Soils in transition: dynamics and functioning of fungi

Wal, A. van der

Chapter 4

Constraints on development of fungal biomass and decomposition processes during restoration of arable sandy soils

Annemieke van der Wal, Johannes A. van Veen, Agata S. Pijl, Richard C. Summerbell and Wietse de Boer

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Abstract

In an earlier study we reported the apparent stabilization of a low fungal biomass in ex-arable lands during the first decades after abandonment. It was hypothesized that the lack of increase of fungal biomass was due to constraints on development of fungi with persistent hyphae such as lignocellulolytic basidiomycetes and ericoid mycorrhizal fungi. With respect to the former group, the slow increase of the pool of lignocellulose-rich organic matter was expected to be the major constraint for their development. To study this, we enriched soil samples of one arable land, of two recently abandoned arable lands, of one older abandoned arable land and of heathland with carbon substrates that differed in composition (glucose, cellulose and sawdust). In addition, we combined the effect of carbon addition on fungal biomass development in arable and recently abandoned lands with inoculation of 1% of soil from the older abandoned site and the heathland. All treatments induced a fast increase and a subsequent rapid decline in fungal biomass in the arable and ex-arable fields. Denaturing Gradient Gel Electrophoresis (DGGE) band patterns and enzyme activities did show differences between the carbon treatments but not between the recent and older abandoned field sites, indicating a similarly responding fungal community even after three decades of land abandonment and irrespective of soil inoculation. Identification of fungi by sequencing and culturing confirmed that decomposition processes were mostly dominated by opportunistic fungi in arable and ex-arable fields. In the heathland, only a very slow increase of microbial activity was observed after addition of carbon and sequencing of DGGE bands showed that ericoid mycorrhiza (ERM) fungi were responsible for carbon decomposition. We conclude that an increase of enduringly present fungal hyphae in ex-arable land may only be possible when a separate litter layer develops and/or when suitable host plants for ERM fungi become established.

Introduction

Saprotrophic fungi and bacteria play a vital role in the cycling of elements by converting organic matter to carbon dioxide with a simultaneous release of nutrients essential for plant growth. Two different decomposition strategies are basically applied by soil microbes. Degradation of lignin-rich organic matter such as straw, litter and wood is mostly carried out by slow-growing (K-selected) saprotrophic basidiomycetes (Boddy and Watkinson, 1995; Frankland, 1998; De Boer et al., 2005). These fungi are favored by lignin-rich substrate since they are able to degrade lignin using extracellular enzymes and are thereby getting access to cellulose and hemi-cellulose (Leonowicz et al., 1999; Shah and Nerud, 2002). Bacteria and fast growing opportunistic fungi, on the other hand, are stimulated by easy accessible carbon sources that do not require a complex metabolic route. Bacterial decomposers have been shown to be the major decomposers of simple water-soluble components of the soil organic matter (Waldrop and Firestone, 2004). In addition, bacteria are important in utilization of plant root exudates and degradation of readily accessible cellulose (Grayston et al., 1996; Priha et al., 1999; Lynd et al., 2002). Recent studies indicated that so-called sugar fungi with opportunistic characteristics (r-selected species) may also significantly contribute to degradation of root exudates (Butler et al., 2003; Treonis et al., 2004). Other studies have indicated that decomposition of easily degraded substrates by fungi is only important under certain conditions such as high substrate concentrations and low pH (Arao, 1999; Griffiths et al., 1999). The carbon accessibility and composition of the organic matter may thus contribute to the relative importance of K-selected versus r-selected microbial decomposers.

In a former study on fungal biomass development in a chronosequence of ex-arable lands, we observed that the saprotrophic fungal biomass was very low just after cessation of cultivation and increased significantly during the first two years after abandonment (Van der Wal et al., 2006b). It was argued that fungal growth was stimulated by the absence of agricultural management activities and that the quick fungal response was brought about by opportunistic fungi feeding on the relatively easily degraded plant residues. After this quick initial response, no further increase was apparent for at least 3 decades and the fungal biomass remained at a far lower level than that of the *Calluna*-dominated heathland which is expected to be the end-successional stage of ex-arable site vegetation development given the climatic and management (grazing) conditions. We hypothesized that the stabilization phase of fungal biomass is due to a very slow increase in total soil carbon and to lack of development of other functional fungal groups, in particular lignocellulose-degrading basidiomycetes and ericoid mycorrhizal fungi. According to this hypothesis, a further increase in fungal biomass will occur only when the input of low quality, lignin-rich material increases or when dwarf-shrubs

enter the vegetation. In heathland systems, the amount of organic matter is higher and the composition different (lignin-rich) compared to that of ex-arable soils (Van der Wal et al., 2006b).

In the current study, the constraints on development of the saprotrophic fungal biomass in former agricultural fields were investigated. We addressed four specific questions. Firstly, is fungal biomass increase in former agricultural land constrained by the amount of substrate or by its composition? Secondly, can slow growing K-selected fungi be stimulated by addition of a carbon source and can they consequently attain an enduringly increased level of fungal biomass? Thirdly, do fungi from recently abandoned arable soil differ from fungi occurring in older abandoned soil and in natural heathland soils with respect to decomposer functions? And finally, are there constraints hampering the increase of lignocellulose degrading fungi in ex-arable lands? For this study, one arable site, two recently abandoned arable sites, one older abandoned arable site and one heathland site were enriched with different carbon substrates (glucose, cellulose and wood). At different time intervals, we determined the increase in biomass, functioning and community composition of fungi.

Material and methods

Soils and sampling

Experiment 1: constraints for saprotrophic fungal biomass development in ex-arable land

For this experiment, four sites in the central part of the Netherlands were selected from the chronosequence study that has been described previously (Van der Wal et al., 2006b). The sites consisted of one arable and two recently (2 years) abandoned arable fields with a low fungal biomass. These fields are hereafter referred to as 'young fields'. In addition, an extendedly (21 years) abandoned site with a relatively high fungal biomass was chosen as reference site, designated the 'old' field. A summary of the soil characteristics and fungal biomass levels of these sites is given in Table 1. In September 2003, at least 100 soil cores (3.5 cm diameter and 10 cm deep) were randomly collected in a 25 x 25 m plot in each of the selected sites. For each site, the soil cores were bulked, homogenized, sieved (4 mm mesh) and stored at 4˚C for not more than one week until the start of the experiment.

Experiment 2: differences in fungal decomposition functions between ex-arable land and heathland

As a follow-up of the first experiment, one of the ex-arable sites (Telefoonweg) was selected for comparison with a heathland site (Mossel) (Table 1). Heathland is the target for nature

restoration of the ex-arable lands selected for this study. Soil sampling (September 2004) and sample processing was as described above.

Table 1: Soil characteristics (0-10 cm)* and fungal biomass (ergosterol content) of the field sites under study.

Field name	Years since abandonment	Former crop or crop at sampling	pH	C g kg^{-1}	C:N ratio	% Sand > 53 µm	Ergosterol content mg kg^{-1}	Soil texture
Rondweg	Arable site	Maize	5.94	22.40	15.90	90.8	0.33	Sand/ coarse sand
Assel	2002	Maize	5.17	25.60	23.80	93.2	0.42	Coarse sand
Telefoonweg	2002	Maize	5.41	28.60	22.30	88.0	0.54	Coarse sand
Dennenkamp	1982	Rye, potatoes, asparagus	5.60	34.30	19.90	86.2	1.36	Sand/ coarse sand
Mossel	Reference	Heathland	3.90	64.00	30.40	86.9	6.08	Coarse sand

* This is a mineral horizon for all sites except for the Mossel site where the 0-10 cm layer consisted of an organic (F/H; mean thickness 3 cm) and a mineral horizon.

Experimental design

Experiment 1: constraints for saprotrophic fungal biomass development in ex-arable land

Soil samples were enriched with carbon substrates which differed in composition. Glucose was chosen as a simple decomposable substrate and α-cellulose (Sigma) as an intermediate degradable substrate. Birch sawdust (size of 0.5 - 2 mm), representing a mixture of interlinked cellulose, hemicellulose and lignin, was chosen as the most difficultly degradable substrate. Glucose, cellulose and sawdust were added to the soil by hand mixing to give a final concentration of 2 mg C g^{-1} dry soil. Mixed soil without added carbon served as control. $(NH_4)_2SO_4$ was added to all treatments to create an added C: N ratio of 20, since this resembles the soil C: N ratio in the (ex)-arable fields (Table 1). Besides the effect of the carbon sources on fungal biomass development in young ex-arable soils, we also examined the combined effect of carbon treatments with inoculation of 1% (1.25 g) of soil from the relatively fungus-rich, older abandoned soil (Dennenkamp). Water content of all the soils was adjusted to the most humid soil which was equivalent to 70 – 75 % of the water-holding capacity. Portions of 125 gram of moist soil were transferred to Petri dishes (14 cm diameter), spread evenly and incubated at 20° C. Petri dishes were sealed with Parafilm (Pechiney Plastic Packaging, Measha WI, USA) to minimize water loss. Dishes were weighed during incubation to adjust for any water loss if needed. Per treatment and per time interval 4 replicate samples per site were incubated. Incubation was for 2, 4, 8 and 16 weeks.

Experiment 2: differences in fungal decomposition functions between ex-arable land and heathland

Soil samples from the ex-arable (Telefoonweg) - and heathland (Mossel) site were enriched with two carbon substrates, cellulose and birch sawdust (size of 0.5 - 2 mm). Based on the

results of the first experiment, cellulose was chosen as the carbon substrate to be used to determine the increase of biomass of opportunistic fast-growing fungi and sawdust was chosen to determine the increase of biomass of slow-growing lignocellulose degraders. Cellulose and sawdust were added to the soil by hand mixing until a concentration of 2 mg C g^{-1} dry soil was attained. Controls without added carbon were included as well. $(NH_4)_2SO_4$ was added to all treatments to give a C: N ratio of 20 to allow comparison of the results with those of the first experiment. For soil from the ex-arable site, the effect of heathland soil inoculation (1%) was also examined. With this inoculation treatment we were able to investigate if we could induce the development in the abandoned arable fields of a saprotrophic fungal community that is characteristic of that seen in the restoration target sites (heathlands). Soils were incubated as described above at their corresponding field moisture content levels, as measured on the day of sampling. These were 17.2% (w/w) for the Telefoonweg site and 26.9% (w/w) for the Mossel site. Soil samples were analyzed after 2, 5 and 16 weeks of incubation. Per treatment and per time interval 4 replicate samples per soil were incubated.

Determination of fungal biomass and bacterial numbers

Ergosterol is a sterol found primarily in fungal cell membranes, though it also occurs in certain microalgae and protozoa (Grant and West, 1986; Newell, 1992; Rzama et al., 1994). In soil, only fungal membranes significantly contribute to the ergosterol content, and ergosterol correlates positively with microscopic measurements of hyphal lengths and fungal PLFA (Stahl and Parkin, 1996; Van der Wal et al., 2006b). Therefore, ergosterol was used as an indicator of fungal biomass development. Ergosterol was measured using two protocols: a disruptive extraction without saponifcation (Gong et al., 2001) and an extraction including saponification (Bååth, 2001). The simple Gong method was used for the first experiment as it has been shown to be as good as the more laborious saponification method for soils with a low organic matter content (de Ridder-Duine et al., in press). The second experiment included an organic-rich heathland soil and therefore use of the Bååth method was needed.

Bacterial numbers were enumerated in microscopic counts of cells stained by 4′,6-diamidino-2-phenylindole (DAPI). Staining was essentially as recommended by Porter et al. (1980), but with modifications allowing use with soil samples. Briefly, microscopic slides were prepared with a 10 times diluted soil suspension of 5 g fresh soil and 50 ml of 0.25 g l^{-1} KH_2PO_4. Slides were coated with gelatin to make sure that the soil suspension was evenly spread in the wells. After the slides were stained with DAPI (2 μg ml^{-1} in demineralized water) for 5 min, one drop of anti-fading solution (1 part glycerol: 1 part phosphate-buffered saline (PBS; 10 mM Na-phosphate and 130 mM NaCl, pH 7.5) and 0.5% ascorbic acid) was

put on each well before the cover slide was placed on top of the slide. Bacterial cells were counted using a Leitz epifluorescence microscope at 10 x 100 magnification.

Soil pH, moisture, nitrogen mineralization and respiration

The pH was measured in suspensions with a soil (in dry weight): water ratio of 1: 2.5. Soil water content was determined as weight loss after overnight drying at 105˚C. The net mineralization or immobilization of nitrogen was determined for each incubation period as the difference between initial and final amounts of soil mineral N (NH_4^+-N + NO_3^--N). Soil mineral N was extracted by shaking 10 g soil (dry weight) in 50 ml 1 M KCl for 2 hours. Concentrations of NH_4^+-N and NO_3^--N in the KCl extract were determined colorimetrically using a Traacs 800 auto-analyzer. Ammonium was measured colorimetrically as a reaction-product of sodium salicylate, sodium nitroprusside and sodium hypochlorite (Wall et al., 1975). Nitrate, after reduction to nitrite in a copper-cadmium redactor cell, was measured colorimetrically as a reaction-product of sulfanilamide and N-1-naphtylethylenediamine (Keeney and Nelsen, 1982).

Soil respiration was determined by measuring the amount of CO_2 production in 40 g fresh soil incubated for 48 h at 20˚C (Van der Wal et al., 2006b). CO_2 concentrations (1 ml headspace gas) were analyzed using a gas chromatograph (Carlo Erba GC 6000) equipped with a hot wire detector (HWD 430). Helium was used as a carrier gas.

Cultivation and identification of cellulose and wood colonizing fungi

In the second experiment, five pieces of cellulose and five sawdust particles were taken out of each replicate at the end of each incubation period and placed on water agar (containing 20 g l^{-1} agar (Baker), 1 g l^{-1} KH_2PO_4, 0.1 g l^{-1} NH_4Cl, and, to inhibit bacterial growth, 50 mg l^{-1} chloramphenicol and 100 mg l^{-1} streptomycin sulphate). The inoculated plates were sealed with Parafilm and incubated at 20°C until fungal mycelia grew out of the inoculum particles. Fragments of mycelium were then transferred to potato dextrose agar (PDA) agar plates (composition: 7.5 g l^{-1} agar (Merck), 19.50 g l^{-1} PDA (Oxoid), 50 mg l^{-1} chloramphenicol and 100 mg l^{-1} streptomycin sulphate), sealed with Parafilm and incubated at 20°C. Pure cultures were used for identification of fungal genera (Domsch et al., 1980).

DNA isolation, PCR-DGGE and cloning

DNA was isolated from 0.25 g fresh weight soil samples using the PowerSoil DNA isolation sample kit according to the manufacturer`s instructions (MOBIO Laboratories, Inc.). Partial small subunit rDNA fragments were amplified using the primers FR1-GC and FF390 (Vainio and Hantula, 2000). PCR reactions were performed in 25 µl reaction mixtures containing 200

µM of each dNTP, 5 U of Expand High Fidelity DNA polymerase (Boehringer, Mannheim, Germany), 0.6 µM of each of the two primers, 1 µl template DNA and using the manufacturer's recommended buffer conditions. The temperature 'touch-down' cycling program for PCR consisted of an initial denaturation step of 94°C for 4 min, followed by 8 cycles (92°C for 30s, 55°C for 60s (decrease by 2°C every second cycle: 'touch-down'), 68°C for 120s), followed by 27 cycles (92°C for 30s, 47°C for 60s, 68°C for 45s + 1s/cycle) and followed by a final incubation step of 68°C for 10 min. PCR products were analyzed on DGGE gels with a gradient of 40 to 55% denaturant (100% denaturant = 7 M urea and 40% formamide) that were prepared as described by Kowalchuk and Smit (2004). Selected bands were cut from the DGGE gel and immediately used for PCR amplification with primers FR1 (without GC clamp) and FF390. Prior to cloning, the PCR product was purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The purified PCR product was ligated into a pGEM-T vector system (Promega). Ligation mixtures were transformed into JM109 *E. Coli* cells (Promega) and cultured on LB/ampicillin/IPTG/X-Gal plates. Per plate, 4 white colonies were checked for the expected fragment using PCR-DGGE reactions as described above. Sequencing was performed by Greenomics™ (Plant Research International BV, Wageningen, the Netherlands). To identify fungal species, similarities between obtained sequences were compared with nucleotide sequences available in GenBank by using the nucleotide BLAST (blastn) program (http://www.ncbi.nlm.nih.gov/BLAST/).

Enzyme assays

Additional analyses on enzyme activities for lignin and cellulose degradation were performed on remaining soil samples of the shortest (2 weeks) and longest (16 weeks) incubation period. Laccase activity was measured as indicator for lignin degradation (Leonowicz et al., 1999). It was measured by oxidation of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) according to Bourbonnais and Paice (1990), but modified for soil samples. Briefly, 4 g fresh soil was shaken in 6 ml milliQ ultrapure water for 1 hour. Supernatant was transferred to Eppendorf cups and after centrifuging for 10 min. at 10000 rev min^{-1}, the supernatant was transferred to Sephadex G-25 medium columns to remove salts and soluble inhibitors (Baldrian and Gabriel, 2002). Enzymes were concentrated 10-fold using Microcon centrifugal filters with 10 kDa cut-off (Millipore). The 200 µl reaction mixture contained 0.5 mM ABTS, 0.1 M sodium acetate buffer, pH 5.0 and 20 µl of concentrated extract. The formation of green dye was followed spectrophotometrically at 420 nm using a micro-plate-reader (Synergy™ HT, BIO-TEK). Because laccase activity was very low, we measured absorption in 1 hour intervals for 7 h and checked for linearity of product formation.

Activity of *endo*-1,4-ß-glucanase as indicator of cellulase was estimated using carboxymethyl cellulose dyed with Remazol brilliant blue R(Azo-CM-Cellulose, Megazyme, Bray, Ireland). Enzyme was extracted by shaking 1 gram of soil in 5 ml sodium acetate solution (0.5 M, pH 5) for 1 hour. Supernatant was centrifuged for 10 min. at 10000 rev min^{-1} and filtered over Sephadex G-25 medium columns. The reaction mixture contained 0.2 ml of 2% Azo-CM-Cellulose in milliQ ultrapure water and 0.2 ml enzyme extract. Samples were incubated at 40°C for 24 hours and the reaction was stopped by adding 1 ml of precipitation solution (20% sodium acetate trihydrate and 3% zinc acetate in 100 ml milliQ ultrapure water). The formation of blue dye was measured spectrophotometrically at 590 nm. The values of extinction were read from the calibration curve of Remazol brilliant blue and expressed as μmol glucose/ 24 h/ g dry weight.

The activity of endo-1,4-ß- xylanase (xylanase) as an indicator of hemicellulase activity was estimated using a similar procedure based on observing dye release from birchwood xylan dyed with Remazol brilliant blue (Azo-xylan, Megazyme, Bray, Ireland).

Statistical analyses

All data were analyzed per incubation period by a one-way ANOVA in SPSS for Windows (Release 11.5.0; standard version) with substrate and field-age as factors. The 'young' fields were pooled since they shared a low fungal biomass (see Van der Wal et al., 2006b). The assumption of normality was tested with Shapiro-Wilk statistics and homogeneity of variance was assessed with Levene's test. Differences between groups were tested with Tukey's honestly significant difference test or, when variances were unequal, with Tamhane's T2 test at $P<0.05$. Welch's test was used to test differences between two groups with unequal group sizes. When only one replicate was available from the old field, a one-sample t test was used to compare the means obtained for the young fields with the value obtained for the old field.

Results

Experiment 1: constraints for saprotrophic fungal biomass development in ex-arable land

Ergosterol content significantly increased in young and old fields after addition of each of the test carbon substrates, but the increase induced by cellulose was almost twice as high as that induced by glucose or birch sawdust (Table 2 & Fig. 1). Soil respiration was highest after addition of cellulose in both young and old fields after two weeks of incubation (data not shown). For all substrates and soils, the highest soil ergosterol concentrations recorded were

Table 2: Effect of carbon enrichment on ergosterol content (mg kg^{-1}) in soil of young fields (n=12) and an old field (n=4). Data represent difference between carbon-enriched and non-enriched control samples.

	glucose				cellulose				wood			
times	mean young fields ± SE		mean old field ± SE		mean young fields ± SE		mean old field ± SE		mean young fields ± SE		mean old field ± SE	
t=1	1.76 ± 0.17 *	a k w	1.56 ± 0.05 *	a k w	3.94 ± 0.42 *	a l w	3.53 ± 0.23 *	a l w	1.85 ± 0.18 *	a k w	1.95 ± 0.05 *	a m w
t=2	1.09 ± 0.09 *	a k x	1.14 ± 0.06 *	a k x	2.41 ± 0.12 *	a l x	3.39 ± 0.29 *	b l w	1.54 ± 0.11 *	a m w	1.86 ± 0.08 *	a m w
t=3	0.79 ± 0.07 *	a k x	1.05 ± 0.08 *	a k x	1.60 ± 0.13 *	a l y	1.89 ± 0.30 *	a l x	1.00 ± 0.13 *	a k x	1.12 ± 0.11 *	a k x
t=4	0.37 ± 0.06 *	a k y	0.55 ± 0.04 *	a k y	0.77 ± 0.09 *	a l z	1.45 ± 0.36 *	a k x	0.58 ± 0.11 *	a k,l x	0.59 ± 0.04 *	a k x

Letters a and b denote differences at P<0.05 level between young and old fields within sampling times and within substrate
Letters k,l,m,n denote differences at P<0.05 between substrates within field-age classes and within sampling times
Letters w,x,y,z denote differences at P<0.05 level between sampling times within field-age class and within substrate
Asterisks denote significant differences between carbon substrate addition treatments and the control (no addition) at P<0.05

Table 3: Effect of carbon enrichment on nitrogen mineralization (N mg kg^{-1}) in soil of young fields (n=12) and an old field (n=4). Data represent difference between carbon enriched and non-enriched control samples.

	glucose				cellulose				wood			
times	mean young fields ± SE		mean old field ± SE		mean young fields ± SE		mean old field ± SE		mean young fields ± SE		mean old field ± SE	
t=1	-118.94 ± 4.28 *	a k w	-156.38 ± 1.28 *	b k w	-96.69 ± 3.01 *	a l w	-131.46 ± 2.31 *	b l w	-36.89 ± 3.51 *	a m w	-92.98 ± 5.07 *	b m w
t=2	7.72 ± 10.38	a k x	50.20 ± 8.20 *	b k x	5.01 ± 9.40	a k x	23.52 ± 2.65 *	a l x	-3.29 ± 6.23	a k x	25.54 ± 3.98	b l x
t=3	0.81 ± 13.80	a k x	26.79 ± 13.31	a k x	-8.93 ± 12.95	a k x	5.13 ± 3.58	a k y	0.34 ± 11.76	a k w,x	-9.43 ± 22.81	a k w,x
t=4	24.68 ± 6.14	a k x	20.83 ± 12.25	a k x	14.07 ± 7.24	a k x	18.91 ± 6.58	a k x,y	7.01 ± 3.62	a k x	12.01 ± 3.36	a k x

Letters a and b denote differences at P<0.05 level between young and old fields within sampling times and within substrate
Letters k,l,m,n denote differences at P<0.05 between substrates within field-age classes and within sampling times
Letters w,x,y,z denote differences at P<0.05 level between sampling times within fields and within substrate
Asterisks denote significant differences between carbon substrate addition treatments and the control (no addition) at P<0.05

those obtained after the shortest of the incubation periods used. During further incubation, ergosterol content declined. The decline seen was similar for all fields except that in the cellulose treatment, the decrease in ergosterol occurred more slowly in the old field than in the younger fields (Table 2 & Fig. 1). The transfer of soil from the old field to the young fields did not result in a significantly different ergosterol content in any of the treatments ($P > 0.05$, data not shown).

Bacterial numbers were highest after addition of glucose in the first incubation period in both young and old fields, although this could not be confirmed statistically (data not shown). For cellulose, the increase of bacterial cells was higher in the old field (9.9E+08, calculated as the difference between carbon-enriched and non-enriched samples) than in the young fields (2.4E+08) at the first sampling time, but the difference between field types decreased rapidly over longer incubation.

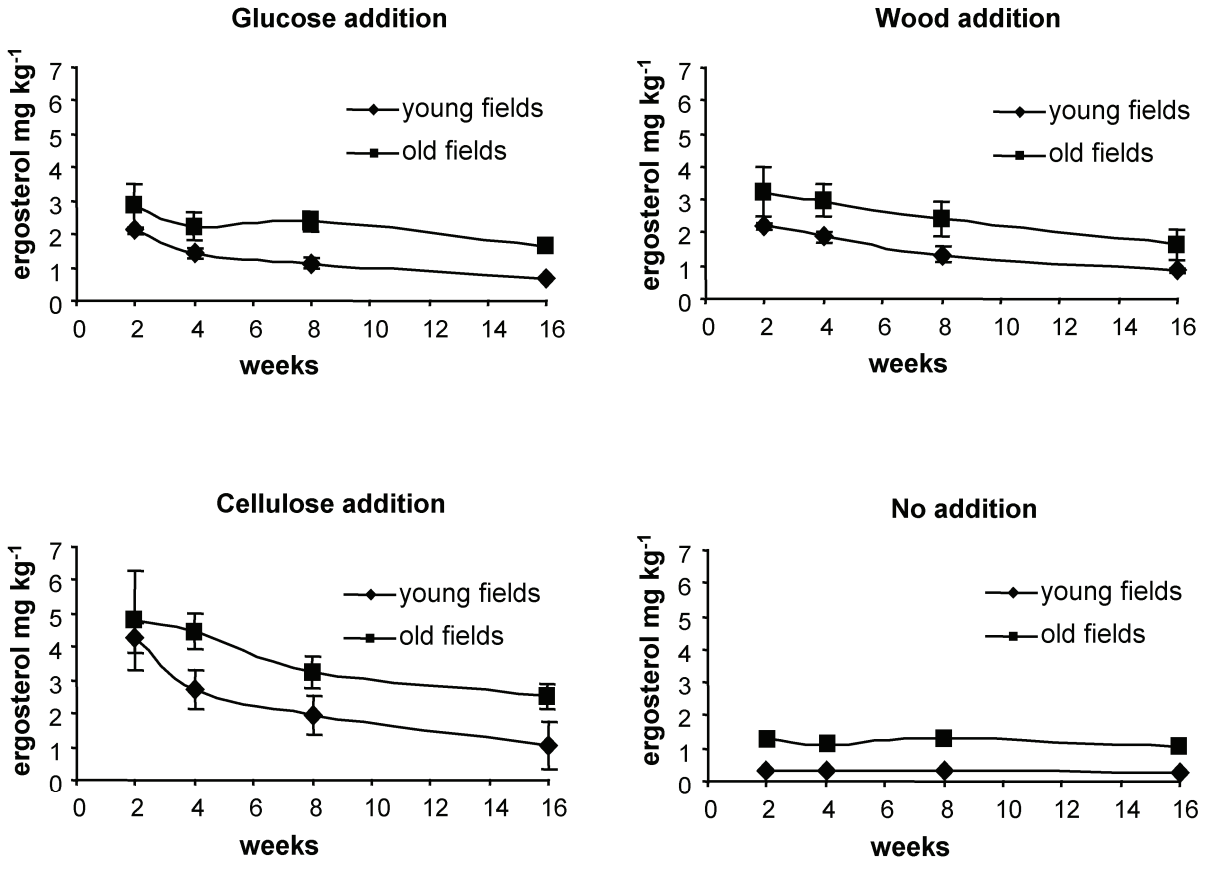

Figure 1: The relation between time of incubation and ergosterol content (mg kg^{-1}) in soil samples from young fields (n=12) and an old field (n=4) amended with glucose, cellulose or wood, as well as in corresponding controls.

All substrate additions resulted in a short-term immobilization of nitrogen in all soils (Table 3). This immobilization was highest for the glucose addition treatment, followed by the cellulose and wood addition treatments in that order. In the subsequent incubation period, net nitrogen mineralization became apparent in both young and old fields. This was highest for glucose and lowest for wood. The values for short-term N immobilization and for subsequent N mineralization in the old field soil were both higher than those obtained for the young fields.

Measurements of enzymatic activity revealed a rapid response for cellulase and hemicellulase in all soils after addition of cellulose and wood, although variability between replicates was high (Table 4). After the longest incubation period, cellulase activity could be seen to have decreased in these two treatments, with the strongest decrease occurring in soils amended with wood. Hemicellulase activity was particular strongly increased by wood amendment in both young and old fields, but it was significantly higher in the latter category than in the former. As was seen with cellulase activity levels, hemicellulase activities were dramatically lower at the time of final sampling in the soils amended with wood. Laccase activity remained low in all treatments (Table 4). Glucose addition did not induce a clear pattern of response in any of the enzymatic activities.

DGGE banding patterns (which were repeatedly obtained from replicate soil samples) formed by fungal 18S rDNA gene fragments were strikingly similar for young and old fields after 2 weeks of incubation, indicating a similar fungal community (Fig. 2). The control and the glucose treatment had similar banding patterns differing strongly from those seen with the wood and cellulose treatments. Cloning and sequencing of dominant DGGE bands proved to be difficult and we were therefore not able to sequence all the bands of interest. However, some information could be extracted from the successfully sequenced bands. After glucose addition, one band became more intense than it was in the control treatments. This band was identified as corresponding to a member of the yeast genus *Cryptococcus*. Other dominant bands in the control and glucose treatment revealed ≥ 98% homology to members of the fungal genera *Fusarium* and *Mortierella*. One band was identifiable only as belonging to the genus *Humicola*. The cellulose and wood treatment yielded very similar banding patterns. In both treatments ≥ 98% homology of band sequences was observed to the fungal genera *Humicola*.

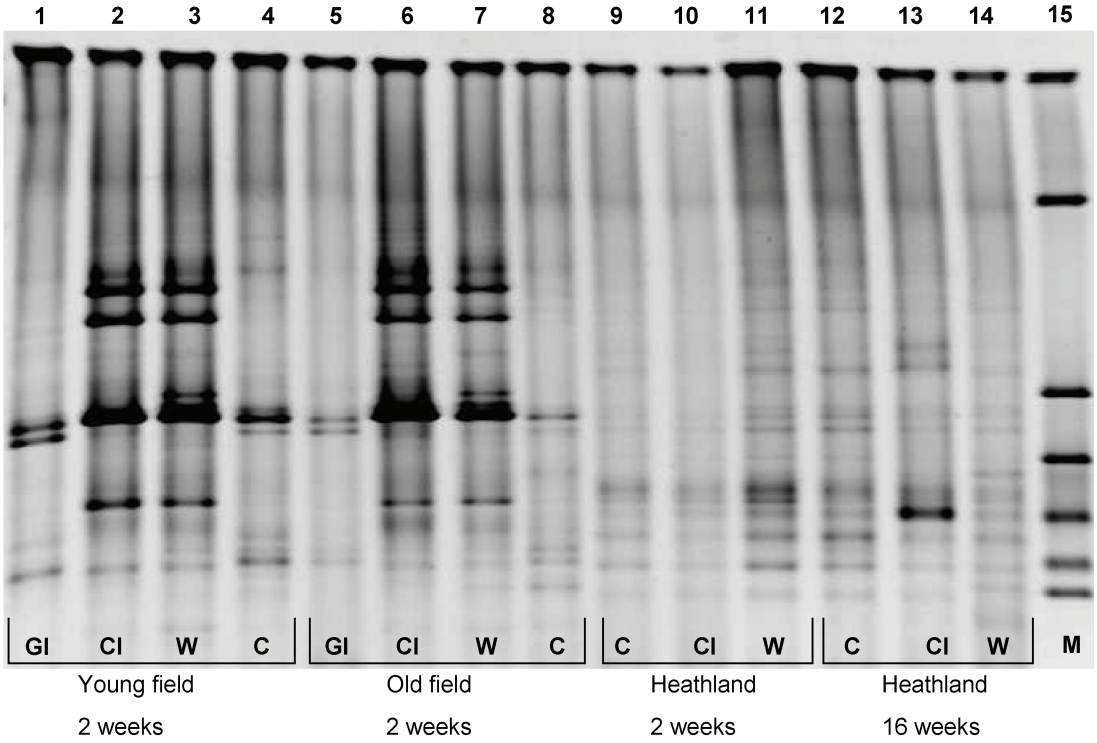

Figure 2: Fungal PCR-DGGE analysis (lane 1-8) of soil incubated for 2 weeks from a young field and an old field after addition of glucose (Gl), cellulose (Cl), or wood (W); (C) indicates no addition. Fungal PCR-DGGE analysis (lane 9-14) of soil incubated for 2 and 16 weeks from a heathland field after addition of cellulose (Cl) or wood (W); (C) indicates no addition. Fungal marker (M) is presented in lane 15.

Experiment 2: differences in fungal decomposition functions between ex-arable land and heathland

Ergosterol content did not significantly increase after addition of cellulose or sawdust to the heathland soil. Even after 16 weeks of incubation there was no significant difference between the treated samples and the controls (Table 5 & Fig. 3). Although we observed an increase in ergosterol content after 5 weeks of incubation, this was not significant since ergosterol content in the untreated heathland soil also increased (Fig. 3). Interestingly, after 5 weeks of incubation green fungal colonies developed on cellulose particles, yet no increase in ergosterol was observed. However, soil respiration at the heathland site in the cellulose treatment was highest after 5 weeks of incubation; the difference in respiration levels among different time periods was statistically significant. These results indicate that microbial activity was highest in the second incubation period (data not shown). In contrast, as in the first experiment, the young field showed a large short-term increase of ergosterol after addition of cellulose and wood. Ergosterol then decreased in the young field as incubation proceeded. The addition of heathland soil to the young field did not result in a significantly different response in ergosterol content in any of the treatments ($P > 0.05$, data not shown).

Table 4: Effect of carbon enrichment on laccase (µmol ABTS /24 h/g), cellulase (µmol glucose/24 h/g) and hemicellulase (µmol glucose/24 h/g) activity in soil of young fields (n=6) and an old field (n=2). Data represent difference between carbon-enriched and non-enriched control samples.

		glucose				cellulose				wood			
enzyme	times	mean young fields ± SE		mean old field ± SE		mean young fields ± SE		mean old field ± SE		mean young fields ± SE		mean old field ± SE	
laccase	t=1	0.01 ± 0.00	a k w	0.01 ± 0.00	a -	0.01 ± 0.00	a k w	0.00 ± 0.01	a -	0.01 ± 0.00	a k w	0.03 ± 0.00	a -
	t=4	0.00 ± 0.00	a k w	0.01 ± 0.01	b -	0.00 ± 0.00	a k w	0.00 ± 0.00	a -	0.00 ± 0.00	a k x	0.00 ± 0.00	a -
cellulase	t=1	0.75 ± 0.88	a k w	-0.45 ± 5.00	a -	34.38 ± 18.36	a k,l w	21.83[1]	a -	33.17 ± 5.11	a l w	32.29 ± 9.55	a -
	t=4	12.28 ± 5.56	a k w	6.24 ± 0.96	a -	11.20 ± 2.58	a k w	14.40 ± 0.48	a -	-0.15 ± 0.48	a k x	3.36 ± 0.00	a -
hemicellulase	t=1	-1.29 ± 1.89	a k w	1.49 ± 0.75	a -	7.60 ± 5.65	a k w	24.87[1]	a -	87.38 ± 31.32	a k w	248.71 ± 42.96	b -
	t=4	5.86 ± 4.90	a k w	1.47 ± 2.95	a -	5.41 ± 3.81	a k w	2.95 ± 0.00	a -	3.20 ± 3.22	a k x	4.42 ± 0.00	a -

Letters a and b denote differences at P<0.05 level between young and old fields within sampling times and within substrate
Letters k,l,m,n denote differences at P<0.05 between substrates within field-age classes and within sampling times
Letters w,x,y,z denote differences at P<0.05 level between sampling times within young fields and within substrate
Asterisks denote significant differences between carbon substrate addition treatments and the control (no addition) at P<0.05
No test for homogeneity of variance could be performed between substrates and between sampling times within old fields, since n<3 (indicated with '-')
[1] No SE is given, since only one sample was measured

Table 5: Effect of carbon enrichment of soil samples on ergosterol content in mg kg^{-1} in soil of a young field (n=4) and a heathland (n=4). Data represent difference between carbon-enriched and non-enriched control samples.

	cellulose				wood			
times	mean young fields ± SE		mean heathland ± SE		mean young fields ± SE		mean heathland ± SE	
t=1	3.46 ± 0.35 *	a k x	0.54 ± 0.55	b k x	2.06 ± 0.25 *	a l x	0.98 ± 0.20	b k x
t=2	1.63 ± 0.86	a k x,y	1.88 ± 0.78	a k x	2.10 ± 0.13 *	a k x	-0.09 ± 0.74	b k x
t=3	0.62 ± 0.11 *	a k y	0.88 ± 0.60	a k x	0.47 ± 0.04 *	a k y	0.98 ± 0.26	a k x

Letters a and b denote differences at P<0.05 level between young and heathland within sampling times and within substrate
Letters k and l denote differences at P<0.05 between substrates within field-age classes and within sampling times
Letters x,y,z denote differences at P<0.05 level between sampling times within fields and within substrate
Asterisks denote significant differences between carbon substrate addition treatments and the control (no addition) at P<0.05

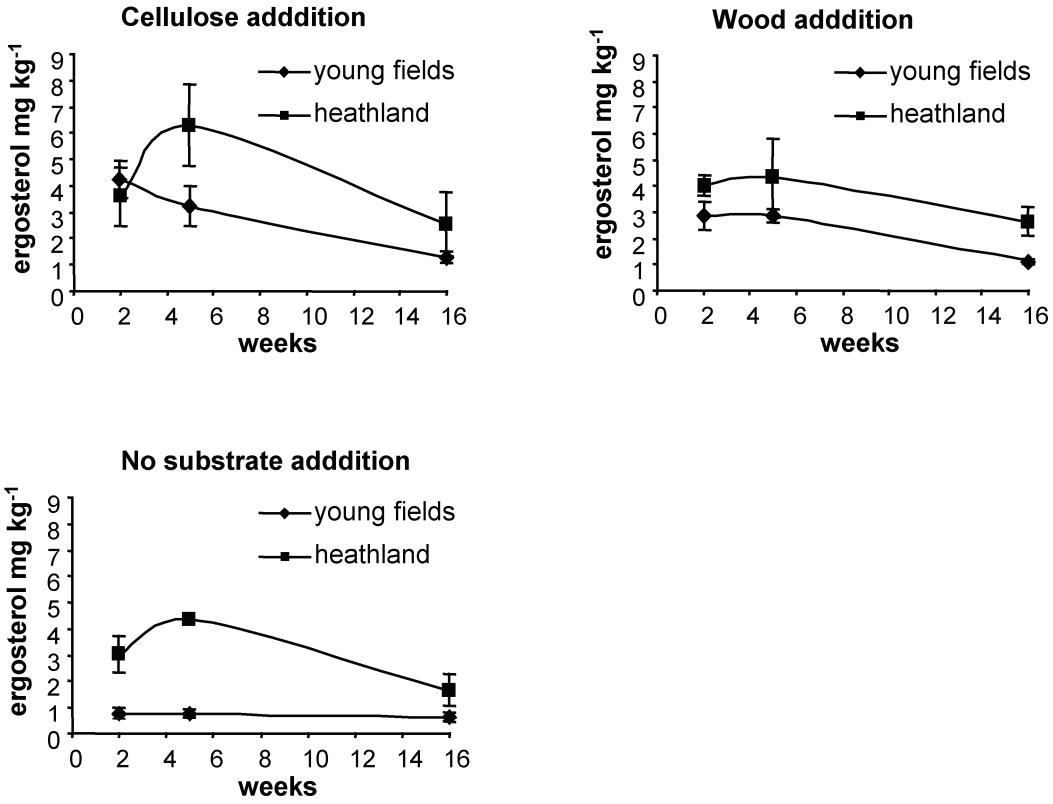

Figure 3: The relation between time of incubation and ergosterol content (mg kg^{-1}) in soil samples from a young field (n=4) and heathland (n=4) amended with cellulose or wood, as well as in corresponding controls.

Bacterial numbers did not significantly differ between the treatments and the controls in both fields (data not shown).

Short-term nitrogen immobilization was not significantly increased after addition of cellulose or wood to heathland soil. At this site, nitrogen immobilization appeared to be highest in the final incubation period for both carbon substrates, but the difference between treatment and control samples was significant only for cellulose (Table 6). The dynamics of mineral nitrogen in the ex-arable land was completely different: as in the first experiment, a significant short-term immobilization occurred for both substrates (Table 6).

As in the first experiment, laccase activity was very low in both soils, even in the sawdust treatment. The response of cellulase levels to the substrate additions differed strongly between the soils. The ex-arable soil showed a rapid increase of cellulase activity and a drop during subsequent incubation, whereas the heathland soil showed a slower but more consistent cellulase response in both carbon treatments (Table 7). Hemicellulase activity increased in both substrate treatments in heathland soil after 5 weeks of incubation.

Table 6: Effect of carbon enrichment of soil samples on nitrogen mineralization in N mg kg^{-1} in soil of a young field (n=4) and a heathland (n=4). Data represent difference between carbon-enriched and non-enriched control samples.

	cellulose				wood			
times	mean young fields ± SE		mean heathland ± SE		mean young fields ± SE		mean heathland ± SE	
t=1	-79.70 ± 2.04 *	a l x	-8.54 ± 2.47	b k x,y	-33.12 ± 0.67 *	a k x	-2.49 ± 4.79	b k x,y
t=2	10.75 ± 4.31	a k y	1.17 ± 6.94	a k x	-4.83 ± 1.61	a l y	13.39 ± 8.97	a k x
t=3	13.78 ± 2.52	a k y	-25.52 ± 8.18 *	b k y	2.48 ± 2.63	a l z	-20.73 ± 6.13	b k y

Letters a and b denote differences at P<0.05 level between young and heathland within sampling times and within substrate
Letters k and l denote differences at P<0.05 between substrates within field-age classes and within sampling times
Letters x,y,z denote differences at P<0.05 level between sampling times within fields and within substrate
Asterisks denote significant differences between carbon substrate addition treatments and the control (no addition) at P<0.05

Table 7: Effect of carbon enrichment of soil samples on laccase (µmol ABTS /24 h/g) activity in soil of a young field (n=4) and a heathland (n=4). Cellulase (µmol glucose/24 h/g) and hemicellulase (µmol glucose/24 h/g) activity in soil of a young field (n=2) and a heathland (n=2). Data represent difference between carbon-enriched and non-enriched control samples.

		cellulose				wood			
enzyme	times	mean young fields ± SE		mean heathland ± SE		mean young fields ± SE		mean heathland ± SE	
laccase	t=1	0.01 ± 0.00	a k x,y	0.00 ± 0.00	a k x	0.00 ± 0.00 *	a k x	0.00 ± 0.00	b k x
	t=2	0.00 ± 0.00 *	a k x	0.00 ± 0.00	a k x	0.00 ± 0.00	a k x,y	0.00 ± 0.00 *	b k y
	t=3	0.01 ± 0.00	a k y	0.00 ± 0.00	b k x	0.00 ± 0.00 *	a l y	0.00 ± 0.00	b l x
cellulase	t=1	33.56 ± 6.23		4.89 ± 2.72		56.58 ± 0.48		12.50 ± 1.63	
	t=2	9.11 ± 0.96		42.11 ± 9.47		1.92 ± 4.32		28.43 ± 2.11	
	t=3	20.44 ± 2.79		48.43 ± 13.69		-2.32 ± 3.25		28.43 ± 4.21	
hemicellulase	t=1	6.32 ± 4.46		-17.69 ± 29.48		164.99 ± 168.34		-14.32 ± 14.32	
	t=2	8.92 ± 7.43		117.91 ± 18.53		16.35 ± 8.92		229.92 ± 4.21	
	t=3	10.41 ± 10.41		64.34 ± 0.00		-1.49 ± 2.97		45.83 ± 7.93	

Letters a and b denote differences at P<0.05 level between young and heathland within sampling times and within substrate
Letters k and l denote differences at P<0.05 between substrates within field-age classes and within sampling times
Letters x,y,z denote differences at P<0.05 level between sampling times within fields and within substrate
Asterisks denote significant differences between carbon substrate addition treatments and the control (no addition) at P<0.05

In the first experiment, the DGGE patterns obtained in heathland soil after cellulose and sawdust addition were completely different from those obtained from the arable soils (Fig. 2). After 2 weeks of incubation, the patterns from amended soils were similar to control patterns. This was still the case after 16 weeks of incubation, although one band increased in intensity in the cellulose treatment (Fig. 2). This band was, after sequencing, identified as a member of the large clade including both *Byssoascus striatosporus* and *Oidiodendron tenuissimum*. Sequencing of bands from the control treatment revealed fungi with ≥ 98% homology to the genera *Hymenoscyphus*, *Mortierella*, *Cryptococcus* and *Chaetomium*. One band was identified as belonging to the genera of either *Thelebolus*, *Hyphozyma* or a closely related organism with affinities to the order Thelebolales. In the cellulose treatment a band was obtained with ≥ 98% homology to the genus *Hymenoscyphus*, and, as in the control treatment, one band was identified as either *Thelebolus* or *Hyphozyma*. In the wood treatment bands were obtained with ≥ 98% homology to the genera *Hymenoscyphus*, *Oidiodendron* and *Cryptococcus*. One band was identified as belonging to the genera of either *Thelebolus* or *Hyphozyma* and one band as a pyronemataceous ascomycete related to the genera *Aleuria*, *Wilcoxina*, *Cheilymenia* or *Otidea*.

Fungi growing from cellulose pieces retrieved from the young field predominately belonged to the zygomycetous genera *Zygorhynchus* and *Mortierella* and the ascomycetous genera *Acremonium* (section *Chaetomioidea*, related to the cellulolytic genus *Chaetomium*), *Humicola*, *Penicillium* and *Trichoderma*. Wood yielded the same zygomycetous genera again as well as *Mucor spp*., while among the ascomycetes, the predominant genera were *Acremonium*, *Humicola*, *Trichoderma*, and *Thielaviopsis*. Fungi isolated from cellulose pieces in the heathland differed from those from young fields by yielding the zygomycota *Mucor* and *Absidia*, as well as members of the *Sporothrix inflata* species complex. The genus *Humicola* was not found in samples from heath. Wood pieces from heath predominantly yielded the zygomycetous *Zygorhynchus*, *Mortierella* and *Umbelopsis*, as well as the ascomycetous *Acremonium*, *Penicillium*, *Trichoderma* and *Sporothrix*.

Discussion

Effect of carbon additions on saprotrophic fungal biomass development in ex-arable land

All carbon additions induced a rapid increase of fungal biomass in both the young and old ex-arable fields. This indicates that the increase in fungal biomass in the field soils is constrained by the availability of biodegradable carbon sources. Except in some zones in the rhizosphere,

limited carbon availability is common in soils (Wardle, 1992), and increase of soil fungal biomass after addition of carbon sources has been shown frequently (Griffiths et al., 1999; Schutter and Dick, 2001; Waldrop and Firestone, 2004).

The ex-arable soils have a low fungal: bacterial ratio and, therefore, fungi are expected to be confronted with intense competition for carbon by bacteria (De Boer et al., 2005). We hypothesized that the extent of fungal biomass increase upon carbon addition would depend on the complexity of the carbon source (sawdust > cellulose > glucose), as bacteria are in general less well equipped than fungi to degrade crystalline cellulose (which we used in this experiment) and are less efficient degraders of lignin (Hatakka, 2001; De Boer et al., 2005). Therefore, the lower response of fungi to glucose than to cellulose is in accordance with the expectation of strong bacterial competition. At the same time, fungal biomass increase after addition of glucose was significant, indicating that bacteria were not able to monopolize the use of this substrate. An explanation for this could be that at high substrate loadings, as were applied in our study (2 mg C g^{-1} soil), the competition for carbon between bacteria and fungi may not have occurred immediately and, therefore, fungi may have had the opportunity to use at least part of the substrate (Griffiths et al., 1999).

Despite the differences in complexities of the substrates, the response times of fungi in the different treatments showed minimal differences. Hence, it seems that fast-growing fungi were the main group stimulated by all treatments. The fact that the response time of fungal biomass to sawdust addition was not essentially different from that seen with the other substrates may reflect the availability of cellulose and hemicellulose on the surfaces of the wood chips (Daniel, 2003). This scenario is supported by the relatively high short-term activity of cellulase and hemicellulase in the sawdust treatments (Table 4). Low laccase activities indicated that lignin modification was not required for organisms to gain access to the other wood polymers. The sawdust that was used as a wood source has a large surface: volume ratio, maximizing the potential accessibility of cellulose and hemicellulose. Such a situation is clearly beneficial for cellulolytic opportunistic fungi. Larger wood chips might have been a better substrate for the increase of biomass of lignocellulolytic basidiomycetes.

The rapid decline in ergosterol for all substrates after the first incubation period indicates a rapid turnover of fungal hyphae, which is typical for fast-growing opportunistic fungi. These organisms devote proportionally more energy to reproductive effort than do fungi forming persistent hyphae (Cooke and Rayner, 1984). The decline in ergosterol was slightly slower for the older ex-arable land than for the arable and recent ex-arable lands (Table 2). Hence, in the older ex-arable land, fungi with relatively persistent hyphae may have made a small but appreciable contribution to decomposition of the substrates.

Another difference between the older ex-arable land and the other sites was the stronger short-term immobilization of nitrogen in the former. Since, in general, increases in bacterial counts in the old field were higher than those obtained from the young field, the relatively high N immobilization in the former was likely due to incorporation of nitrogen into bacterial biomass. Hence, contrary to our expectations, bacteria appeared to make a greater contribution to decomposition in the older ex-arable land than in the recently abandoned sites. Bacterial numbers at the start of the experiment were 1.94E +08 in the older ex-arable land and 1.42E +08 in the young fields.

Community composition and decomposition functions of fungi in ex-arable land

The remarkable similarity between the short-term fungal biomass responses of young and old fields to the carbon additions parallels the similarity in DGGE band patterns obtained from the two types of fields. These findings suggest that fungally driven decomposition functions in the old ex-arable field have not yet diverged from those in the arable and recently abandoned sites (Fig. 2). The cellulose and wood treatment yielded almost identical band patterns, which differed strongly from those obtained in the glucose treatment. The pattern obtained in the glucose treatment resembled that obtained with the control. Interestingly, the fungal community characteristic of the control soil remained unchanged after addition of glucose, while at the same time ergosterol content increased, indicating that fungi with good abilities to grow on glucose were already dominant in arable and ex-arable land. This conclusion was also supported by the sequence information that revealed that the dominant fungi tended to be so-called sugar fungi such as the fast-growing zygomycetes *Zygorhynchus* and the soil yeasts in the genus *Cryptococcus*. Such r-strategists can be expected to predominate when an easily degradable carbon source becomes available in an environment where a relatively scarce resident microflora is present (Cooke and Rayner, 1984). This applies to the soils under study as the initial fungal biomass was very low. The rapid appearance of new bands in the cellulose and sawdust treatments indicates that the glucose-consuming fungi were not efficient in the exploitation of cellulose, leaving an opportunity for growth of other fungi. The cellulose-degrading fungi seen probably also used hemicellulose, since hemicellulase activity increased in the sawdust treatment without the appearance of additional DGGE bands. The near-exclusive isolation of a relatively uniform group of fungal genera characterized by an opportunistic growth strategy from both cellulose and wood pieces seems to support the proposed role of r-strategist fungi in these treatments. However, culturing natural substrates on general fungal growth media selects to some extent for fast-growing species and an absence of outgrowing k-strategic fungi can not exclude their

presence and activity in the substrata tested (Garrett, 1951). Yet, sequencing of DGGE bands did not reveal specialized lignocellulolytic basidiomycetes in any of the treatments.

Community composition and decomposition functions of fungi in heathland

The response of fast-growing fungi to carbon additions, as observed in the ex-arable sites, was completely absent in the heathland soil. This is remarkable, since both sequencing of DGGE bands and identification of culturable isolates indicated that some fast-growing fungi found in the ex-arable sites were also present in the heathland soil. The lack of response of such opportunistic fungi may be due inhibition by the low soil pH or by phenolic compounds originating from *Calluna* roots (Read, 1991; Aerts, 2002). Although the presence of fast-growing fungi was confirmed in DGGE patterns obtained from the heathland soil, this soil yielded a band pattern completely different from that obtained with the young field (Fig. 2). Moreover, sequencing revealed the presence of different fungal genera in heathland. Sequencing of dominant bands in the control treatment revealed a fungus with ≥ 98% homology to the genus *Hymenoscyphus*, and one belonging or closely related to the genus *Oidiodendron*. Some *Hymenoscyphus* species (recently classified in *Rhizoscyphus*) as well as one particular *Oidiodendron* species, *O. maius* (Sigler and Gibas, 2005), form ericoid mycorrhizae (ERM) with plants in the families *Ericaceae* and *Epacridaceae* (Smith and Read, 1997). Such sequences, therefore, are not unexpected in heathland. After addition of carbon in this soil type, the DGGE band patterns remained similar to those of the control. In the cellulose treatment, however, one band was more intense after 16 weeks of incubation than at earlier time points, and proved to be a fungus in the ascomycetous family Myxotrichaceae, related to the two members of this family represented in the 18S rDNA sequence database, *Byssoascus striatosporus* and *Oidiodendron tenuissimum*. Though one member of this family, *O. maius*, has been shown to be an ericoid mycobiont, the remainder appear to be saprotrophs often involved in the breakdown of cellulose (Domsch et al., 1993; Sigler and Gibas, 2005). Even if the sequence obtained was that of a mycobiont rather than a saprotroph, the response to cellulose can still be explained. ERM fungi are slow-growing, facultative symbionts which are known to possess saprotrophic abilities (Bending and Read, 1997; Cairney and Burke, 1998). The consistency of the banding pattern seen during incubation and the increase in intensity of a band potentially related to ERM fungi, together with a slow increase in soil respiration, N immobilization, cellulase activity and hemicellulase activity, suggests that ERM fungi may be responsible for the low decomposition rate of the carbon added to the heathland soil samples. This would imply that they are able to exhibit slow decomposition without the need to be associated with a plant. Based on the enzymatic activities seen, only cellulose and hemi-cellulose but not lignin could have been decomposed by these ERM fungi,

although it is shown that some ERM fungi have some abilities to degrade lignin (Bending and Read, 1997).

Perhaps even more remarkable than the lack of response of fast-growing fungi to carbon addition in heathland is the fact that no ligninolytic wood- degrading basidiomycetes appeared to become established in the sawdust treatment, even though the low pH of the heathland soil has been shown to favor production of ligninolytic enzymes (Shah and Nerud, 2002). It may be that selective inhibition of phenolic compounds originating from *Calluna* plants underlies the inability of ligninolytic basidiomycetes to develop in this type of habitat (Leake and Read, 1989; Read, 1991). Lignocellulolytic fungi may need an established biomass in large wood pieces or a distinct litter layer from which they colonize wood fragments in the mineral heathland soil via mycelial cords (Boddy, 1993). Such hyphae have little interaction with the soil environment and may overcome the inhibition induced by the phenolic compounds.

Comparison of DNA- and cultivation-based fungal community composition

We used two different methods to identify fungal genera, one molecular and one based on cultivation. Several fast-growing genera, e.g. *Penicillium*, *Mucor* and *Trichoderma*, that grew out from sawdust and cellulose particles on agar were not detected by PCR-DGGE, although DNA was extracted from soil including the substrate particles. This may indicate that these genera were only growing in and on the substrate particles and were not able to colonize the soil, which may have partly complicated DNA extraction. This growth strategy is typical for opportunistic fungi as they produce survival propagules, e.g. spores, rather than exploratory hyphae when nutrient conditions become unfavorable (Cooke and Rayner, 1984). ERM-related fungi were detected in the heathland soil by PCR-DGGE but not by the cultivation method. However, this does not imply that the substrate particles were not colonized by these fungi as there presence may have been masked by fast-growing fungi. Therefore, the DGGE profiling method is appropriate for studying the dominant members of the microbial community (Muyzer et al., 1993) and the culturing method clearly also contributes to the identification of fungi that are present in and on substrate particles.

Constraints on development of lignocellulolytic fungi and ericoid mycorrhizal in ex-arable land

Our attempts to bring about an increase in the biomass of k-strategy fungi in ex-arable land by addition of carbon substrates in combination with soil inoculations were not successful. An increase in biomass of non-ruderal fungal types may be important for a more rapid transition of abandoned arable land into heathlands. ERM associated with *Calluna* plants are able to use

fungal chitin as a source of N for plant nutrition (Kerley and Read, 1997). Long-term N immobilization in fungal biomass may thus stimulate the colonization of ex-arable lands by *Calluna*. In addition, a relatively high amount of fungal biomass can give protection against nitrogen leaching and can store soil carbon more effectively than can micro-organisms with a rapid biomass turnover (Bailey et al., 2002; Vinten et al., 2002). This storehousing of nitrogen will then increase the stability of the soil ecosystem.

The strong response of opportunistic, fast-growing fungi to all carbon additions, including sawdust, in both recent and older abandoned fields, may have prevented colonization by fungal species introduced with the soil inoculum. The inoculated fungi may not have been able to capture readily available carbon in competition with an opportunistic microflora (Lang et al., 1997). The monopolization of substrate use by r-strategist fungi that was apparent in the current experiments may also occur in the field. This could be the reason why fungal biomass remains low in reclaimed farmland soil for several decades (Van der Wal et al., 2006b).

Colonization of ex-arable land by hyphae of lignocellulolytic basidiomycetes may depend on a decrease in the range of opportunities for fast-growing fungi to exploit degradable substrates. These opportunities are likely to decrease in intact litter with a high lignin content, since in this situation, degradation or modification of lignin becomes essential for access to the co-occurring cellulose and hemi-cellulose polymers. The development of a lignin-rich litter layer may be a prerequisite for the development of lignocellulolytic basidiomycetes. The ERM fungi which appeared to be responsible for the slow decomposition of the added substrates in heathland soil were not able to establish themselves when heathland soil inoculum was transferred to arable and ex-arable soils. It is possible that the density of ERM fungi in the soil inoculum was too low to allow them to dominate the decomposition processes occurring in the ex-arable soil in the absence of their normal *Calluna* hosts. Other factors, e.g. the relatively high soil pH, may also have had a negative effect on establishment of ERM fungi. In conclusion, the introduction of slow-growing ERM fungi and lignocellulolytic basidiomycetes into farmlands in reclamation in order to foster the development of a microbial community conducive to regeneration of the natural ecosystem is difficult, and may only be possible if the dominating vegetation of dwarf shrubs and a distinct litter layer are present. This poses clear constraints to a relatively rapid (i.e. within a few decades) transition from arable land to the desired natural environment.

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