therefore suspect the same potential for leaf litter decomposition between uninvaded and eradicated plots but differing organic matter decomposition potential due to sustained alteration to the Mortierellomycetes, Geoglossomycetes, and Umbelopsidomycetes.

We also found that pathotrophic fungi partially recovered following garlic mustard eradication. There were two classes of pathogens that were only found in the invaded and eradicated plots (pathogenic Eurotiales and Olpidiales). The Eurotiales included 11 *Penicillium* species, *Aspergillus citriporus, Sagenomella diversispora*, and three *Talaromyces* species with varying animal and plant virulence. Of particular interest, we found that the sole member of the Olpidiales, *Olpidium brassicae* was never found in the uninvaded plots, but it was present across the invaded and eradicated plots. We previously reported that *Olpidium brassicae* was exclusively found in garlic mustard invaded plots (Anthony et al. 2017), and so this taxon is a stable member of the invaded landscape and does not go locally extinct with garlic mustard eradication. *O. brassicae* can transmit plant viruses that spill over into neighboring plants, but this fungus is not known to cause disease in the host plant (Hartwright et al. 2010). *O. brassicae* also produces zoospores that can remain dormant for up to 20 years (Campbell 1985). It is also important to acknowledge that despite these fungi being annotated as pathotrophs, it is possible that they are actually living as saprotrophic fungi. We cannot discern the ecology of pathotrophic fungi with flexible trophic modes based on DNA metabarcoding. Nonetheless, the prevalence of novel pathotrophic fungi is consistent with earlier work (Anthony et al. 2017) and could have negative impacts on plant and soil animal recovery.

We previously found a shift in the dominant trophic guild from EMF in reference uninvaded forested areas to saprotrophic and pathotrophic fungi with garlic mustard invasion (Anthony et al. 2017). We found the same pattern in the first-year samples of this current study, but this was gone by year three. In the first year, the average cover of adult garlic mustard was 14 plants m^2 (5% relative abundance), while in the third year it dropped to 6 plants m^2 (3% relative abundance). Since adult plants are larger and produce more of the secondary chemicals than first year plants (unpublished data; Supplementary Fig. 2), decreased adults in the third year could allow the fungal community 'to recover' in the invaded plots. Other work has also shown that the density of garlic mustard scales with the impacts of invasion on AMF richness (Lankau 2011) and AMF community composition (Burke et al. 2011). Since garlic mustard invasions tend to be patchy across an invaded forest, it is likely that times of low garlic mustard cover result in rapid recovery of fungal guild relative abundances. This could be a potentially good time to introduce native species, especially ectomycorrhizal tree seedlings that rely more heavily on mycorrhizas (Bennett et al. 2017).

Conclusion: abiotic–biotic soil recovery

Remediation should consider how both abiotic (e.g. edaphic properties) and biotic (e.g. community composition) attributes change (or not) in concert during restoration. Soil abiotic and biotic elements shape each other through environmental filtering and microbial metabolism, and this system informs overall response to management (Bradshaw 1996). We applied this framework to our study and found that eradicated sites did not recover or even rehabilitate after three years of garlic mustard removal (Fig. 4). Rather, eradicated sites remained indistinguishable from invaded ones. Of course, there are many abiotic and biotic elements that we did not measure, but basic soil properties and fungal communities are good indicators of soil function in temperate forests. We therefore conclude that garlic mustard management in southern New England should anticipate eradication programs requiring longer than 3 years to restore soils. Work on arbuscular mycorrhizal fungi at a single site showed that even after 6 years, AMF communities did not fully recover following garlic mustard eradication (Lankau et al. 2014). Environmental stressors can permanently alter soil systems (Bradshaw and Chadwick 1980), but we do not know what the long-term impacts of invasion are on soils. Future work will need to track the long-term (> 10 years) outcomes of eradication in order to determine if restoration is possible. We also think future work should address whether eradicated areas are equally prone to invasion as uninvaded or currently invaded areas, whether tree seedlings survive as well in the eradicated landscape despite different fungal taxonomic makeup (including AMF species that have not yet been studied in detail or across multiple forested areas), and whether eradicated soils can regain some of their lost soil C. These important questions can help land managers determine whether the eradicated landscape, despite not being restored, adequately supports native biodiversity and ecosystem function.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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