Global Change Biology

Global Change Biology (2011) **17**, 2184–2195, doi: 10.1111/j.1365-2486.2010.02376.x

Fungal community composition and function after long-term exposure of northern forests to elevated atmospheric CO_2 and tropospheric O_3

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Abstract

The long-term effects of rising atmospheric carbon dioxide (CO_2) and tropospheric O_3 concentrations on fungal communities in soil are not well understood. Here, we examine fungal community composition and the activities of cellobiohydrolase and *N*-acetylglucosaminidase (NAG) after 10 years of exposure to 1.5 times ambient levels of CO_2 and O_3 in aspen and aspen–birch forest ecosystems, and compare these results to earlier studies in the same long-term experiment. The forest floor community was dominated by saprotrophic fungi, and differed slightly between plant community types, as did NAG activity. Elevated CO_2 and O_3 had small but significant effects on the distribution of fungal genotypes in this horizon, and elevated CO_2 also lead to an increase in the proportion of *Sistotrema* spp. within the community. Yet, although cellobiohydrolase activity was lower in the forest floor under elevated O_3, it was not affected by elevated CO_2. NAG was also unaffected. The soil community was dominated by ectomycorrhizal species. Both CO_2 and O_3 had a minor effect on the distribution of genotypes; however, phylogenetic analysis indicated that under elevated O_3 *Cortinarius* and *Inocybe* spp. increased in abundance and *Laccaria* and *Tomentella* spp. declined. Although cellobiohydrolase activity in soil was unaffected by either CO_2 or O_3, NAG was higher (~29%) under CO_2 in aspen–birch, but lower (~18%) under aspen. Time series analysis indicated that CO_2 increased cellulolytic enzyme activity during the first 5 years of the experiment, but that the magnitude of this effect diminished over time. NAG activity also showed strong early stimulation by elevated CO_2, but after 10 years this effect is no longer evident. Elevated O_3 appears to have variable stimulatory and repressive effects depending on the soil horizon and time point examined.

Keywords: elevated carbon dioxide, elevated ozone, enzyme activities, FACE, fungal communities, long-term

Received 21 April 2010 and accepted 4 October 2010

Introduction

Soil fungal communities carry out key carbon (C) and nitrogen (N) cycling functions, and in doing so influence the long-term productivity of terrestrial ecosystems. Fungi inhabiting forest floor and soil depend on plant-derived organic substrates for growth, and therefore environmental factors that affect the production and biochemistry of these substrates have the potential to affect community composition and function. Two such factors are the rising concentrations of atmospheric carbon dioxide (CO_2) and O_3 in the Earth's atmosphere (IPCC, 2007). Generally, higher CO_2 stimulates plant growth, at least in the short-term (Poorter *et al*., 1996; Delucia *et al*., 1999; Karnosky *et al*., 2003). Increased growth aboveground results in greater woody biomass and greater litter-fall, whereas belowground, significant increases in fine-root biomass have been recorded (Kubiske *et al*., 1998; King *et al*., 2005). Ozone is an atmospheric pollutant whose concentration is also rising (IPCC, 2007; Finlayson-Pitts & Pitts, 1997). In contrast to CO_2, elevated atmospheric O_3 typically leads to reduced plant growth and increased plant mortality (Karnosky, 1996; King *et al*., 2005). Both CO_2 and O_3 also may cause changes in plant growth that alter plant tissue biochemistry (Liu *et al*., 2005). Through their effects on plant growth and tissue biochemistry, rising atmospheric concentrations of CO_2 and O_3 could therefore indirectly affect the size, composition, or physiological activity of soil fungal communities (Chung *et al*., 2006; Andrew & Lilleskov, 2009).

Fungal communities in soil are species rich assemblages, and their composition can exhibit both rapid between season turnover, as well as slower long-term change as forest ecosystems mature (Frankland, 1998). In addition to their dynamic nature, fungal communities are ecologically heterogeneous, including species of free-living saprobes as well as species of plant-associated mycorrhizal mutualists (O'Brien *et al*., 2005; Edwards & Zak, 2010). Because of the vital role that symbiotic fungi play in providing their host plants with growth-limiting

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nutrients, the effect of rising [CO_2] on mycorrhizal communities has been a prominent area of research (Fransson *et al*., 2001; Treseder, 2004; Parrent *et al*., 2006; Parrent & Vilgalys, 2007). Treseder (2004) reported that the extent of mycorrhizal colonization generally tended to increase under elevated CO_2, while Fransson *et al*. (2001) and Parrent *et al*. (2006) observed that elevated CO_2 can lead to changes in the relative abundance of some ectomycorrhizal species. These changes in mycorrhizal community composition may reflect species-specific abilities to maintain an adequate nutrient supply to the host, and hence have a functional component (Godbold *et al*., 1997; Gorissen & Kuyper, 2000). Elevated ozone can also increase ectomycorrhizal colonization of host species, and alter the relative abundance of species within the ectomycorrhizal community (Kasurinen *et al*., 1999, 2005; Grebenc & Kraigher, 2007) despite O_3 having a generally negative impact on primary productivity (Andersen, 2003; King *et al*., 2005).

The effect of CO_2 on saprotrophic community composition is poorly understood, although one study (Strnadová *et al*., 2004) found no elevated CO_2 effect. Generally, CO_2 effects on saprotrophic communities have been considered from a functional perspective; Hall *et al*. (2006) found that chemical changes to senescing leaves brought about by elevated CO_2 had little effect on litter decomposition, though decomposition in an elevated CO_2 environment increased the rate of litter decay. In contrast, Cotrufo & Ineson (1996) and Parsons *et al*. (2004) found that changes in leaf chemistry caused by elevated CO_2 resulted in slower litter decomposition. The effect of O_3 on saprotrophic fungal communities appears to be completely unknown, although Parsons *et al*. (2004) found no significant O_3 effect on leaf litter decay.

The FACTS-II Aspen Free Air Carbon Dioxide Exposure (FACE) experiment, which began in 1997, provides a unique opportunity to examine the long-term effects of elevated CO_2 and O_3 on plant communities and their associated microorganisms (Dickson *et al*., 2000). During the first 5 years of FACTS-II, plant productivity was stimulated 25–45% by elevated CO_2, whereas elevated O_3 reduced productivity by 13–23% (King *et al*., 2005). Early FACTS-II studies have also provided evidence of higher microbial extracellular enzyme activities [e.g., cellulases and *N*-acetylglucosaminadase (NAG)] in the soil under elevated CO_2, and have shown that both CO_2 and O_3 can affect fungal community composition (Larson *et al*., 2002; Chung *et al*., 2006). The aforementioned studies were conducted in the 2nd and 5th year of fumigation, respectively. More recent studies of soil C storage and ectomycorrhizal fungal sporocarp productivity suggest that CO_2 and O_3 effects may be transient, as evidenced by apparent convergence of community and ecosystem properties over time (Talhelm *et al*., 2009; Andrew & Lilleskov, 2009). In this study, we examined fungal community composition and the activity of extracellular enzymes involved in C and N acquisition in the forest floor and upper soil horizon of the two fastest growing plant communities in the FACTS-II FACE experiment (aspen and aspen–birch) over the course of the 10th growing season. We reasoned that: (1) altered substrate availability under CO_2 and/or O_3 will drive compositional changes in the fungal community; (2) microbial communities beneath plants exposed to elevated CO_2 should be characterized by higher extracellular enzyme activities than those beneath plants developing under ambient CO_2; O_3 should elicit the opposite effect; and (3) functional and compositional effects of the fumigation treatments will be seen in both the forest floor (Oe) and upper soil (Ap). We also examined the long-term effect of elevated CO_2 and O_3 on the activity of key C and N acquiring extracellular enzymes through time-series analysis derived from observations collected throughout this decade-long experiment.

Methods

Field site and experimental design

The FACTS-II FACE experiment (45°40.5′N, 89°37.5′E, 490 m elevation) consists of 12, 30 m diameter plots, which were planted in 1997 with trembling aspen (*Populus tremuloides* Michx.), paper birch (*Betula papyrifera* Marsh.), and sugar maple (*Acer saccharum* Marsh.). One half of each plot is planted with aspen genotypes of different CO_2 and O_3 sensitivity, one quarter of each plot is planted with aspen and sugar maple, and the remaining quarter with aspen and birch. All sections were planted at a density of 0.95 stems m^{-2}, for a total of 670 stems per 30 m diameter plot. We report here results for the aspen and aspen–birch communities.

Soils are Alfic Haplorthods with a sandy loam Ap horizon overlaying a sandy clay loam Bt horizon. Initial soil chemical and physical properties have been summarized by Dickson *et al*. (2000). Factorial CO_2 and O_3 treatments were arranged in a randomized complete block design with three replicates. Fumigation gases are delivered to 32 vertical vent pipes spaced uniformly around the perimeter of each 30 m plot. Fumigation occurs during the daylight hours for the duration of the growing season. In 2007, atmospheric CO_2 concentrations averaged 397.4 ± 14.7 μmol mol^{-1} in the ambient treatment and 484.0 ± 16.6 μmol mol^{-1} (mean ± SD) in the elevated CO_2 treatment. Ozone concentrations averaged 38.6 ± 7.33 nmol mol^{-1} in the ambient treatment, and 44.6 ± 7.36 nmol mol^{-1} in the elevated ozone treatment.

Forest floor and soil sampling

In May, July, and October 2007, we collected five Oe horizon and five Ap horizon (0–5 cm) samples from the central area of the aspen and aspen–birch communities in all 12 FACE rings. Oe horizon was collected from within five randomly placed

10 cm × 10 cm quadrats, and Ap horizon was sampled with a 2.5 cm diameter corer from directly beneath the Oe in the center of each quadrat. The five Oe samples in each FACE ring section were combined to form one composite sample; it was shredded with sterile DNase-free scissors and manually homogenized. The individual Ap samples from each FACE ring section were likewise combined and manually homogenized. Sampling resulted in 48 samples per time point, 144 samples in total. Samples were stored on ice until enzyme assays were conducted.

DNA extraction, polymerase chain reaction and cloning

A subsample was taken in the field from each homogenized Oe or Ap sample and placed into a preweighed PowerMax-Soil™ lysis tube (Mo Bio, Carlsbad, CA, USA). Lysis tubes were flash-frozen in liquid N_2 and stored on dry ice for overnight transport to the laboratory, where they were subsequently held at −80 °C before DNA extraction. Lysis tubes were weighed before DNA extraction to calculate Oe or Ap dry mass, after moisture content correction. DNA was extracted using the Mo Bio PowerMax™ Soil DNA kit, as per the manufacturer's instructions. DNA was eluted in a final volume of 3000 μL.

Ribosomal rDNA ITS 2 and approximately 600 bases of the large-subunit rDNA (28S) was selectively amplified from DNA extracts using the primers 58A2F (5′-ATC GAT GAA GAA CGC AG-3′, Martin & Rygiewicz, 2005) and LR3 (5′-CCG TGT TTC AAG ACG GG-3′, Hopple & Vilgalys, 1994). 58A2F is a general fungal primer and was designed to minimize co-amplification of nonfungal DNA, whereas LR3 amplifies eukaryotic DNA. Each PCR reaction contained 50–200 ng of DNA, 200 nM dNTPs, 1X PCR buffer, including 1.5 mM $MgCl_2$ (Roche, Alameda, CA, USA), 0.5 μM of each primer, and 50 μg of bovine serum albumin. After an initial denaturation step of 3 min at 94 °C, 35 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 90 s were followed by a final extension at 72 °C for 15 min. All PCR was conducted using Stratagene PCR cyclers (La Jolla, CA, USA); quality of PCR product was assessed by visualization of ethidium bromide-stained 1.5% agarose gels. Triplicate PCR products from each horizon of the three replicate stands in each ecosystem ($n = 3$) were combined and purified (UltraClean PCR Clean-up, Mo Bio) before cloning into pCR®2.1-TOPO® using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA). Forty-eight clones for each soil horizon in each of the aspen and aspen–birch sections of each FACE ring were selected to create a library of 24 × 96 clones. Clones were grown overnight in Luria broth supplemented with 10% glycerol, and sent to SeqWright (Houston, TX, USA) for bidirectional sequencing with M13F and M13R primers. Sequence quality was assessed by visual inspection, and full-length contiguous sequences for each clone were constructed in Geneious 3.7.0 (Biomatters Ltd., Auckland, New Zealand).

Definition of Operational Taxonomic Units (OTUs)

In this study, we used two complementary approaches to define operational taxonomic units. The ribosomal fragment that we amplified was typically 1000–1200 nucelotides in length, and includes a small fragment of the highly conserved rDNA 5.8S, the hypervariable internal transcribed spacer 2 (ITS2), and a 5′ fragment of the rDNA 28S, including variable regions D1 and D2. Initially, we created an alignment of all sequences using MAFFT (Katoh *et al*., 2002) within Geneious. The rDNA 5.8S, ITS2, and variable regions D1 and D2 were edited out of this alignment, as were gaps and ambiguous positions in the rDNA 28S. This reduced alignment was used to generate a similarity matrix in MEGA 4.0 (Tamura *et al*., 2007) which we used to define OTUs based on ⩾99% similarity using the furthest neighbor algorithm in DOTUR (Schloss & Handelsman, 2005). BLAST searches were then used to determine the likely taxonomic identity of these OTUs (Ascomycota, Basidiomycota, Eukaryota). Fungal OTUs were used in the creation of the phylogenies used for phylogenetic community analysis. Subsequently, for each of the OTUs that included more than one sequence, we repeated the alignment procedure using the full length of the amplified rDNA 18s–ITS2–rDNA 28S (including D1 and D2), and again used DOTUR but with a ⩾7% similarity cutoff. In many cases, rDNA 28S OTUs were split into two or more subgroups, and for clarity, we refer to these as genotypes. Genotypes were used to create the 'species' matrix input for canonical ordination. Nonparametric estimates of species richness (Chao I and ICE) were also based on the genotype matrix, and were determined with ESTIMATES (Colwell, 2009). Representative genotype sequences are archived in GenBank under accession numbers HQ432958–HQ433223.

Extracellular enzyme assays

We determined cellobiohydrolase (EC 3.2.1.91) and 1,4-β-*N*-acetylglucosaminisase (EC 3.2.1.14) activities in both Ap and Oe samples. Enzyme assays used methylumbelliferone-(MUB) linked substrates (Saiya-Cork *et al*., 2002) and were expressed as nanomole of the substrate released per hour per gram of dry soil. Assays used 1 g of Ap soil or 0.5 g of finely chopped Oe horizon. Assays were run in triplicate.

Statistical analyses

We used the UniFrac metric, which is a measure of phylogenetic dissimilarity (Lozupone & Knight, 2005) to cluster samples based on their OTUs. UniFrac was used to examine the influence of soil horizon, overstory plant composition, and the four treatments on the distribution of fungal evolutionary lineages. Jackknifing with 100 replicates was used to assess the stability of UniFrac clusters. To assess soil horizon and plant community effects, OTU data was pooled across treatments within each horizon and field block ($n = 12$). To assess CO_2 and O_3 treatment effects, OTUs were pooled across plant communities and across the entire site within each horizon ($n = 8$). Input phylogenies were created for Ascomycota and Basidiomycota separately, using a maximum likelihood (ML) approach (RAXML, Stamatakis *et al*., 2005). RAXML was implemented via the CIPRES portal (Miller *et al*., 2009) with a General Time Reversible model (GTR + γ) and model parameters estimated from the data. The influence of alternate topologies on UniFrac was assessed by repeating the analyses with an alternate phylogeny created using neighbor-joining (NJ) and the Kimura two-parameter model in MEGA 4.0.

UniFrac was implemented at http://128.138.212.43/UniFrac/index.psp.

We used canonical correspondence analysis (CCA; Ter Braak, 1987) to examine the magnitude and significance of plant community type, CO_2, and O_3 treatments on the distribution and abundance of fungal genotypes. Ascomycota and Basidiomycota were included together in the genotype matrix. Genotypes represented by a single sequence were not included in the analysis, and the remaining genotypes were weighted in accordance with their frequency, using the automatic 'downweight rare species' function in CANOCO 4.54 (Biometris, Wageningen, the Netherlands). The significance of the CCA was assessed via Monte Carlo permutation tests (Manly, 1991), with 500 permutations restricted to account for the split-plot nature of the experiment.

We used a mixed effects model in an analysis of variance (ANOVA) for a split-plot randomized complete block design. CO_2, O_3 and their interaction were considered as fixed whole-plot effects, and plant community, time, and horizon as fixed split-plot effects. Block and block interactions were considered random effects. ANOVA was performed using PROC MIXED in SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). All variables were log transformed to normalize variance after inspection of residual plots. We examined the temporal trends in cellobiohydrolase and 1,4-β-*N*-acetylglucosaminisase activity by combining this study with previous published (Larson *et al*., 2002; Chung *et al*., 2006) and unpublished datasets (2005 and 2009, D. R. Zak, unpublished results). Significance of CO_2, O_3 and their interaction were again determined as whole plot effects in ANOVA, with time and plant community as split-plot effects.

Results

CCA

One thousand nine hundred and seventy-five rDNA clones were successfully sequenced, and BLAST searches placed 1877 (95%) of these in the fungal subphyla Ascomycota and Basidiomycota. DOTUR and alignments divided the Ascomycota into 176 genotypes, and the Basidiomycota into 128 genotypes (Table 1). Genotypes were typically recovered from either the forest floor or the soil horizon; <10% were recovered from both. Ascomycota and Basidiomycota also showed contrasting distributional patterns, with Ascomycota density and diversity highest in the litter layer, and Basidiomycota density and diversity highest in the upper soil (Table 1). Overall, aspen and aspen–birch forest types showed similar levels of diversity, although we recovered more genotypes under ambient atmospheric conditions than either elevated CO_2 or elevated O_3 (Table 1).

The forest floor community was characterized by a combined total of 210 ascomycete and basidiomycete genotypes. No genotype was recovered from all samples; the most widely distributed ascomycete was recovered from 18 of the 24 ring sections, and the most widely distributed basidiomycete from 16. One hundred and thirteen genotypes were represented by a single sequence and a further 11 were recovered from a single ring section. As a result of this high degree of distributional heterogeneity, exploratory detrended correspondence analysis (DCA, not shown) indicated a relatively low degree of community similarity within the forest floor; the primary axis had a gradient length of 7.58 SD and accounted for 14.2% of the species data, whereas the secondary axis had a gradient length of 3.07 SD and accounted for a further 7.3%. The high variance of the primary axis was largely due to a single sample dominated by two ascomycete genotypes not found across the rest of the site. With this sample removed, the primary DCA axis was 4.15 SD in length and accounted for 12.0% of the variance, whereas the secondary axis was largely unchanged. Despite the high

Table 1 Observed and estimated numbers of fungal ribosomal (5.8S–ITS2–28S) genotypes recovered from soil and forest floor of young aspen and mixed aspen-birch forests developing under ambient and elevated (e) levels of CO_2 and O_3

	Ascomycota				Basidiomycota			
	Clones	Genotypes	Chao 1 (SD)	Simpson (SD)	Clones	Genotypes	Chao 1 (SD)	Simpson (SD)
Overall	833	176	376 (58)	39.4 (4.2)	994	128	280 (54)	22.4 (0.4)
Stratification								
Litter	676	143	356 (77)	31.1 (0.8)	337	69	122 (23)	7.5 (0.6)
Soil	157	51	109 (73)	19.4 (1.0)	657	75	348 (156)	16.5 (0.5)
Forest ecosystem								
Aspen	429	110	299 (52)	26.0 (1.0)	481	89	281 (83)	19.5 (0.8)
Birch	404	119	206 (32)	35.9 (2.0)	513	79	348 (156)	16.4 (0.5)
Atmospheric treatment								
Ambient	223	94	221 (49)	47.9 (2.8)	259	68	160 (44)	24.1 (1.2)
eCO_2	225	84	153 (28)	22.3 (2.4)	203	44	90 (28)	15.8 (1.7)
eO_3	193	69	107 (18)	23.5 (6.0)	256	51	74 (13)	14.9 (1.1)
$eCO_2 + eO_3$	192	61	88 (15)	32.7 (1.6)	276	51	89 (23)	16.2 (0.9)

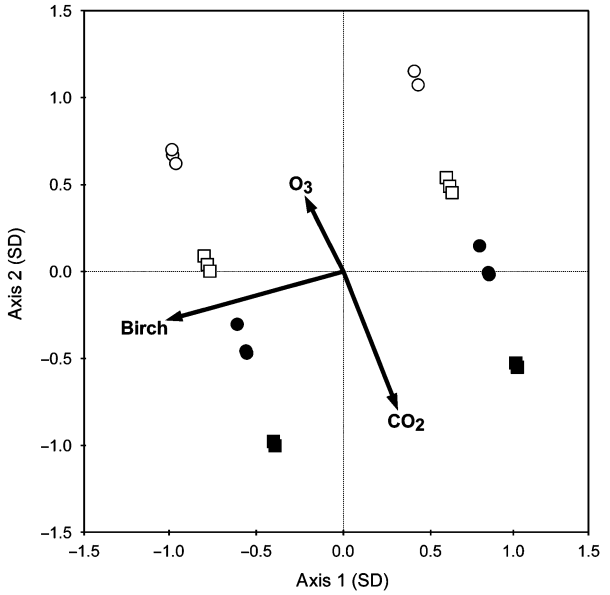

Fig. 1 Ordination diagram based on canonical correspondence analysis of similarity of forest floor fungal communities with respect to three environmental factors (proportion of birch in the overstory, atmospheric concentrations of CO_2 and O_3). Each sample is an individual FACE ring section; Ambient CO_2 and O_3, open squares; elevated CO_2 and ambient O_3, closed squares; Elevated O_3 and ambient CO_2, open circles; elevated CO_2 and O_3, closed circles. The first axis accounts for 8.3% of the variation in fungal community data and the second axis for a further 6.0%.

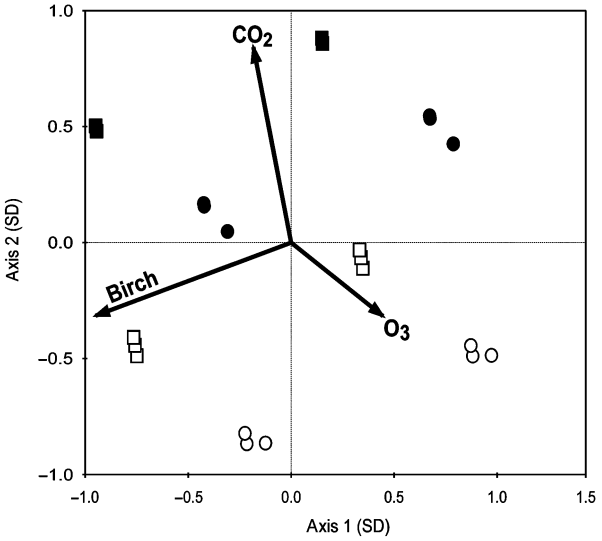

Fig. 2 Ordination diagram based on canonical correspondence analysis of similarity of forest floor fungal communities with respect to three environmental factors (proportion of birch in the overstory, atmospheric concentrations of CO_2 and O_3). Each sample is an individual FACE ring section; Ambient CO_2 and O_3, open squares; elevated CO_2 and ambient O_3, closed squares; Elevated O_3 and ambient CO_2, open circles; elevated CO_2 and O_3, closed circles. The first axis accounts for 5.5% of the variation in fungal community data and the second axis for a further 4.4%.

heterogeneity of the forest floor fungal community, CCA nevertheless indicated a small but significant correlation between fungal community composition, forest type, and levels of CO_2 and O_3 (Fig. 1). The primary canonical axis, which was strongly correlated with the proportion of birch in the community ($r^2 = 0.92$) accounted for 8.3% of the variance in species distributions and was highly significant ($P = 0.004$). The secondary CCA axis, which accounted for a further 6% of the species variance, was correlated with the concentration of CO_2 ($r^2 = 0.73$). Atmospheric O_3 was weakly correlated with both canonical axes, and orthogonal to CO_2 (Fig. 1). As a result of this, CCA suggested that in both the aspen and the aspen–birch ecosystems, the ambient CO_2 and O_3 communities and the elevated $CO_2 + O_3$ communities were the most similar in composition.

The soil fungal community was characterized by a combined total of 126 genotypes. As with the forest floor, no genotype was recovered from all samples; the most common ascomycete was recovered from 9, and the most common basidiomycete from 14 of the 24 ring sections. Nearly 63% of all soil genotypes were recovered from a single ring section. The primary axis of a DCA was 4.96 SD in length and captured 14.2% of the variance in species distributions; a second axis was 3.35 SD and captured a further 9.6% (not shown). As with the forest floor community, CCA indicated that the primary axis was largely driven by differences between the aspen and aspen–birch communities, and differences along the second axis by elevated CO_2 (Fig. 2). Unlike the forest floor analysis however, neither the primary nor the overall CCA was statistically significant (primary axis, $P = 0.73$; overall, $P = 0.69$).

Phylogenetic analyses

For the Ascomycota, the rDNA 28S data matrix contained 236 taxa (including 76 in-group reference taxa) aligned in 680 positions. Two hundred and sixty ambiguous or undersampled sites were excluded from the analysis, and of the remaining 420, 167 were conserved and 217 parsimony informative. Both ML and NJ phylogenies suggested that Pezizomycotina (*sensu* Spatafora *et al.*, 2006) dominated the ascomycete assemblages in both the soil and forest floor; only seven OTUs placed within Orbiliomycetes, Saccharomycotina, or Taphrinomycotina, and none of these was either abundant or widely distributed. Within Pezizomycotina, Sordariomycetes, Capnodiales, Dothidiales, and Pleosporales (Dothideomycetes) were well supported in both NJ and ML analyses (Supporting Information, Figs S2

and S3). Eurotiomycetes and Lecanoromycetes were poorly resolved in the NJ analysis, but well resolved and supported in ML. Pezizomycetes were split into three well-supported basal clades in both analyses. Altogether, these groups accounted for 65% of the Pezizomycotina OTUs. In both analyses, the remaining 35% of OTUs cluster together with Leotiomycete reference sequences, but the group as a whole was poorly resolved and weakly supported.

The Basidiomycota data matrix contained 273 taxa (including 165 in-group reference taxa) aligned in 684 positions. Two hundred and sixty-two ambiguous or under sampled sites were excluded, and of the remaining 422, 202 were conserved and 194 parsimony informative. Both NJ and ML approaches indicated that Agaricomycotina (*sensu* Hibbett, 2006) dominated the basidiomycete assemblage, representing 95% of all OTUs (Supporting Information, Figs S3 and S4). Within the Agaricomycotina, Tremellales and Filobasidiales (Tremellomycetes) were well supported, as were Auriculariales, Sebacinales, Trechisporales, Thelephorales, *Sistotrema* and Ceratobasidium (Cantharellales) and Boletales. Russulales were moderately well supported in ML, but not NJ, although both analyses supported Russulaceae and *Peniophora*. Corticiales were not well supported in either analysis; two environmental OTUs clustered with *Phanerochaete*, whereas another three clustered separately with *Hyphodontia* and *Phlebiopsis*. These 10 orders together accounted for 60% of the Agaricomycotina OTUs. The remaining 40% of Agaricomycotina OTUs placed within the Agaricales, although this order had low bootstrap support in NJ and was unresolved in ML. Within Agaricales, *Cortinarius* and *Inocybe* dominated, each accounting for 25% of the diversity of this order.

UniFrac distinguished forest floor and soil Pezizomycotina communities with strong Jackknife support (Fig. 3a). This divergence reflected the lower diversity of ascomycetes generally within the soil, as well as the preferential distribution of the Pezizomycetes, 94% of which were recovered solely from soil. Within the forest floor, all communities clustered together with high Jackknife support (Fig. 3a). There was some indication of a plant community effect, with aspen and aspen birch communities forming two distinct clusters; however, the nodes defining these had very low support (Fig. 3a). Soil ascomycete communities were more heterogeneous, but as with the forest floor, no clear differentiation of aspen and aspen–birch communities was observed (Fig. 3a). Tree topology (NJ or ML) had no effect on the analysis.

UNIFRAC also clearly distinguished between the forest floor and soil Agaricomycete communities, and both forest floor and soil communities formed strongly supported clusters (Fig. 3b). Within forest floor, the agaricomycete communities split into three subsets with moderate to high degrees of Jackknife support. However, there was no relationship between these and either plant community type or spatial location within the FACE site (Fig. 3b). Within the soil cluster, no internal nodes were well supported and there was no plant community effect (Fig. 3b).

UNIFRAC provided no evidence of a treatment gas effect on the forest floor Pezizomycotina communities (Fig. 4a). In contrast, UNIFRAC clustered the Agaricomycete communities developing under elevated CO_2 together with strong Jackknife support (Fig. 4b). Moreover, these communities and the community developing under ambient conditions cluster together and apart from the elevated O_3 community with moderate Jackknife support (Fig. 4b). In soil, UNIFRAC clustered the Pezizomycotina communities developing under ambient, elevated CO_2, and elevated O_3 conditions together with low to moderate Jackknife support (Fig. 4a). Agaricomycete soil communities cluster into ambient O_3 and elevated O_3 subgroups, but only with low to moderate support (Fig. 4b).

Extracellular enzyme activity in the 10th year

Cellobiohydrolase activity was significantly higher in the Oe than Ap horizon (mean Oe, 1432.4 nmol h^{-1} g^{-1}; mean Ap, 35.24 nmol h^{-1} g^{-1}, $P<0.0001$). In both horizons, and within both plant communities, cellobiohydrolase activity was highest in May and declined monotonically and significantly throughout the growing season (Table 2). Within the Oe horizon, cellobiohydrolase activity was 10% higher, and within the Ap horizon 26% higher, under mixed aspen–birch than under aspen, and these differences were statistically significant (Table 2). Elevated CO_2 had no significant effect on cellobiohydrolase activity in either Oe or Ap (Table 2). In contrast, elevated O_3 significantly reduced cellobiohydrolase activity ($\sim$ 17%) in the Oe horizon of both plant communities (Table 2).

NAG activity was significantly higher in Oe than Ap horizon (mean Oe, 1208.5 nmol h^{-1} g^{-1}; mean Ap, 89.2 nmol h^{-1} g^{-1}, $P<0.0001$). As with cellobiohydrolase, NAG activity in both horizons and plant communities also tended to decline over the course of the growing season, although the magnitude of this decline was only significant in the Oe horizon (Table 2). Plant community also significantly influenced NAG activity (Table 2), although contrasting results were obtained in the Oe and Ap horizons: in Oe, NAG activity was $\sim$10% higher under mixed aspen–birch than aspen; in Ap, NAG activity was $\sim$18% higher under aspen than mixed aspen–birch. Neither CO_2 nor O_3 had significant effects

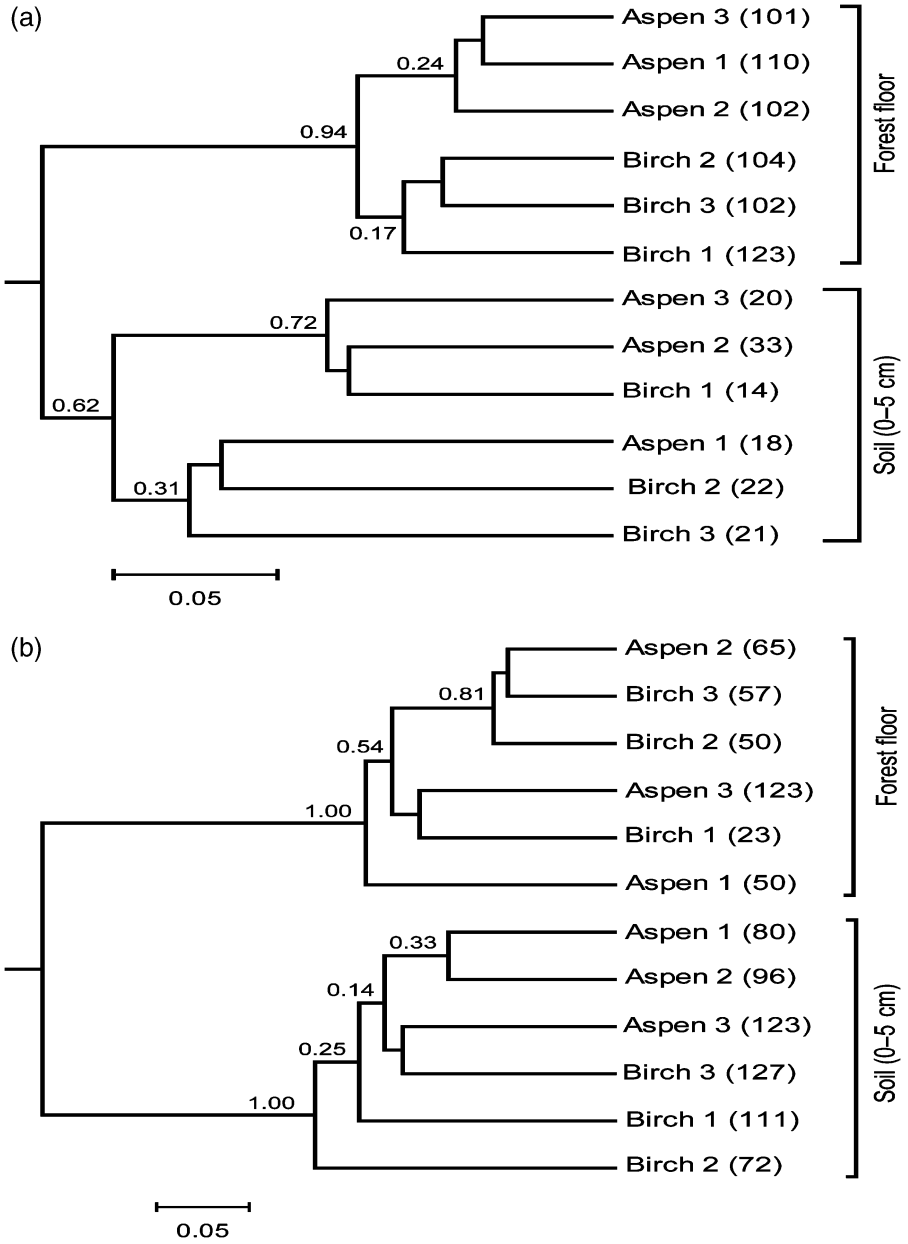

Fig. 3 UPGMA cluster analysis depicting the similarity of aspen and aspen–birch (birch) fungal communities in forest floor and soil horizons based on UniFrac analysis of rDNA 28S lineages. (a) Pezizomycotina; (b) Agaricomycetes. Nodal support is indicated as proportion of Jackknifes. Number of Operational Taxonomic Units per community is in parentheses.

on NAG activity in the Oe horizon (Table 2). In the Ap horizon, significant interactions were observed between CO_2, O_3 and plant community type (Table 2). Elevated CO_2 increased NAG activity in soil beneath mixed aspen–birch (mean ambient = 64.8 nmol h^{-1} g^{-1}; mean elevated CO_2 = 91.5 nmol h^{-1} g^{-1}). In contrast, elevated CO_2 decreased NAG activity under aspen (mean ambient = 103.3 nmol h^{-1} g^{-1}; mean elevated = 85.1 nmol h^{-1} g^{-1}). NAG activity under elevated O_3 exhibited a complex time interaction whereby under aspen, elevated O_3 lead to higher NAG activity in July and October, whereas under aspen–birch, elevated O_3 resulted in higher NAG activity in May and July.

Long-term temporal trends in cellobiohydrolase and NAG activity

There was no significant temporal trend in cellobiohydrolase activity (Fig. 5), and mean cellobiohydrolase activity over time was similar in both plant communities. Despite minor year-to-year variation, cellobiohy-

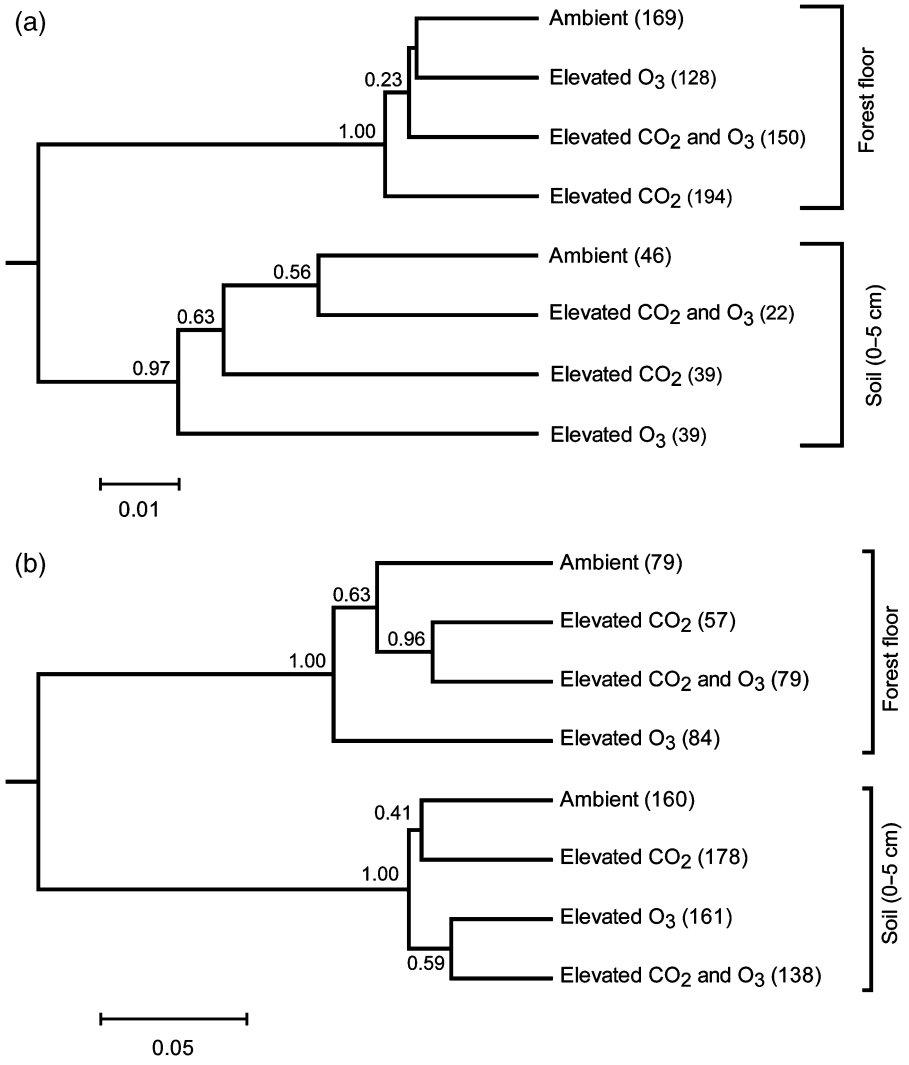

Fig. 4 UPGMA cluster analysis depicting the similarity of aspen and aspen–birch (birch) fungal communities in forest floor and soil horizons under ambient, elevated CO_2, elevated O_3, and elevated CO_2 and O_3, based on UNIFRAC analysis of rDNA 28S lineages. (a) Pezizomycotina; (b) Agaricomycetes. Nodal support is indicated as proportion of Jackknifes. Number of Operational Taxonomic Units per community is indicated in parentheses.

drolase activity in the Ap horizon under ambient CO_2 and O_3 was steady from 1999 to 2009, and averaged $19.8 \pm 4.67\,\text{nmol}\,h^{-1}\,g^{-1}$ (mean and SD). The long-term average under elevated O_3, $20.5 \pm 6.53\,\text{nmol}\,h^{-1}\,g^{-1}$, did not differ significantly from ambient O_3 ($P = 0.87$). Mean long-term cellobiohydrolase activity was significantly higher under elevated CO_2 (35.3 vs. $19.8\,\text{nmol}\,h^{-1}\,g^{-1}$; $P = 0.02$). Elevated CO_2 and O_3 exhibited a significant interaction, and average cellobiohydrolase activity under $CO_2 + O_3$ ($22.2 \pm 16.4\,\text{nmol}\,h^{-1}\,g^{-1}$) was not significantly different from ambient. There were no significant time interactions.

NAG activity in the Ap horizon tended to increase between 1999 and 2009 (Fig. 6), and this was significant ($P < 0.0001$). NAG activity was significantly higher under elevated CO_2 (85.5 vs. $103.1\,\text{nmol}\,h^{-1}\,g^{-1}$, $P = 0.04$), and was unaffected by O_3 ($P = 0.20$). NAG activity also showed a marginally significant $CO_2 \times$ Year interaction ($P = 0.09$); the magnitude of the difference between ambient and elevated CO_2 decreased over time, and in 2007 and 2009, activity was equivalent or slightly higher under ambient CO_2 than in the elevated CO_2 treatment (Fig. 6).

Discussion

We hypothesized that by affecting the quantity and quality of plant-derived organic substrates, exposure to elevated CO_2 or O_3 would indirectly affect the composition of the fungal community in these devel-

Table 2 Statistical significance of the effects of elevated CO_2, elevated O_3, plant community type (species) and time during the growing season on the activities of cellobiohydrolase and *N*-acetylglucosaminidase in soil in the 10th year of FACE

	Cellobio-hydrolase		*N*-acetylglucos-aminidase	
Parameter	Oe	Ap	Oe	Ap
CO_2	0.231	0.807	0.344	0.362
O_3	**0.050**	0.276	0.490	0.426
$CO_2 \times O_3$	0.499	0.188	0.546	0.684
Species	**0.050**	**0.008**	0.082	0.058
$CO_2 \times$ Species	0.392	0.819	0.832	**0.037**
$O_3 \times$ Species	0.117	0.713	0.943	0.730
$CO_2 \times O_3 \times$ Species	0.591	0.326	0.404	0.610
Time (month)	< **0.0001**	**0.004**	<**0.0001**	0.164
$CO_2 \times$ Time	0.877	0.678	0.360	0.816
$O_3 \times$ Time	0.244	0.306	0.122	0.855
$CO_2 \times O_3 \times$ Time	0.818	0.690	0.617	0.232
Species × Time	0.242	0.650	0.544	0.142
$CO_2 \times$ Species × Time	0.546	0.202	0.510	0.145
$O_3 \times$ Species × Time	0.295	0.862	0.317	**0.038**
$CO_2 \times O_3 \times$ Species × Time	0.820	0.830	0.378	0.211

P-values ≤ 0.05 are deemed significant and highlighted in bold.

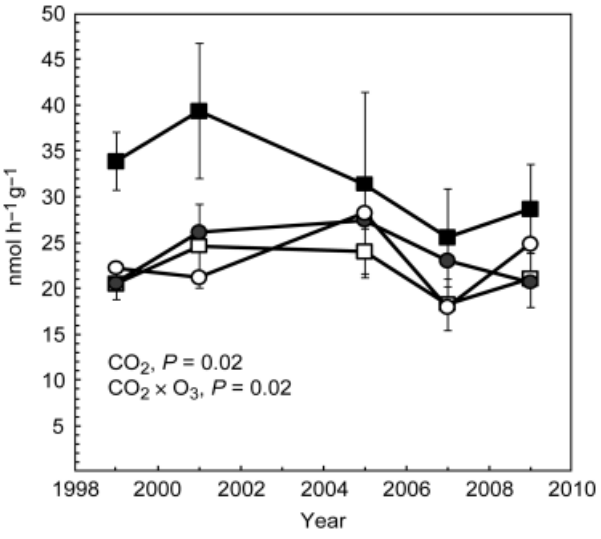

Fig. 5 Long-term response of cellobiohydrolase in the soil horizon to elevated CO_2 and O_3. Open squares, ambient CO_2 and O_3; closed squares, elevated CO_2; open circles, elevated O_3; closed circles elevated CO_2 and O_3. Data is pooled across seasons within years and across plant communities.

oping northern hardwood forests. To test this hypothesis, we used a combination of classic analytical ordination and phylogenetic analyses, and considered both the overall community of ascomycetes and basidiomycetes in forest floor and soil horizons, and each of these dominant subphyla separately. Our results indicate that although natural factors such as stratification and plant community type exert a dominant influence on the diversity, distribution, and abundance of fungal species and lineages, elevated CO_2 and O_3 also have the potential to alter fungal community composition and function. Moreover, our results suggest that elevated CO_2 and O_3 may act differently on saprotrophs and ectomycorrhizal fungi, and that the magnitude of the effect may change during forest development.

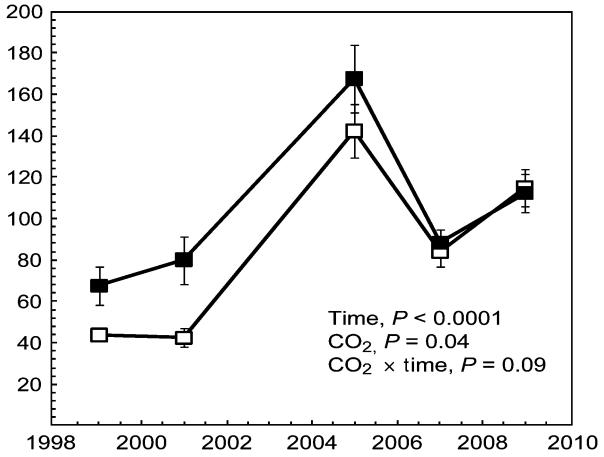

Fig. 6 Long-term response of *N*-acetylglucosaminidase in the soil horizon to elevated CO_2. Open squares, ambient CO_2; closed squares, elevated CO_2. Data is pooled across seasons within years and across plant communities.

Structure and function of the fungal communities

Our ribosomal gene library indicated a genetically and evolutionarily diverse fungal community. Ascomycota and Basidiomycota dominated this community, and within the two groups alone we recovered $\sim$300 genotypes representing species from at least 20 orders (Table 1). The true richness is likely even higher (Supporting Information, Fig. S1). Few genotypes were widely distributed, and none were recovered from all 24 ring sections. Similar levels of richness were recovered from both forest types, but nearly twice as any genotypes were recovered from the forest floor as from soil (Table 1). This difference is attributed to a greater richness of ascomycete genotypes in the forest floor than in soil, and moreover reflects ecological difference between forest floor and soil fungal communities (Lindahl *et al*., 2007). Within the forest floor, the ascomycete assemblage was dominated by Sordariomycetes, Dothideomycetes, Eurotiomycetes, and Leotiomycetes, lineages characterized by endophytic, parasitic, and saprotrophic ecologies (Spatafora *et al*., 2006). In contrast, although OTUs from these

lineages were also recovered from the soil, the ascomycete assemblage here was also notable for a diversity of Pezizomycetes, which may be mycorrhizal (Spatafora *et al*., 2006). A similar functional stratification was seen in the Basidiomycota, with OTUs from ectomycorrhizal lineages (e.g. *Cortinarius*, *Inocybe*, *Amanita*, Russulaceae, *Tomentella*) overwhelmingly recovered from soil, and saprotrophic lineages, (e.g. *Sistotrema*, *Clitocybe*, *Mycena*, *Collybia*) from forest floor. This presumed functional difference in primary mode of C acquisition and role in N cycling is evidenced by the two orders of magnitude difference in cellobiohydrolase activity between forest floor and soil, and by a much higher proportion of N-acquiring enzyme activity in the soil.

Plant community composition was the second most important driver of fungal community composition and a significant factor in community function. The influence of plant community type presumably reflects host- and substrate-specificity of some saprotrophic and mycorrhizal species (Lodge & Cantrell, 1995; Andrew & Lilleskov, 2009). Significant plant community effects were not observed in the phylogenetic analyses (Fig. 3), although there was a tendency for aspen and aspen–birch Pezizomycotina communities to cluster apart. Generally, the fungal communities of these two developing forests were indistinguishable in terms of evolutionary lineages. Plant effects were seen most clearly in the canonical ordinations, where they accounted for between 5% and 8% of the difference in genotype distribution and abundance (Figs 1 and 2), although the effect was only significant in the forest floor. Yet despite the minor effects on community composition, plant community had a strong effect on decomposition processes, with cellobiohydrolase activity significantly higher in both horizons under mixed aspen–birch and NAG showing a complex soil horizon $\times$ plant community interaction (Table 2). It remains uncertain if these functional differences result solely from the minor differences in fungal community composition, or if fungal biomass, or even different rates of functional gene expression also play a role. Nevertheless, it does suggest a degree of functional plasticity within the fungal community.

Exposure to elevated levels of atmospheric CO_2 or O_3 appears to have more subtle effects on the fungal community. Atmospheric gas effects had a small effect on the distribution and abundance of the most frequently encountered species, as evidenced by the ordinations (Figs 1 and 2). In the phylogenetic analyses, Pezizomycotina communities in the forest floor were unaffected by the treatments, whereas in the soil, Pezizomycotina communities exhibited a nested structure with no clear relationship to treatments (Fig. 4). In contrast, phylogenetic analysis of the Agaricomycete forest floor community strongly suggests that plant exposure to elevated CO_2 can lead to lineage sorting (Fig. 4). The main difference between the elevated and ambient CO_2 communities appears to be a greater abundance of *Sistotrema* in the former. Although this difference does not appear to affect cellobiohydrolase activity, it is possible that a change within the Agaricomycete community reflects an increase in the proportion of recalcitrant compounds within the forest floor (Lindroth *et al*., 2001). Further studies of functional gene expression and phylogenetics will be required to help determine the importance and effectiveness of these species during the later, lignin-dominated stages of litter decomposition.

Phylogenetic analysis also suggested that the agaricomycete community in the soil was affected by the treatments (Fig. 4). In soil, two distinct, weakly to moderately well-supported clusters were observed, and plant exposure to elevated O_3 appears to be the driver in this differentiation. The ectomycorrhizal communities developing under elevated O_3 were characterized by higher proportions of *Cortinarius* and *Inocybe* species, and lower proportions of *Laccaria* and *Tomentella*. This community change also may have functional consequences; although the timing of peak activity differed between aspen and aspen–birch communities, elevated O_3 was associated with higher levels of NAG activity (Table 2).

Functional responses to elevated CO_2 and O_3 in the root zone may change over time

The apparent effects of elevated atmospheric and tropospheric gases on microbial function may differ depending on which functions are considered, and moreover, on the time point at which the observations are made. Our results indicate that early responses to elevated CO_2 and O_3 may not be sustained over time as forest ecosystems continue to develop. For example, although Larson *et al*. (2002) and Chung *et al*. (2006) found evidence of reduced cellulolytic activity in the Ap under elevated O_3, our study found no evidence that O_3 suppresses cellulolytic activity, either in the 10th year of the experiment (Table 2), or indeed, as a whole when all temporal data are analyzed (Fig. 5). A similar response was initially observed with elevated CO_2; it strongly stimulated cellulolytic activity in the Ap horizon, this effect was offset by elevated O_3, and there was little evidence of significant differences between plant communities. While generally higher soil cellulolytic activity under elevated CO_2 is still evident after 10 years, the magnitude of the CO_2 stimulation appears to diminish over time (Fig. 5). Moreover, during the last two sampling periods (2007 and 2009), a CO_2 main

effect was obscured by plant community differences, wherein cellulolytic activity was higher under aspen–birch than aspen. This apparent trend towards a strengthening plant community-specific response over time is consistent with the fact that birch has become competitively dominant under elevated CO_2 (Zak *et al*., 2007).

The temporal conditionality of microbial functional response is seen even more clearly with NAG (Fig. 6). NAG breaks down chitin, the second most abundant polysaccharide in nature and an important source of organic N (Kang *et al*., 2005). Like cellobiohydrolase, NAG activity shows seasonal variability and annual fluctuations (Fig. 6). Unlike cellobiohydrolase, whose activity was relatively stable year-to-year, NAG activity tended to increase over time (Fig. 6). Moreover, like cellobiohydrolase, NAG activity was strongly stimulated by elevated CO_2, and slightly lower under elevated O_3, early in the FACTS-II experiment. By year 10 however, NAG response to elevated CO_2 differed between plant communities, being higher in aspen–birch and lower in aspen. Moreover, as noted above, elevated O_3 is now associated to higher levels of NAG activity. Higher levels of NAG activity may reflect increased mycorrhizal fungal activity due to greater host-plant demand for N; a recent ^{15}N tracer study has shown that while elevated CO_2 has generally increased total N acquisition over time, birch have become much more effective foragers of 'new' N than aspen (Zak *et al*., 2007). Higher levels of NAG activity may also indicate greater fungal biomass; however, neither soil microbial nor fungal biomass have displayed significant responses to CO_2 or O_3 (Larson *et al*., 2002; Chung *et al*., 2006). Even so, fine root biomass has clearly increased over time in all treatments (Pregitzer *et al*., 2008) and it is likely that mycorrhizal fungal biomass has therefore also increased. Moreover, the most recent root biomass data also show that the inhibitory effects of O_3 have decreased over time, presumably as a result of compensatory growth by O_3-tolerant aspen genotypes (Pregitzer *et al*., 2008). Interspecific differences in foraging efficiency, combined with a smaller difference in fine root biomass between elevated CO_2 and ambient treatments over time and compensatory aspen root growth under elevated O_3 may therefore underlie the altered effect of O_3 and the diminished and increasingly community-specific, long-term response of NAG to elevated CO_2.

Conclusions

In our decade-long experiment, stratification of the fungal community between forest floor and soil horizons and differing plant communities had a greater influence on fungal community composition and function than did elevated CO_2 and O_3. Nevertheless, our results demonstrate that plant exposure to elevated concentrations of tropospheric O_3 or CO_2 can lead to small, but persistent changes in fungal community function, and that these may be related to concomitant changes in community composition. Moreover, our results further suggest that O_3 and CO_2 may affect different parts of the fungal community, and that their functional effect may decline or even change entirely over time. Generally, if developing forests and their associated fungal communities respond under natural field conditions in a similar manner, the accumulation of CO_2 and O_3 in the Earth's atmosphere will likely affect saprotrophic and ectomycorrhizal species to differing degrees, with complex and largely unexplored long-term ecosystem consequences.

Acknowledgements

Our work was supported by Grants (DE-FG02-93ER61666 and DE-FG02-95ER62125) from the Office of Science (BER), US Department of Energy and the US Forest Service Global Change Research program. We also gratefully acknowledge Dave Karnosky, Kurt Pregitzer, George Hendry, John Nagy, Keith Lewin, and Judd Isebrands, whom were instrumental in establishing and maintaining the Rhinelander FACE facility, and Lauren Cline, Kirsten Hofmockel, and Rebecca Mau for unpublished enzyme data.

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Supporting Information

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

Figure S1. Species accumulation curves (•), and the calculated ICE (■) and Chao 1 (triangle) estimates of fungal diversity under aspen and aspen birch at FACE II. Top panel; Ascomycetes. Bottom panel; Basidiomycetes.

Figure S2. Neighbor Joining tree showing the phylogenetic relationship between Pezizomycotina operational taxonomic units and GenBank derived reference sequences. Bootstrap support >75% is indicated above nodes, and strongly supported branches (> = 90%) are indicated with bold type.

Figure S3. Maximum Likelihood Consensus tree of the Pezizomycotina from the FACE II site. Bootstrap support >65% is indicated above nodes, and strongly supported branches (> = 90%) are indicated with bold type.

Figure S4. Neighbor Joining tree showing the phylogenetic relationship between Basidiomycotina operational taxonomic units and GenBank derived reference sequences. Bootstrap support >75% is indicated above nodes, and strongly supported branches (> = 90%) are indicated with bold type.

Figure S5. Maximum Likelihood Consensus tree of the Basidiomycotina from the FACE II site. Bootstrap support >65% is indicated above nodes, and strongly supported branches (> = 90%) are indicated with bold type.

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