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Plant–soil interactions regulate the identity of soil carbon in invaded ecosystems: implication for legacy effects

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Summary

1. Introduced, invasive plants can alter local soil chemistry and microbial communities, but the underlying mechanisms and extent of these changes are largely unknown. Based on characteristics associated with invasiveness in plants, it was hypothesized that introduced species that produce large amounts of litter with distinctive secondary compounds can a) alter the chemistry of both extractable and bulk carbon in the soil, b) shift microbial communities towards microbes better able to metabolize the compounds in the litter and c) cause soil carbon chemistry and microbial communities to shift to relatively uniform, novel states at multiple sites.

2. Composition of phenolics in senescent tissues (leaves and roots) of *Polygonum cuspidatum* was compared to the composition of extractable phenolics and non-extractable bulk organic carbon in soils under and adjacent to large, long-established stands of *P. cuspidatum* at four sites in the eastern U.S. Rates of degradation of phenolics, activities of enzymes associated with the breakdown of phenolics and shifts in microbial community composition were also measured at the sites.

3. Soils under *P. cuspidatum* stands contained twice as much phenolics as adjacent soils, but the composition of phenolics differed greatly between soils under stands and senescent tissues of *P. cuspidatum*. Flavonoids and proanthocyanidins constituted >90% of the identified phenolics in *P. cuspidatum* tissues, whereas monophenolic compounds accounted for >90% of the phenolics in soils under stands. Soils under and adjacent to stands also exhibited distinctive compositions of relatively persistent bulk organic carbon; composition differed less between soils under stands at different sites than between soils under and adjacent to stands at the same site.

4. Soils under *P. cuspidatum* had 2·8 times greater abundance of fungi than soils adjacent to stands, and fungal markers showed clear separation of soils under and adjacent to *P. cuspidatum*. However, the potential activity of enzymes that degrade polyphenols was lower in soils under stands. Exogenously applied, chemically complex polyphenols persisted in both *P. cuspidatum*-invaded and adjacent non-invaded soils, whereas less complex compounds rapidly disappeared from both soils.

5. *Synthesis*. Results suggest that interactions between plant inputs, abiotic reactions and biotic transformations may create and maintain new states in invaded soils that are chemically and biologically less diverse. In the case of polyphenol-rich, fast-growing invasive species, these interactions may alter the composition of bulk soil organic matter that has relatively slower turnover rates, resulting in legacy effects. Restoration could thus require, not just removal of the species, but also post-removal interventions such as soil amendments.

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Introduction

One of the major effects of introduced plant species may be to create novel states in the ecosystems where they spread (Hobbs, Higgs & Hall 2013). The impacts of invasive plants on ecosystem properties include strong effects on the diversity of native species (Hejda, Pysek & Jarosik 2009), soil carbon, nutrient dynamics (Ehrenfeld 2003; Laungani & Knops 2009) and organismal interactions (Carpenter & Cappuccino 2005). However, which attributes of invasive species facilitate the creation and maintenance of new ecosystem properties is less understood (Ehrenfeld 2010; Drenovsky *et al.* 2012), as is how long new states may persist after these species are removed (Elgersma *et al.* 2011; Suding *et al.* 2013).

One important trait that can confer a competitive advantage on invasive plant species is high or efficient uptake of resources (Funk & Vitousek 2007; Drenovsky *et al.* 2008; Tharayil *et al.* 2009) that enables plants to produce a large amount of biomass under a range of resource availabilities (Reinhart *et al.* 2006). Many invasive plants are functionally (Strauss, Webb & Salamin 2006; van Kleunen, Weber & Fischer 2010; Drenovsky *et al.* 2012) and chemically distinct (Cappuccino & Arnason 2006; Penuelas *et al.* 2010; Schaffner *et al.* 2011; Macel *et al.* 2014) from the native species they displace. Introduced species that produce large quantities of chemically distinctive litter can affect soil processes including cycling of carbon (Liao *et al.* 2008; Tamura & Tharayil 2014) and nitrogen (Ehrenfeld 2003; Dassonville *et al.* 2011; Tharayil *et al.* 2013), species interactions (Wolfe *et al.* 2008) and microbial communities (Batten *et al.* 2006; Callaway *et al.* 2008; Cantor *et al.* 2011; Yannarell *et al.* 2011). These effects on soils may persist and hamper the recovery of native species even after the removal of the invasive species (Marchante *et al.* 2009; Kulmatiski 2011; Flory & Bauer 2013; Dickie *et al.* 2014; Grove, Parker & Haubensak 2015). However, which attributes of invasive species contribute to such legacy effects is less well understood (Suding *et al.* 2013), and this could be due in part to limited understanding of the persistence and transformation of the compounds added to soils by invasive plants.

Studies that link plant invasion to the chemical inputs of the invader have often assumed relatively unaltered persistence of plant inputs in soils, a prediction that has proven difficult to validate. However, several microcosm experiments that have controlled for direct resource competition provide evidence that invader-associated chemical/biotic interference reduces the performance of native competitors (Callaway & Aschehoug 2000; Wolfe *et al.* 2008). The role of soil biota in facilitating both positive and negative plant-to-plant interactions is also becoming more evident (van der Putten *et al.* 2013; Bardgett & van der Putten 2014). Since plant inputs such as litter, root exudates and leachates fuel the metabolism of heterotrophic soil biota, the ability of the microbial community to modulate the influence of invasive plants on native ecosystems could be partly regulated by the chemical inputs of the invasives. Despite this likely influence of plant inputs on biotic and edaphic processes in invaded ecosystems, our knowledge of the significance of plant inputs in changing plant–soil feedbacks remains fragmentary.

Plant phenolics are likely to be a suitable class of compounds in which to study the transformation of soil chemistry and microbial communities by introduced, invasive species. Phenolic compounds in plants include monophenols, which have a single phenol ring, and polyphenolics such as lignins, tannins and flavonoids (Seigler 2002). Phenolics can constitute as much as 30% of plant dry mass, and lignin alone constitutes approximately 30% of the organic carbon in the biosphere (Boerjan, Ralph & Baucher 2003). Phenolics can mediate interactions between plants and other plants, animals and microbes (Hattenschwiler & Vitousek 2000; Badri *et al.* 2013; Weston & Mathesius 2013). Soil heterotrophs generally lack the ability to synthesize phenolics, reducing ambiguity about the source of these compounds in soils.

Polygonum cuspidatum Sieb. & Zucc. (aka. *Fallopia japonica* (Houtt.) Ronse Decr., *Reynoutria japonica* Houtt. and Japanese knotweed), is a perennial, herbaceous plant species native to Japan. The species was introduced to Europe and North America for use as an ornamental and as fodder (Adachi, Terashima & Takahashi 1996; Barney *et al.* 2006) and has since escaped from cultivation and spread widely on both continents. *Polygonum cuspidatum* is listed as an Invasive Alien Species of Concern in Europe, which regulates its possession, growing, sale and transport. The species is regulated by law in eight states in the United States and has escaped cultivation in 30 others. The species is common in disturbed habitats such as abandoned crop fields, pastures and roadsides and in riparian habitats including forest gaps (Barney *et al.* 2006) and can affect native fauna such as invertebrates (Maerz, Blossey & Nuzzo 2005). *Polygonum cuspidatum* often forms nearly monotypic stands whose density can exceed 25 stems m^{-2} and that can produce more than 1 kg m^{-2} $year^{-1}$ of litter that is rich in secondary compounds, particularly phenolics (Fan *et al.* 2009; Miyagi *et al.* 2010). *Polygonum cuspidatum* can alter spatial (Dassonville *et al.* 2007) and temporal (Tharayil *et al.* 2013) variability of mineral nutrient availability, which can in turn increase its competitive ability (Parepa, Fischer & Bossdorf 2013a). The species has thus been described as constructing a novel niche (Dassonville *et al.* 2011). The extensive range of *P. cuspidatum* in the

eastern United States makes this an appropriate species and region in which to investigate the effects of introduced, invasive plant species on soil chemistry via input of distinctive secondary compounds coupled with transformation of these compounds in soil.

It was specifically predicted that the high input of polyphenol-rich litter by *P. cuspidatum* will not only influence the chemistry of extractable soil carbon fraction, but also the chemistry of the non-extractable bulk soil carbon that has a relatively slower turnover rate. Further, it was predicted that compared to adjacent soils, soil under long-established stands of *P. cuspidatum* would have (i) a composition of phenolics more similar to that in senescent tissues of *P. cuspidatum*; (ii) greater abundance of microbes with high ability to metabolize phenolics as measured by soil enzymatic activities and degradation potential; and (iii) distinctive and relatively uniform compositions of soil phenolics across multiple sites.

Materials and methods

SAMPLE COLLECTION

Differences between soil chemistry and microbial communities under and adjacent to stands of *P. cuspidatum* were tested at four sites in the eastern United States, three in abandoned agricultural fields and one in riparian forest (Table 1; Tharayil *et al.* 2013). The criteria for the selection of sites were presence of a stand of knotweed that was at least 20 years old and actively expanding (Maerz, Blossey & Nuzzo 2005; Siemens & Blossey 2007; Aguilera *et al.* 2010; Tharayil *et al.* 2013), apparent lack of recent disturbance, well developed and drained soil (water table >0·75 m below the soil surface during the active growing season) and relative homogeneity of texture, bulk density and pH between invaded and non-invaded soils within a site (Table 1). Sites were also selected for proximity to collaborating institutions and to be widely spaced within the region.

Soil samples were collected during May and June 2010. At each site, five 1 × 1 m plots were randomly located 6–10 m inside and five more plots 4–6 m outside the stand of *P. cuspidatum*, with the constraint that plots be at least 8 m apart. Four soil cores 10 cm diameter × 5 cm deep were collected at the corners of a 50 × 50 cm area in the centre of each plot after removing surface litter and organic matter layer. Previous research at similar sites indicated that effects of knotweed on soil processes were strongest in the upper 0–5 cm of the soil (Tharayil *et al.* 2013). Inside stands, the density of stems of *P. cuspidatum* was >20 stems m^{-2}, and soil cores were always <5 cm from the nearest stem, so that all cores included soil from the rhizosphere. Knotweed rhizomes have high concentrations of secondary metabolites including polyphenolic compounds (Miyagi *et al.* 2010), so any cores that cut through rhizomes >3 mm in diameter were discarded (surface feeding roots of *P. cuspidatum* are less than 1 mm). Cores from the same plot were combined in a plastic bag and kept on ice. Samples were transported within several hours to the laboratory, where they were homogenized, passed through a 2-mm sieve and cleaned of any visible fine roots. Homogenized soils and the fine roots removed from the soil cores were stored at −80 °C until analysis.

In order to assess the chemical input from the senesced leaf litter, five stems of knotweed were selected 1 m away from each sampling plot, and three leaves at the top of each stem were tagged during soil sampling. These leaves were collected during November 2010, soon after senescence. Leaves with any visible herbivore damage or signs of decomposition were discarded. Remaining leaves were pooled, air dried at 22 ± 2 °C in the laboratory and stored at −20 °C until analysis.

EXTRACTION OF PHENOLICS

Phenolic compounds derived from leaf or root litter can include a free fraction stored in cell vacuoles and a fraction bound to cell wall components through ester or ether linkages. We used sequential extractions of litter and soil to separate these fractions (Fig. S1, Supporting information). Tissue samples were ground to <250 μm particle size in a ball mill. Samples of ground litter (250 mg) and soil (2 g) were then sonicated with 10 mL of water followed by 10 mL of methanol : propanol (1 : 1; v/v) to remove the strongly polar and weakly polar free phenolic fractions, respectively. After cooling on ice, samples were subjected to 20 cycles of 1 minute of sonication alternating with 1 minute of cooling. Any floating residue of decomposed litter was removed at the start of the water extraction.

Following the extraction of free phenolics, the extracted litter was incubated at 22 °C with 10 mL of 1 N NaOH on a rotary shaker for 20 h to release the ester-bound phenolic compounds. After acidification, the supernatant was further partitioned to ethyl acetate using liquid-liquid extraction (see Supporting Information

Table 1. Study site characteristics

Site	Habitat	Soil texture	Db*	pH	Coordinates	Dominant plants adjacent to stand
Amherst, MA	Old field	Silt loam	1·10	6·0	42°24′N, 72°31′W	*Aesculus* sp., *Asclepias* sp., *Dactylis* sp., *Elytrigia repens*, *Galium* sp., *Lepidium* spp., *Oxalis stricta*, *Plantago* spp., *Rhus glabra*, *Schedonorus phoenix*, *Setaria pumila*, *Trifolium* sp., *Vicia* sp.
Northampton, MA	Riparian forest	Sandy loam	1·25	5·6	42°19′N, 72°35′W	*Acer negundo*, *A. saccharinum*, *Carya* sp., *Osmunda cinnamomea*, *Populus deltoides*, *Ulmus rubra*, *Vitis labrusca*
Cayuta, NY	Old field	Silt loam	1·17	6·6	42°17′N, 76°42′W	*Asclepias* sp., *Dactylis* sp, *Chrysanthemum* sp., *Lepidium* spp., *Plantago* spp., *Schedonorus phoenix*, *Solidago* sp., *Vicia* sp.
Cashiers, NC	Old field	Loam	1·21	5·3	35°08′N, 83°02′W	*Robinia pseudoacacia* L., *Solidago* sp., *Rosa multiflora*, *Rubus* sp., *Impatiens capensis*, *Trifolium repens*, *Festuca* spp., *Plantago* spp.

*Mean bulk density (g cm^{-3}) at 0–15 cm depth (n = 5 per site).

for details). The per cent recovery of all phenolic compounds for liquid-liquid extraction was >78 ± 10% (mean ± SE). The water extract, methanol extract and the ethyl acetate fraction were completely dried under N_2 and stored at −20 °C until analysis.

Condensed tannins from knotweed litter were extracted and purified using a Sephadex LH20 resin following Tharayil *et al.* (2011). The extractable and cell wall bound tannins in the non-extracted knotweed litter were quantified using an acid-butanol assay as in Tharayil *et al.* (2011), with purified *P. cuspidatum* tannins as a standard. Acid-butanol assay is a less suitable method quantify tannin content of soils as this assay extracts significant amount of interfering compounds from soil organic matter, resulting in large overestimation of proanthocyanidins. Hence, the concentration of tannins in soils were quantified after depolymerizing proanthocyanidins in the presence of excess phloroglucinol and analysing the cleaved monomers and their phloroglucinol-adducts using liquid chromatography mass spectrometry (see Supporting Information for details).

ANALYSIS OF PHENOLICS

The strongly polar, weakly polar and ester-bound fractions of phenolics extracted from leaf and soil samples were analysed with different combinations of gas or liquid chromatography and mass spectrometry (Fig. S1). Operating parameters of all chromatographs and spectrometers used in the study are detailed in the Supporting Information. The flavonoids in each fraction were identified using an ultra-performance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer (Q-ToF; Premier, Waters, Milford, MA, USA). Samples were analysed in both positive and negative ionization modes. Compound identities were established based on accurate mass (<5 ppm error) and structural fragmentation of parent ions by collision-induced dissociation (CID) with argon (Figs S2 and S3). Following identification, amounts of dominant flavonoids were quantified with an ultra-fast liquid chromatography coupled to a triple-quadrupole mass spectrometer (LCMS 8030; Shimadzu Scientific, Columbia, MD, USA) with an ESI interface. Before this analysis, multiple reaction monitoring (MRM) was optimized for the major flavonoid compounds (Table S1).

Both identities and quantities of flavonoids were confirmed using commercial authentic standards. The MRMs of glycosylated flavonoids that lacked authentic standards were optimized using product-ion scans at collision energies of 18, 25 and 32 V and quantified using their respective aglycones. Identities of these compounds were confirmed by searching the fragmentation spectra obtained through CID with tandem mass spectral data bases (METLIN; RIKEN tandem mass spectral data base). The limits of detection of all aglycones and of quercitrin using the optimized MRM transitions (Table S2, Fig. S2) in a sample matrix were <10 pg on column.

The monophenols in each fraction were analysed using gas chromatography (Martens 2002). Compounds were derivatized using 200 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) at 60 °C for 40 min and analysed using an Agilent 7980A gas chromatograph system coupled with a 5975 C Series mass detector (Agilent Technologies, Santa Clara, CA, USA) operated at 70 eV fragmentation voltage. Compounds were identified by comparing mass fragmentation patterns with Wiley 9th + NIST08 MS Libraries and by retention time match with authentic standards.

BULK ORGANIC CARBON IN SOILS

Less than 15% of the bulk organic C in soils can generally be extracted through solvent extraction and base hydrolysis. Also, most of the bulk C generally consists of humic materials that cannot be extracted in native form (Otto and Simpson 2006). Hence, diffuse reflectance infra-red Fourier transform (DRIFT) spectroscopy was used to compare bulk soil carbon profiles under and adjacent to stands of *P. cuspidatum*. Each soil sample was dried at <40 °C and powdered to particles <10 μm using a ball mill. DRIFT spectra of the samples were collected using a spectrometer (Perkin-Elmer Spectrum One, Waltham, MA, USA) in transmission mode, equipped with a deuterated triglycine sulphate detector. Using second-order derivatives (Fig. S3), 33 peaks corresponding to major functional groups were identified for principal component analysis (PCA) based on relative peak heights, calculated as the ratio of the individual intensity of each peak to the sum of the intensities of all selected peaks within a sample (Haberhauer & Gerzabek 1999; Tharayil *et al.* 2011).

SOIL BACTERIA AND FUNGI

Measurements on soil microbiota focused on functional attributes of soil bacteria and fungi that would link litter inputs to soil chemistry. Measurements included microbial biomass, microbial community structure based on PCR-DGGE, potential activity of four soil enzymes that could indicate C and N cycling, and potential rates of degradation of three polyphenols that were abundant in knotweed litter.

Biomasses of bacteria and fungi in soil samples were respectively quantified on the basis of concentrations of muramic acid and ergosterol. Muramic acid was extracted from soils via acid hydrolysis and quantified with high-pressure liquid chromatography (HPLC) after derivatization with ortho-phthaldialdehyde (OPA) reagent as described in Tharayil *et al.* (2013; see also Supporting Information). Ergosterol was extracted via base hydrolysis and quantified with HPLC as described in Tamura & Tharayil (2014; see also Supporting Information).

Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE; Smalla *et al.* 2007) was used to profile the structure of microbial communities in soil samples. Total DNA was extracted with the Fast DNA Soil Spin Kit (QBIOgene, Carlsbad, CA, USA) following the manufacturer's instructions. DNA quality was checked using a 0·7% agarose gel and Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA was then quantified with a Nanodrop fluorospectrometer (Thermo Scientific). Genomic DNA was amplified with bacterial primers specific to the V3 region of the 16S rRNA gene [Lane 1991; primers PRBA338F (5′-*CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG* GAC TCC TAC GGG AGG CAG CAG-3′) and PRUN518R (5′-ATTACCGCGGCTGCTGG-3′)] or to the fungal ITS region [Gardes & Bruns 1993; primers ITS1 (5′ *CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G* CTT GGT CAT TTA GAG GAA GTA A 3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC – 3′)] with a GC clamp. See Supporting Information for details on PCR and DGGE conditions.

The potential activities of β-1,4-glucosidase (BG, EC 3.2.1.21), and β-1,4-N-acetylglucosaminidase (NAG, EC 3.2.1.14) were respectively quantified using 4-methylumbelliferyl-β-D-glucopyranoside and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide as substrates. β-1,4-glucosidase and NAG are bacterial enzymes that are involved in the mineralization of cellulose and fungal chitin, respectively. Enzyme activities were measured in 1 g subsamples of soil with 150 mL of 50 mM acetate buffer (pH 5·5) in 96-well plates read in a microplate flourometer (Synergy 2, Winooski, VT, Ex-355 nm, Em-450 nm; Triebwasser *et al.* 2012). The activity of peroxidase (PER, EC 1.11.1.7) and phenol oxidase (POX, EC 1.10.3.2) in the same soil slurries was estimated using L-DOPA as substrate (Suseela *et al.* 2014) and absorbance at 450 nm in a spectrophotometer (Jasco V-550, Easton, MD, USA). Peroxidase and POX are extracellular fungal enzymes that are primarily responsible for the degradation of polyphenols in soil.

The ability of the microbiota in soils under and adjacent to stands of *P. cuspidatum* to degrade compounds abundant in senescent knotweed litter was studied by incubating tannins, quercitrin and emodin. The incubation system consisted of 40-mL glass vials with open-top septa caps containing 2 g of sand (40–100 mesh, ACROS Organics, New Jersey, USA). After the vials and caps were autoclaved, 0·5 mg of the compound to be incubated was added to sand followed by 300 μL of the soil slurry (1 : 10, soil water, ~10 mg soil). The vials were then incubated at 22 °C. Head space CO_2 accumulation was sampled daily in a subset of vials. The caps were opened and vials were flushed with air when the headspace CO_2 concentration rose above 15,000 ppm. A subset of 4 vials was destructively sampled from each soil and polyphenol treatment at 0, 1, 2, 4, 8 and 16 days. For sampling, the soils were extracted by adding 5 mL of a 1 : 1 mixture of methanol : propanol assisted by sonication. The extractable and residual (non-extractable) concentration of phenolics in the soil was quantified as described above.

STATISTICAL ANALYSIS

Effects of zone [under (invaded) or adjacent to (non-invaded) a stand of *P. cuspidatum*; fixed factor] and site (random factor) on concentrations of phenolics, ergosterol and muramic acid and on soil enzyme activities were tested using mixed-model restricted maximum likelihood (REML) analysis. Differences between individual means were tested *post hoc* using Tukey's HSD. Ability of the concentrations of soil flavonoids and monophenols to predict soil peroxidase activity was tested with linear regressions. Compositions of phenolics and DRIFT peaks were analysed using principal component analysis (PCA) after appropriate transformation and scaling to meet the assumption of homoscedasticity. Presence and absence of bands comprising the fingerprint patterns generated by PCR-DGGE was scored and analysed using the software program Bionumerics (Applied Maths, Sint-Martens Latern, Belgium, www.applied-maths.com). Bands were included in the analysis if their intensity was >3% of the total intensity. Within- and between-subject fingerprint comparisons were performed with cluster analysis of Dice Similarity Indices using an unweighted pair group method with arithmetic means (UPGMA). Principle components analysis was also used to ordinate sites and zones based on these indices for fungal markers. Statistical analyses were performed using SAS v9.2 (SAS Institute Inc, Cary, NC, USA). Figures were prepared using SigmaPlot v12.5 (Systat Software, Inc, San Jose, CA, USA) and JMP v12Pro (SAS Institute Inc).

Results

PHENOLICS IN SENESCENT TISSUES

Phenolics constituted about 15% of the total dry mass of newly senescent leaves of *P. cuspidatum* (Fig. 1). The condensed tannins (proanthocyanidins) in *P. cuspidatum* litter were primarily procyanidins (proanthocyanidins made exclusively of catechin monomers). Tannins were the most abundant class of phenolics and comprised 10·6% of total leaf dry mass. Flavonoids made up 3·5% of total leaf dry mass, and 42–58% of these flavonoids were glycosides of quercetin or resveratrol, or anthocyanins. Emodin, resveratrol and their glycosides were unique to *P. cuspidatum* roots and made up 0·5–1% of root dry mass. Monophenols accounted for less than 1% of leaf mass and were dominated by ferulic, sinapic and coumaric acids. Total amount of phenolics in solvent extracts was seven times higher than in water extracts but was only half the amount associated with cell walls.

PHENOLICS IN SOILS

Concentrations of both flavonoids and monophenols were over two times higher in invaded soils than in soils adjacent to stands of *P. cuspidatum* (Fig. 2). The relative difference between soils under and adjacent to stands in concentrations of flavonoids was much greater than difference in monophenols, but absolute amounts of flavonoids were much lower than amounts of monophenols (Fig. 2). Soils adjacent to stands generally had no detectable flavonoids except at the forested site, where soils contained quercetin, rutin and kaempferol.

Thirteen ester-bound monophenols accounted for over 95% of the total phenolics identified in soils. Most of these 13 compounds differed markedly in concentration between soils under and adjacent to stands (Fig. S4; $F_{1,6} = 29{\cdot}15$; $P = 0{\cdot}002$). Soils under *P. cuspidatum* had 2 to 20 times

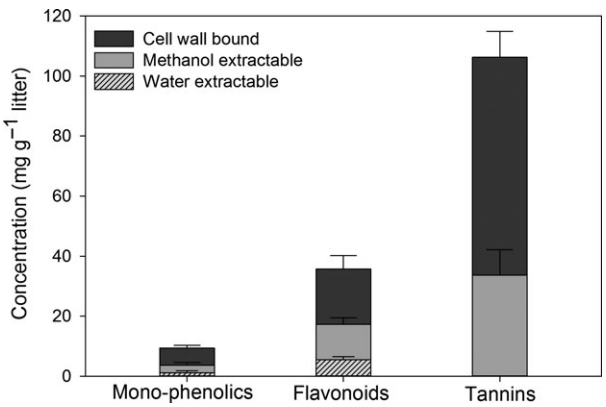

Fig. 1. Concentrations (mg g^{-1} dry mass of leaf; mean + SE, based on site means) of types of phenolics in senescent leaves of *Polygonum cuspidatum*.

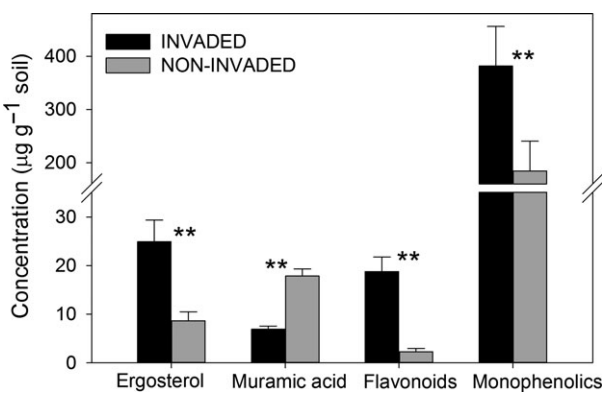

Fig. 2. Concentration (μg g^{-1} dry mass of soil; mean + SE, based on site means) of ergosterol, muramic acid, flavonoids and monophenols in soils under (invaded) and adjacent (non-invaded) to stands of *Polygonum cuspidatum*. Symbols above pairs of bars show whether means differed between zones: no symbol – $P > 0{\cdot}05$; ** – $P < 0{\cdot}01$.

higher concentrations than adjacent soils of protocatechuic acid, acetovanillone, acetosyringone and syringaldehyde. These four compounds made up 62% of the total monophenols in invaded soils but only 22% of those in non-invaded soils, which had higher concentrations of all the other relatively abundant ester-bound phenolics except vanillin. Results thus did not support the prediction that compositions of phenolics would be more like those in senescent tissues of *P. cuspidatum* in invaded soils than in soils adjacent to stands of *P. cuspidatum*.

Consistent with the prediction that soils under stands of *P. cuspidatum* would have distinctive compositions of phenolics, principal component analysis of soils based on concentrations of the 13 dominant ester-bound monophenols showed a clear separation of soils under and adjacent to stands (Fig. 3). This analysis was also consistent with the prediction that soils under *P. cuspidatum* would have relatively uniform soil chemistry, since invaded soils were ordinated much more closely together than non-invaded soils (Fig. 3). Non-invaded soils also showed a separation between the forested site and the sites in old fields, whereas invaded soils did not.

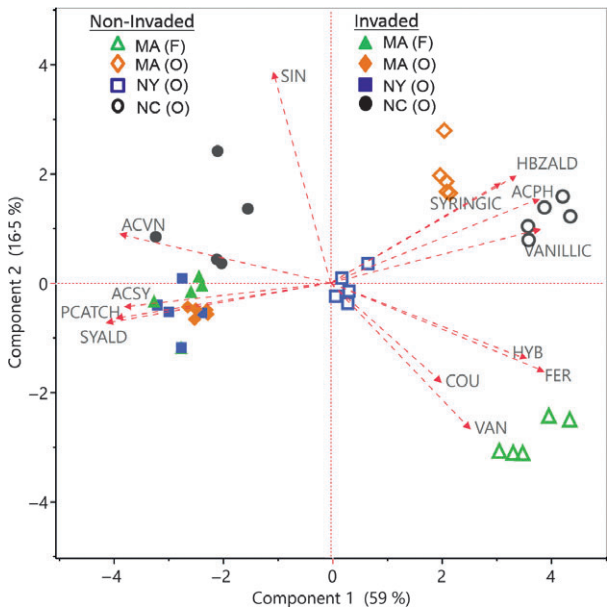

Fig. 3. Principal component analysis of soils under (invaded) and adjacent (non-invaded) to stands of *Polygonum cuspidatum* at four sites (O – abandoned agricultural field; F – riparian forest) and states in the United States (MA – Massachusetts; NC – North Carolina; NY – New York), based on composition of monophenols (n = 3). Scores of each site and loading of each variable in the first two principal components were overlaid to obtain the biplot. Eigenvectors of endpoints relative to each component are represented by biplot rays. Abbreviations for monophenols: ACPH- acetophenone; ACSY- acetosyringone; ACVN – acetovanillone; COU – coumaric acid; FER-ferulic acid; HBZALD – hydroxybenzaldehyde; PCAT – protocatecheuic acid; SIN – sinapic acid; SYALD – syringaldehyde; SYR- syringic acid; VAN – vanillin; VANILLIC – vanillic acid.

BULK ORGANIC CARBON COMPOSITION OF SOILS

Principal component analysis of soils based on bulk soil carbon composition similarly showed that soils under *P. cuspidatum* had distinctive and relatively uniform chemistry compared to soils adjacent to stands (Fig. 4). Invaded soils under stands were clearly separated from and more tightly clustered than non-invaded soils adjacent to stands. Again, the strong separation of the forested site from the old field sites observed in non-invaded soils was absent in invaded soils.

ABUNDANCE, DIVERSITY AND FUNCTIONAL ACTIVITY OF SOIL MICROBIOTA

Concentrations of ergosterol, a measure of fungal biomass, were 2·8 times higher in invaded soils than in soils adjacent to stands of *P. cuspidatum* (Fig. 2; $F_{1,3}$ = 38·73; P = 0·008), while concentrations of muramic acid, a measure of bacterial biomass, were 61% lower in invaded soils than in soils adjacent to stands ($F_{1,3}$ = 47·55; P < 0·001). Bacterial 16S rRNA gene PCR-DGGE fingerprints under and adjacent to stands at all sites showed numerous, indistinct bands (Fig. S5). Fingerprints of fungal communities showed more distinct bands (Table S2), which could be used to compare community composition between samples (Table S3). Principal component analysis based on Dice Similarity Indices showed the same main pattern as seen

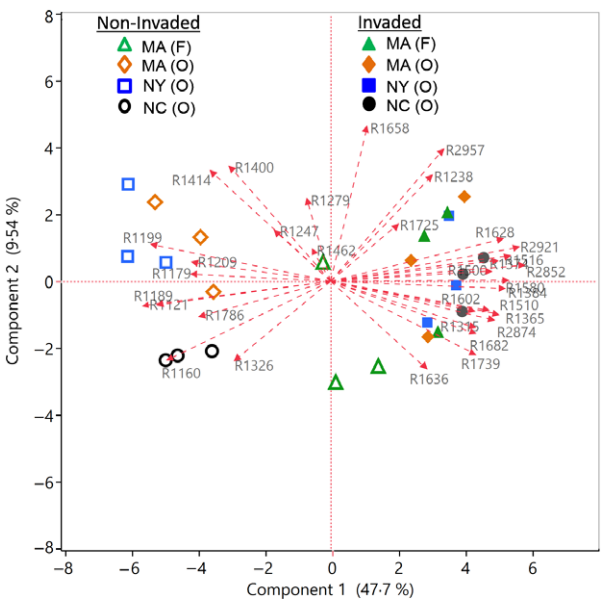

Fig. 4. Principal component analysis of soils under (invaded) and adjacent (non-invaded) to stands of *Polygonum cuspidatum* at four sites (O – abandoned agricultural field; F – riparian forest) and states in the United States (MA – Massachusetts; NC – North Carolina; NY – New York), based on composition of bulk organic carbon as measured by the relative intensities of 33 dominant DRIFT peaks. Scores of each site and loading of each variable in the first two principal components were overlaid to obtain the biplot. Eigenvectors of endpoints relative to each component are represented by biplot rays.

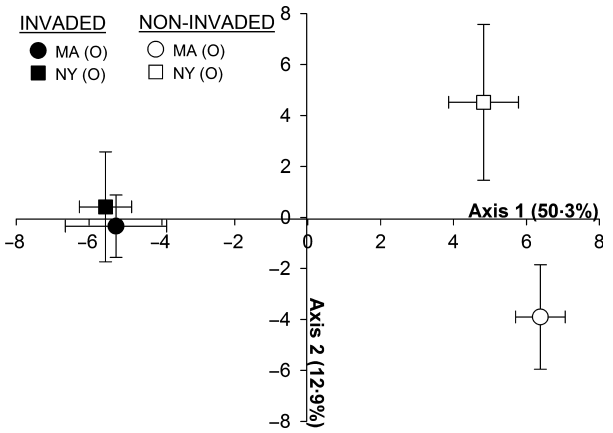

Fig. 5. Principal component analysis of soils under (invaded) and adjacent (non-invaded) to stands of *Polygonum cuspidatum* at two sites in abandoned agricultural fields in two states (MA – Massachusetts; NY – New York), based on fungal PCR-DGGE fingerprints. Points show mean ± SE, based on replicates within sites.

for soil chemistry, although only two of the four sites were sampled (Fig. 5): fungal communities in soils under *P. cuspidatum* were clearly separated from and more clustered than those in adjacent soils.

Contrary to predictions, the potential activity of several classes of enzymes likely to be involved in the microbial breakdown of litter or leachates of *P. cuspidatum* was not consistently higher in invaded soils than in soils adjacent to stands (Fig. 6a). Activity of β-N-acetylglucosaminidase was 70% higher in invaded soils than in non-invaded soils ($F_{1,3}$ = 90·09; P = 0·003). However, activities of β-glucosidase ($F_{1,3}$ = 26·42; P = 0·014) and possibly of peroxidase ($F_{1,3}$ = 5·26; P = 0·06) were higher in non-invaded soils. Moreover, activity of peroxidase was more negatively related to the abundance of soil flavonoids than to the abundance of monophenols (Fig. 6b).

The potential rate of degradation, as measured by the per cent of the initially added polyphenols that could be recovered after incubation, varied with compound type (Fig. 7). The rate of degradation of quercitrin was similar in inocula from soils under and from soils adjacent to stands of *P. cuspidatum* (P = 0·19), whereas degradation of emodin (P < 0·01) was faster in soils under stands.

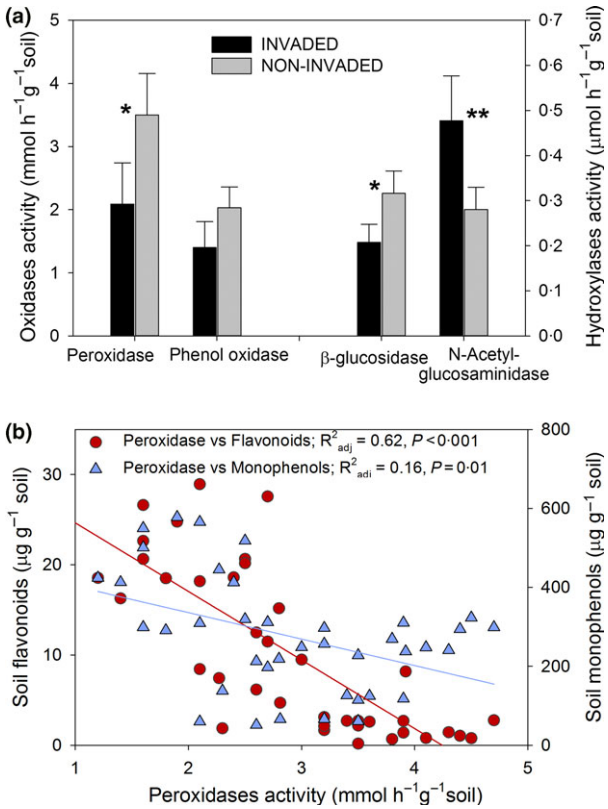

Fig. 6. (a) Potential activities (mean + SE, based on site means) of four enzymes in soils under (invaded) and adjacent (non-invaded) to stands of *Polygonum cuspidatum*. Symbols above pairs of bars show whether means differed between zones: no symbol – P > 0·05; *P = 0·05–0·01; **P < 0·01. (b) Relationships between concentrations of flavonoids and monophenols and potential peroxidase activity in soils. Points are individual samples from all sites and positions. Lines show linear regressions (flavonoids – R^2 = 0·62, P < 0·001; monophenols – R^2 = 0·16; P = 0·01).

Discussion

CONTRAST BETWEEN LITTER AND SOIL CHEMISTRY

Results strongly supported the prediction that invasion by *P. cuspidatum* in the eastern United States can alter soil phenolic chemistry to a novel, relatively uniform state. Compared to soils adjacent to stands, soils under stands of *P. cuspidatum* at the four study sites in three U.S. states had distinctive and relatively uniform compositions of dominant extractable phenolics and bulk organic carbon. The soil type of the site influenced the magnitude but not the direction of phenol accumulation, with sandy soils in riparian forest retaining lower amounts of phenolics compared to silt-rich soils in the other three sites. One likely explanation for the relative similarity in composition of extractable phenolics in soils under stands of *P. cuspidatum* is that these compounds may be highly species-specific (Kraus *et al.* 2003; Vanholme *et al.* 2010; Tharayil *et al.* 2011). Replacement of plant communities that vary in species composition at different sites with nearly monospecific stands of a single, new species with distinctive chemical inputs might thus typically cause compositions of soil phenolics at sites to become more similar. The extractable phenolic compounds that are released during the early stages of tissue decomposition (Suseela *et al.* 2014) represent the major biologically available fraction of phenolics in soil (Martens 2002) and can serve as allelochemicals, aid in nutrient acquisition and mediate organismal interactions (e.g. Thorpe, Archer & DeLuca 2006; Mandal, Chakraborty & Dey 2010). *Polygonum cuspidatum* has been previously shown to cause homogenization of the content of soil mineral nutrients in invaded landscapes across NW Europe (Dassonville *et al.* 2008).

Compared to their influence on the extractable fraction of soil organic carbon, the influence of plant inputs on the

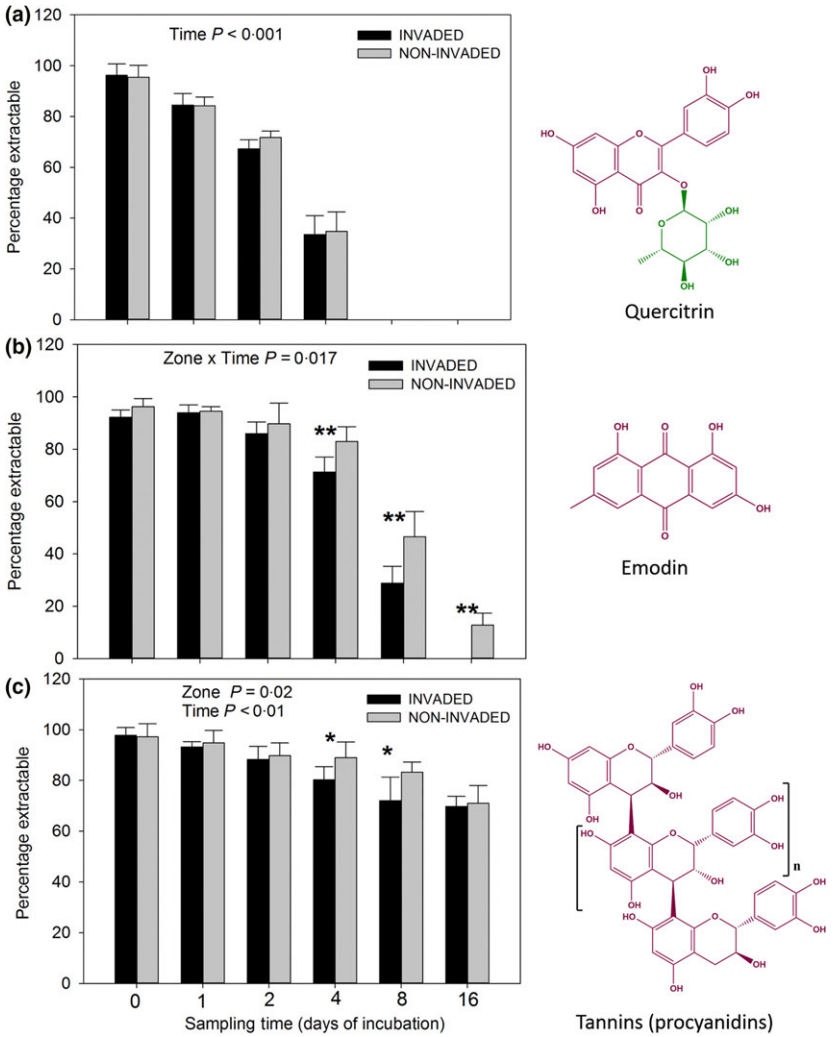

Fig. 7. Per cent of initially added phenolic compounds recovered from sand containing microbial inoculum from soils under (invaded) and adjacent (non-invaded) to stands of *Polygonum cuspidatum* at different sampling times: (a) quercitrin, (b) emodin, (c) tannin (procyanidins) purified from *P. cuspidatum*. The respective molecular structural formulae of the compounds are also shown. Bars show mean ± SE (n = 4). no symbol $P > 0{\cdot}05$; **P = 0·05–0·01; $P < 0{\cdot}01$.

composition of non-extractable bulk soil carbon is thought to be relatively limited (Gentile, Vanlauwe & Six 2011; Mambelli *et al.* 2011). However, the composition of bulk soil carbon under stands of *P. cuspidatum* suggested that soils had changed to a new, relatively uniform state following prolonged invasion. Soils under *P. cuspidatum* were characterized by high absorption at wavelengths of 2852 and 2921 cm^{-1}, characteristic respectively of the symmetric and asymmetric stretches of CH groups found in polyaliphatic compounds (Lammers, Arbuckle-Keil & Dighton 2009); and at wavelengths of 1510 and 1628 cm^{-1}, characteristic of aromatic C=C stretching and skeletal vibrations of lignins (Suseela *et al.* 2013). Lignins and the polyaliphatic compounds that are constituents of waxes, cutin and suberin are relatively less susceptible to microbial degradation (Lorenz *et al.* 2007). At sites in abandoned agricultural fields, non-invaded soils adjacent to *P. cuspidatum* were characterized by high absorption at 1121 and 1190 cm^{-1}, characteristic of polysaccharides; and at 1786 cm^{-1}, characteristic of a C=O stretch found in carboxylic acids. Across sites, soils under *P. cuspidatum* thus had a higher proportion of relatively recalcitrant carbon and a lower proportion of labile carbon than adjacent soils. Though the identified changes in the composition of bulk soil carbon in *P. cuspidatum* invaded soils are not likely to have any direct toxic effect on native vegetation, these changes could alter the composition and function of soil biota. The abundance of polyphenols and other relatively recalcitrant carbon could lead to reduced microbial activity and cycling of C and N under *P. cuspidatum*, consistent with previous observations (Koutika *et al.* 2007; Maurel *et al.* 2010; Tharayil *et al.* 2013). Since many of the recalcitrant plant compounds have relatively slow turnover times (Lorenz *et al.* 2007), prolonged effects of *P. cuspidatum* on this aspect of soil chemistry could be highly persistent and contribute to a legacy effect. Successful restoration of native communities after removal of *P. cuspidatum* in the study sites could therefore require further active interventions such as soil amendments.

ACTIVITY AND DIVERSITY OF MICROBES

Community composition of soil fungi, like soil chemistry, was distinctive and relatively uniform in soils under *P. cus-*

pidatum. Previous work has shown that introduced plant species can change microbial communities in soils (e.g. Batten *et al*. 2006; Yannarell *et al*. 2011). Results here extend this work to show that an introduced plant can cause similar shifts in both molecular-level carbon composition and microbial community structure across geographically distinct locations. As in all studies of invasion ecology in which invasion is not experimentally manipulated, the possibility cannot be completely excluded that the differences found between invaded and non-invaded areas predated rather than resulted from invasion. However, it seems unlikely that causes unrelated to the establishment of *P. cuspidatum* could have produced a similar shift of soil chemistry and microbiota to a state unlike any found where the species had not established at such widely separated sites.

The composition of phenolics in the litter of *P. cuspidatum* could explain its apparent ability to foster a distinctive, relatively uniform community of soil fungi. Biotic decomposition of the complex polyphenolic compounds abundant in *P. cuspidatum* requires the production of peroxidase enzymes that are primarily limited to a few fungal species (Sinsabaugh 2010). This explanation is consistent with the relatively high fungal biomass and low bacterial biomass measured in soils under *P. cuspidatum*. High concentrations of tannins may also favour fungi over bacteria in soil (Winder *et al*. 2013). Although this study was not able to test for change in bacterial community composition, introduced plants can cause such changes (Yannarell *et al*. 2011; Silva *et al*. 2013), and *P. cuspidatum* is reported to alter the activity of soil denitrifying bacteria (Dassonville *et al*. 2011; Bardon *et al*. 2014). *Polygonum cuspidatum* has been shown to strongly benefit from its association with the native soil biota in its introduced European ranges (Parepa, Schaffner & Bossdorf 2013b)

INTERPLAY BETWEEN SOIL CHEMISTRY AND MICROBES

Results did not fulfil the predictions that the composition of phenolics would be more like that in senescent tissues of *P. cuspidatum* or that abundance of microbes with high ability to metabolize phenolics would be greater in invaded soils than in soils adjacent to stands of *P. cuspidatum*. Failure to find close similarity between the compositions of phenolics in litter and in soil under stands could reflect rapid biotic or abiotic transformations of these compounds as they move from litter to soil. For example, flavonoids and tannins accounted for >95% of the phenolics in litter but <6% of the extractable phenolics in knotweed invaded soils. Low persistence of flavonoids in soil might be due to the rapid redox reactions (Tharayil, Bhowmik & Xing 2008) and microbial degradation (Cesco *et al*. 2012) that these compounds tend to undergo in soil matrices. The flavonoid quercitrin, which was especially abundant in senescent leaves but not in soil, can be converted by microbial quercitinase to quercetin (Tranchimand, Brouant & Iacazio 2010), which can then undergo ring cleavage and decarboxylation reactions to form protocatechuic acid (Rao & Cooper 1994). Similarly, protocatechuic acid could be formed during the microbial degradation of catechin (Wang *et al*. 2013; Tharayil & Triebwasser 2010), which is the monomer of the procyanidins that are abundant in knotweed tissues. These biotransformations are consistent with the low abundance of quercitrin and tannins, and the high abundance of protocatechuic acid observed in soils under *P. cuspidatum* stands. However, incubation of tannins and quercitrin in soils under laboratory conditions resulted in only a transient build-up of protocatechuic acid in our study, indicating further degradation of protocatechuic acid under *in vitro* incubation conditions.

The higher relative abundance of monophenols in soils under stands than in tissues of *P. cuspidatum* could be due to enzymatic or abiotic depolymerization of phenolic macromolecules in litter. The monophenols syringaldehyde and acetosyringone are oxidation products of the syringyl units of lignin, and acetovanillone is an oxidation product of the vanillyl units of lignin (Martens 2002; Tamura & Tharayil 2014). Results thus suggest that one should measure the influence of introduced species on soil chemistry, not just by similarity between plant inputs and soil chemistry, but also by similarity between likely products of transformations of chemicals found in litter and soil chemistry.

Transformation of plant inputs in soil might further explain why studies have found stronger allelopathic effects of *P. cuspidatum* in the field (Siemens & Blossey 2007; Murrell *et al*. 2011; Dommanget *et al*. 2014) than in laboratory experiments with leachates (Parepa, Fischer & Bossdorf 2012). In natural ecosystems, the toxic effect of invasive species on natives could in part operate through a metabolism-mediated allelopathy, whereby the initial inputs of the invasive is transformed to more toxic compounds through biotic or abiotic reactions. For example, metabolism-mediated allelopathy is reported in *Rhododendron formosanum*, where catechin in plant litter is metabolized to more phytotoxic protocatechuic acid by soil microbes (Wang *et al*. 2013). Thus, along with their traditionally studied direct effects, the effects of invasive plants on native species may also be mediated by biotic/abiotic transformations of plant inputs and associated alterations in soil nutrient cycling.

Laboratory incubation studies also reflected similar ability of soil biota to degrade compounds that are inputted by *P. cuspidatum*. Inocula from invaded soils and from soils adjacent to *P. cuspidatum* showed similar ability to actively degrade relatively simple phenolic compounds abundant in the litter of *P. cuspidatum*, but inocula from soils under *P. cuspidatum* more rapidly metabolized complex compounds. For instance, quercitrin was rapidly degraded by both inocula, potentially due to the presence of labile rhamnose; while emodin, which is an aglycon, was more rapidly degraded by inocula from soils under stands. In contrast the more chemically complex procyani-

dins resisted microbial degradation to a greater extent by both inocula. The low rate of disappearance of procyanidins during laboratory incubation contrasts with observations in the field, where this compound was rarely detected in soils under *P. cuspidatum*. We attribute the lower detection of procyanidins in field soils to the strong sorption of this compound to soil minerals, especially following a prolonged exposure under field conditions, which prevents extraction of tannins in their native form (Tharayil *et al.* 2013). A similar pattern of apparently low concentrations of lignins in soil matrices have been discussed recently (Hernes *et al.* 2013). Despite this apparent absence, tannins are thought to maintain their biological activity and interfere with soil C and N mineralization (Tharayil *et al.* 2013) through ability to protect proteinaceous substrates from mineralization and also by inactivating microbial exo-enzymes that catalyse decomposition of plant litter (Kraus *et al.* 2003, Triebwasser *et al.* 2012; Triebwasser-Freese *et al.* 2015). Procyanidins are also shown to have an inhibitory effect on bacterial denitrification (Bardon *et al.* 2015). In natural, multi-compound systems the degradation pattern of plant inputs could also be influenced by the companion compounds that could protect or predispose the recalcitrant compounds to microbial degradation (Tharayil, Bhowmik & Xing 2008).

Abiotic transformations and interactions between chemicals released from litter appear likely to mediate the effects of invasive species on soils. Despite having a higher content of polyphenolic substrates than soils adjacent to stands, soils under *P. cuspidatum* had a lower potential peroxidase activity. Tannins input by *P. cuspidatum* can interfere with peroxidase activity, not only by complexation and precipitation reaction, but also by quenching the active enzyme intermediate (porphyrin π-cation radical) through electron donation, without themselves becoming radicals (due to resonance-stabilization; Triebwasser *et al.* 2012). An alternative explanation is suggested by the negative correlation of soil peroxidase activity and soil flavonoids. Flavonoids derived from litter of *P. cuspidatum* can likely form chelates with Fe (II and III; Hider, Liu & Khodr 2001) in the soil. This polyphenol-facilitated stabilization of Fe can slow iron-catalysed Fenton reactions that generate highly reactive hydroxyl radicals (Perron & Brumaghim 2009), resulting in a greater persistence of phenolic compounds in invaded soils.

Overall, our results suggest that the chemical composition of the litter input of an invading species can influence, not only the chemistry of the extractable phenolics, but also the chemical composition of the more persistent bulk soil organic matter. Litter chemistry could form an important mechanism by which introduced species alter the biogeochemistry of systems and create conditions that favour their own dominance. Results from this study indicate that the identity of the plant inputs may seldom remain unaltered in soil and that it is their transformed products that may instead persist. Interactions between plant inputs, abiotic reactions and microbial transformations may create and maintain new chemical and biological states that contribute to invasion, and the ecological homogenization that invasive species can cause above-ground may extend below-ground as well.

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Data accessibility

Data deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.c85gk (Suseela *et al.* 2015).

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Appendix S1. Detailed description of methods.
Table S1. Multiple reaction monitoring optimized for the quantification of flavonoids using LC-MS/MS in negative ionization mode.
Table S2. Comparison of number of bands scored in fungal PCR-DGGE fingerprints.
Table S3. Comparison of average Dice Similarity values within and between sampling sites.
Fig. S1. Design for sequential extractions and detection techniques for phenolic compounds.
Fig. S2. Mass spectrum of the product ions of the prominent compounds that were identified in solvent extracts of senescent leaves of *Polygonum cuspidatum*.
Fig. S3. Second derivative spectra of the C-H stretch region of DRIFT peaks.
Fig. S4. Concentration of the most abundant monophenols in soils under and adjacent to stands of *Polygonum cuspidatum*.
Fig. S5. Bacterial 16S rRNA gene PCR-DGGE fingerprints.
Fig. S6. Dendrograms of pairwise comparisons of fungal PCR-DGGE fingerprints using Dice Similarity indices grouped with UPGMA.