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Stimulation of microbial extracellular enzyme activities by elevated CO_2 depends on soil aggregate size

MAXIM DORODNIKOV*†‡, EVGENIA BLAGODATSKAYA*‡, SERGEY BLAGODATSKY*‡, SVEN MARHAN§, ANDREAS FANGMEIER† and YAKOV KUZYAKOV*

*Department of Agroecosystem Research, University of Bayreuth, Universitätstr, 30, 95440 Bayreuth, Germany, †Institute of Landscape and Plant Ecology (320), University of Hohenheim, August-v.-Hartmann-Str. 3, 70599 Stuttgart, Germany, ‡Institute of Physico-chemical and Biological Problems in Soil Science, RAS, Institutskaya 2, 142290 Puschino, Russia, §Institute of Soil Science and Land Evaluation (310), University of Hohenheim, Emil-Wolff-Str. 27, 70599 Stuttgart, Germany

Abstract

Increased belowground carbon (C) transfer by plant roots at elevated CO_2 may change properties of the microbial community in the rhizosphere. Previous investigations that focused on total soil organic C or total microbial C showed contrasting results: small increase, small decrease or no changes. We evaluated the effect of 5 years of elevated CO_2 (550 ppm) on four extracellular enzymes: β-glucosidase, chitinase, phosphatase, and sulfatase. We expected microorganisms to be differently localized in aggregates of various sizes and, therefore analyzed microbial biomass (C_{mic} by SIR) and enzyme activities in three aggregate-size classes: large macro- (>2 mm), small macro- (0.25–2 mm), and microaggregates (<0.25 mm). To estimate the potential enzyme production, we activated microorganisms by substrate (glucose and nutrients) amendment. Although C_{total} and C_{mic} as well as the activities of β-glucosidase, phosphatase, and sulfatase were unaffected in bulk soil and in aggregate-size classes by elevated CO_2, significant changes were observed in potential enzyme production after substrate amendment. After adding glucose, enzyme activities under elevated CO_2 were 1.2–1.9-fold higher than under ambient CO_2. This indicates the increased activity of microorganisms, which leads to accelerated C turnover in soil under elevated CO_2. Significantly higher chitinase activity in bulk soil and in large macroaggregates under elevated CO_2 revealed an increased contribution of fungi to turnover processes. At the same time, less chitinase activity in microaggregates underlined microaggregate stability and the difficulties for fungal hyphae penetrating them. We conclude that quantitative and qualitative changes of C input by plants into the soil at elevated CO_2 affect microbial community functioning, but not its total content. Future studies should therefore focus more on the changes of functions and activities, but less on the pools.

Keywords: elevated atmospheric CO_2, extracellular enzyme activity, macroaggregates, microaggregates, soil microbial biomass

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Introduction

During the past two centuries, the CO_2 concentration in the atmosphere has increased by 35%, mainly because of fossil fuel combustion and land-use changes (IPCC, 2007). Studies exploring ecosystem responses to elevated CO_2 have gained widespread attention in the last few decades (Pendall *et al*., 2004; van Kessel *et al*., 2006). Most investigations, however, have been focusing on plant biomass responses to elevated atmospheric CO_2, while belowground processes in soils have received less attention. The direct effects of increasing CO_2 on plants include (i) higher net primary productivity (Melillo *et al*., 1993; Schimel, 1995) through accelerated photosynthetic rates (Paterson *et al*., 1997), (ii) changes in plant chemistry such as higher C/N ratio, higher concentrations of starch, sugars, and carbohydrates

Correspondence: Maxim Dorodnikov, Department of Agroecosystem Research, University of Bayreuth, Universitätstr, 30, 95440 Bayreuth, Germany, tel. +499 215 52305, fax +499 215 52315, e-mail: maxim.dorodnikov@uni-bayreuth.de

(Cotrufo *et al*., 1994, 2005), (iii) reduced stomatal conductance of plants resulting in higher water use efficiency (Körner, 2000), and (iv) stimulated root growth (Paterson *et al*., 1997; Rogers *et al*., 1998), which increases rhizodepositions including root exudates (van Veen *et al*., 1991; Cheng, 1999; Phillips *et al*., 2006). In contrast to the response of plant biomass to elevated CO_2, the feedback of belowground components of terrestrial ecosystems, especially soil microorganisms, to atmospheric CO_2 enrichment occurs indirectly through plant-derived deposits; this process is less well-studied compared with the aboveground biomass response. For example, the reviews by Zak *et al*. (2000) and Freeman *et al*. (2004) showed that the response of soil microorganisms to elevated CO_2 is highly variable, regardless of whether total biomass, its activity or effects on the N-cycle were studied.

Microorganisms are the main source of crucial enzymes in the cycling of main nutrients (C, N, P, and S) in soil (Aon *et al*., 2001). Moreover, soil enzyme activities are highly sensitive and could serve as indicators of various changes in the plant–soil system: they integrate information on both the microbial status and the physico–chemical soil conditions (Aon *et al*., 2001). The effect of elevated atmospheric CO_2 on the activity of various soil microbial enzymes has been intensively studied over the last decade (Kandeler *et al*., 1998; Ebersberger *et al*., 2003; Henry *et al*., 2005). For example, it increased the activities of enzymes such as phosphatase and urease that promote P or N cycling (Ebersberger *et al*., 2003). The activity of cellulose-degrading enzymes also tended to increase under elevated CO_2 (Mayr *et al*., 1999), probably due to an increased turnover of fine roots. However, conflicting results have also been reported. Moorhead & Linkins (1997) found that cellulase activity in the surface organic soil horizons of tussock tundra decreased. No effect of elevated atmospheric CO_2 on the soil enzyme activities (phosphatase, α-, β-glucosidase, leucine aminopeptidase, phenol oxidase, peroxidase) was observed in a northern fen bulk soil (Kang *et al*., 2001) or forest soil under temperate trees (Larson *et al*., 2002).

Such inconsistencies can reflect various factors, one of which – the aggregate structure – apparently plays the key role because the accessibility of soil organic matter (SOM) for microbial decomposition can be decreased by occlusion in soil aggregates (Jastrow *et al*., 2007). Thus, microaggregates ($<250\,\mu m$ diameter) formed by primary particles coupled together by plant and microbial debris and by humic materials or polysaccharide polymers better protect SOM against decomposition than do macroaggregates ($>250\,\mu m$ diameter) (Denef *et al*., 2001; Bossuyt *et al*., 2002; Six & Jastrow, 2002). Elevated atmospheric CO_2 could potentially increase the soil aggregation due to increase of root growth (Paterson *et al*., 1997; Rogers *et al*., 1998), helping stabilize aggregates via enmeshment. On the other hand, the byproducts of microbial activity, such as extracellular polysaccharides of bacteria or glycoprotein glomalin of arbuscular mycorrhizal fungi, can form, with the surrounding mineral particles, an organo-mineral sheath around the cells (Chenu, 1993; Rillig *et al*., 1999a) leading to an increase in macroaggregates as an indirect additional effect. Although the turnover of C and N in soil aggregates has received considerable attention, this is not the case for the impact of soil structure on microbial enzyme activities. The indirect effect of elevated atmospheric CO_2 on microbial activity in soil aggregates is even more poorly understood. We expect that increased plant rhizodeposits under elevated CO_2 (van Veen *et al*., 1991; Cheng, 1999; Phillips *et al*., 2006) stimulate enzyme activities in macro- vs. microaggregates, because the microbial community associated with macroaggregates actively mineralizes C and N; especially fungi play an important role in forming and stabilizing macroaggregates (Guggenberger *et al*., 1999; Väisänen *et al*., 2005).

Microbial activity in soil is controlled by the availability of easily decomposable organic substrates (Friedel *et al*., 1996; Klose *et al*., 1999). The reported increase of the available substrates, such as root exudates, under elevated CO_2 (van Veen *et al*., 1991; Cheng, 1999; Phillips *et al*., 2006) could stimulate the microbial activity. However, the sampling of soil for subsequent measurements in a laboratory disables the permanent supply of soil microorganisms with easily decomposable substrate from plant rhizodeposits. This probably levels the possible differences in microbial activity between elevated and ambient CO_2 treatments. We, therefore, assume that 'activation' of the soil microbial community by adding an easily decomposable organic substrate could stimulate microbial growth and boost enzyme activities. Glucose addition to soil generally stimulates microbial growth as indicated by higher soil respiration, microbial biomass, and enzyme activities (Anderson & Domsch, 1973; Lin & Brookes, 2000; Dilly & Nannipieri, 2001). Furthermore, glucose is one of the abundant components of root exudates (Whipps & Lynch, 1983), that is why 'activation' of soil microbial community by addition of glucose provides conditions for microbial growth similar to rhizosphere soil. Because other studies using FACE approach showed increase of microbial activity under elevated CO_2 as shown by increased decomposition rates of litter (Cotrufo *et al*., 2005) or specific growth rates of microbes (Blagodatsky *et al*., 2006), we also expect that glucose application will more strongly affect enzyme activities under elevated vs. ambient CO_2 treatment.

This study applies a highly sensitive method, using a microplate system combined with 4-methylumbelliferone (MUF)-labelled fluorogenic substrates, to measure enzyme activities (Freeman *et al*., 1995; Pritsch *et al*., 2004). We estimated the enzymatic activities representing the main steps of soil biogeochemical nutrient cycles, i.e. C (β-1.4-glucosidase, *N*-acetyl-β-D-glucosaminidase), N (*N*-acetyl-β-D-glucosaminidase), P (phosphatase), and S (sulfatase). These enzyme activities were measured in bulk soil and in three aggregate-size classes (large macro-, small macro-, and microaggregates) under an agricultural ecosystem with spring wheat exposed for 4 years, and oilseed rape exposed for 1 year to elevated CO_2.

Materials and methods

Study site

Soil samples were taken from the free air carbon dioxide enrichment (FACE) facility located in Stuttgart-Hohenheim, Baden-Wuerttemberg, Germany (48°43′N latitude, 9°13′E longitude). The soil is a Gleyic Cambisol (WRB, 1998) without $CaCO_3$. Mean annual temperature is 8.7 °C and average rainfall 680 mm yr^{-1} (mean 1961–1990, meteorological station Stuttgart-Hohenheim). Properties of the soil under ambient and elevated CO_2 treatments were identical: 9% sand, 69% silt, 22% clay; pH 6.8; bulk density (0–10 cm) 1.1 g cm^{-3}; C_{org} 1.59%; N_{tot} 0.17%; C/N ratio 9.1.

The FACE experiment, starting in 2002, included plots with elevated atmospheric CO_2 level (540 ppm, with enclosures), ambient plots (380 ppm, with enclosures), and control plots (ambient CO_2 level, no enclosures) (Erbs & Fangmeier, 2006). Each treatment was replicated five times. Spring wheat (*Triticum aestivum* cv Triso) was annually planted on the plots from 2002 to 2006. In 2007, oilseed rape (*Brassica napus*) was grown on the plots for the first time. Soil was tilled in spring before crop sowing. Beginning in 2003, inorganic NPK fertilizers were applied in equal amounts of 140 kg N ha^{-1}, 60 kg K ha^{-1}, 30 kg P ha^{-1} to each plot under ambient and elevated CO_2 treatments. No organic fertilizers were applied.

Aggregate-size fractionation

Soil was sampled from the top 10 cm from each ambient and elevated CO_2 plot using soil corers (inner diameters: 5 cm) in September 2007, 3 weeks after rape harvest. Soil samples were stored at 7 °C for 1 week before aggregate-size fractionation. Aggregates were isolated according to Kristiansen *et al*. (2006). Soil samples were placed into a ventilation box (room temperature 22 °C) and spread out into a thin layer. The soil was dried to optimal moisture that would allow limited mechanical stress to induce maximum brittle failure along natural planes of weakness. When individual soil clods had reached the desired condition, these were gently manually crumbled to <8 mm. The resulting aggregates were size separated by a brief sieving procedure. Portions of 300 g were transferred to a nest of sieves (2 and 0.25 mm) and shaken three times for 2 min. All visible roots and stones were removed and the aggregates >2 mm were collected (large macroaggregates class). The same procedure was carried out for the material retained on the 0.25 mm sieve, isolating aggregate-size class of 0.25–2 mm (small macroaggregates class). The remaining material passed through the 0.25 mm sieve was identified as aggregate class <0.25 mm (microaggregates class). Preliminary tests showed that the sieving duration was sufficient to quantitatively separate the various aggregate size-classes while minimizing aggregate abrasion during the sieving.

Soil microbial biomass

Subsamples of bulk soil and the three soil aggregate classes were moistened with deionized water to 60% of water holding capacity. This value corresponded to an optimal level for microbial respiration activity determined in preliminary experiments. Soil samples were preincubated for 24 h at room temperature before measurements. Soil microbial biomass (C_{mic}) was measured in bulk soil and isolated soil aggregates using the initial rate of substrate-induced respiration (SIR) (Anderson & Domsch, 1978). Twelve grams of soil (equal to 10 g of dry soil) were amended with glucose (10 mg g^{-1}). After glucose addition, the soil samples were immediately placed into the respiration apparatus, which consisted of 24 plastic tubes, to measure the rate of CO_2 production. Each sample was continuously aerated (100 mL min^{-1}) at 21 °C, and the evolved CO_2 was measured using an infrared detector (Gas Exchange Measurement System 2250, ADC, Great Amwell, UK) connected to the Gas Handling Unit with a flowmeter (ADC). C_{mic} was calculated by mean value of CO_2 evolution rate during first 6 h after glucose amendment using the equation of Anderson & Domsch (1978) and conversion factor 30 suggested by Kaiser *et al*. (1992):

$$C_{mic}\left(\mu g\, g\, soil^{-1}\right) = \left(\mu L\, CO_2\, g\, soil^{-1}\, h^{-1}\right)30. \quad (1)$$

Soil 'activation' and enzyme assays

To detect the actual (basal) and potential ('activated') enzyme activities under ambient and elevated CO_2

treatments the enzymes activities were measured in bulk soil and isolated soil aggregates before and after addition of glucose and nutrients. For 'activation', soil was amended with a powder-mixture containing glucose (4 mg C g^{-1}), talcum (20 mg g^{-1}), and mineral salts: $(NH_4)_2SO_4$ – 1.9 mg g^{-1}, K_2HPO_4 – 2.25 mg g^{-1}, and $MgSO_4 \cdot 7H_2O$ – 3.8 mg g^{-1}. These amounts were sufficient for unlimited microbial growth and were established in another study (Blagodatsky *et al*., 2000). After substrate addition, the soil samples were left for 48 h to induce the microbial growth. The technique of enzyme measurements in substrate-amended and nonamended bulk soil and aggregates was identical.

Extracellular enzyme activities in bulk soil and isolated soil aggregates were measured using fluorogenically labelled substrates according to a modified technique described in Sowerby *et al*. (2005) and Pritsch *et al*. (2004, 2005). Four fluorogenic enzyme substrates based on MUF were used: MUF-β-D-glucopyranoside (MUF-G; EC 3.2.1.21, for the detection of β-glucosidase), MUF-*N*-acetyl-β-D-glucosaminide dihydrate (MUF-NAG; EC 3.2.1.14) for chitinase, MUF-phosphate disodium salt (MUF-P; EC 3.1.3.2) for phosphatase, and MUF-sulfate potassium salt (MUF-S; EC 3.1.6) for sulfatase activity. To dissolve the MUF-substrates, 2 mL of 2-methoxyethanol (Hoppe, 1983) was used. Predissolved MUF-substrates were further diluted with sterile distilled water to give the desired concentrations. All chemicals were purchased from Fluka (Germany).

The soil samples (1 g) were suspended in water (20 mL) and shaken on an overhead shaker for 15 min at room temperature and at maximum speed to ensure thorough mixing. A subsample of the soil suspension (1.0 mL) was added to the 3 mL MUF-substrate solution (containing either 400 μmol MUF-G, or 200 μmol MUF-NAG, MUF-P, or MUF-S), already pipetted in deep-well plates (24-wells × 10 mL, HJ-Bioanalytik GmbH, Mőnchengladtbach, Germany), and incubated at 11 °C for 1 h. The temperature of incubation was chosen based on average annual soil temperature of the experimental site (S. Marhan, personal communication). The calibration solutions were prepared using soil suspension (1 mL) and MUF of different concentrations (0–100 μmol, 3 mL). Deep-well plates with the soil-MUF-substrates and soil-calibration-MUF concentrations were centrifuged (20*g*, 5 min). Thereafter, 0.5 mL of supernatant was pipetted to the 24-well microplates (Becton Dickinson, Franklin Lakes, NJ, USA) containing 1.25 mL sterile distilled H_2O and 0.25 mL of 20 mmol glycine–NaOH buffer solution (pH 11) to stop enzyme reactions. Fluorescence was measured in microplates at an excitation wavelength of 355 nm and an emission wavelength of 460 nm, slit width of 25 nm, with a Victor^3 1420–050 Multilabel Counter (PerkinElmer, Waltham, MA, USA). Calibration curves were included in every series of enzyme measurements. Enzyme activities were expressed as MUF release in micromolar per gram bulk soil/aggregates dry weight and hour (μmol g^{-1} h^{-1}).

Calculation of recoveries and statistical analysis

The study was conducted with samples of eight plots (eight independent replicates corresponded to four ambient CO_2 plots and four of elevated CO_2 plots). Microbial biomass C and enzyme activity (β-glucosidase, chitinase, phosphatase, and sulfatase) were calculated on an oven-dry weight (105 °C) basis. The total C_{mic} content and enzyme activities calculated based on the weight distributions of aggregate-size classes represented the recovery, when compared with the respective values of bulk soil.

The significance of differences between C_{mic} and enzyme activities in bulk soil and the three aggregate-size classes under ambient and elevated CO_2 treatments was examined using Two-way ANOVA with two independent factors 'CO_2' and 'aggregates'. The Fischer LSD *post hoc* test was used to separate treatments at the $P < 0.05$ significance level.

Results

Aggregate distribution and C content

The weight distribution among the aggregate-size classes of the bulk soil was as follows: small macroaggregates (0.25–2 mm) contributed 58–59%, large macroaggregates (>2 mm) 31–34%, and microaggregates (<0.25 mm) 8–10% of the weight of bulk soil (Table 1). The atmospheric CO_2 enrichment did not affect the distribution of aggregates in the different size classes (Table 1). C_{org} content in each aggregate class was similar to the C_{org} of the bulk soil under both CO_2 treatments (Table 1).

Microbial biomass distribution

The microbial biomass content in aggregates (C_{mic}) increased as follows: large macroaggregates < small macroaggregates < microaggregates. Thus, the smallest class (microaggregates), amounting to about 10% of the total weight of a soil sample showed the highest microbial biomass; content C_{mic} (mg C g^{-1} aggregates) was 0.566 ± 0.031 under ambient CO_2 and 0.585 ± 0.004 under elevated CO_2 treatment. The smallest content of C_{mic} 0.340 ± 0.025 and 0.353 ± 0.023 was found in large macroaggregates under ambient and elevated CO_2, respectively. The portion of C_{mic} in the C content of

Table 1 Aggregate-size distribution, their C (C_{tot}) and microbial biomass content (C_{mic}) in bulk soil and soil aggregates in ambient (amb) and elevated (elev) CO_2 concentrations

Aggregate-size classes/soil (mm)	Weights distribution, %		C content, mg g^{-1} soil (aggregates)		Microbial biomass mg C g^{-1} soil (aggregates)		C_{mic}/C_{tot}		Recovery, %	
	amb	elev	amb	elev	amb	elev	amb	elev	amb	elev
>2	34^{Ab}	31^{Ab}	14.89 ± 0.42^{Aab}	14.75 ± 0.30^{Aa}	0.340 ± 0.025^{Aa}	0.353 ± 0.023^{Aa}	0.023	0.024	26.7	25.0
0.25–2	58^{Ac}	59^{Ac}	15.43 ± 0.49^{Aab}	16.25 ± 0.20^{Ab}	0.453 ± 0.016^{Ab}	0.460 ± 0.024^{Ab}	0.029	0.028	60.6	61.8
<0.25	8^{Aa}	10^{Aa}	15.14 ± 0.19^{Aab}	15.38 ± 0.24^{Aab}	0.566 ± 0.031^{Ac}	0.585 ± 0.004^{Ac}	0.038	0.038	10.5	13.4
bulk	100	100	15.57 ± 0.15^{Aab}	16.16 ± 0.48^{Ab}	0.434 ± 0.026^{Ab}	0.438 ± 0.013^{Ab}	0.028	0.027	97.8	100.2

The recovery of microbial biomass-C in soil aggregates was calculated based on the weights distribution of aggregates in bulk soil. Values are averages of four field replicates. Treatments followed by the same letters are not significantly different between elevated and ambient CO_2 (uppercase letters) and aggregate size classes (lowercase letters) at $P \leq 0.05$ (according to two-way-ANOVA and Fischer's LSD test).

aggregates increased as aggregate size decreased (Table 1). Elevated CO_2 tended to increase C_{mic} both in bulk soil and in the three aggregate-size classes, but this increase was not significant. Cumulative recovery of C_{mic} in aggregates was nearly 100% of the bulk soil under ambient and elevated CO_2 (Table 1).

Extracellular enzyme activities

Enzyme activities without substrate amendment. The extracellular enzyme activities were differently distributed through aggregate-size classes as well as in the bulk soil under both CO_2 treatments (Fig. 1). Bulk soil activities (864.6 ± 37.6 and $869.6 \pm 30.6\,\mu mol^{-1}\,g^{-1}\,soil\,h^{-1}$) were the highest for β-glucosidase under ambient and elevated CO_2 treatments, respectively. The lowest activities in bulk soil under both treatments corresponded to sulfatase and amounted to 4.4 ± 0.7 and $4.3 \pm 0.7\,\mu mol^{-1}\,g^{-1}\,soil\,h^{-1}$, respectively (Fig. 1). Elevated CO_2 did not significantly change the activities of β-glucosidase, phosphatase, and sulfatase in bulk soil, but did significantly increase chitinase activity there.

Activities of extracellular enzymes were lower in microcompared with macroaggregates, except β-glucosidase, whose activity was the highest in microaggregates under ambient CO_2 treatment (Fig. 1). CO_2 enrichment decreased the enzyme activities in microaggregates. Significantly lower activities in microaggregates under elevated vs. ambient CO_2 were found for β-glucosidase and chitinase. In contrast to microaggregates, CO_2 enrichment tended to increase activities in macroaggregates, although significantly higher values were detected only for chitinase in large macroaggregates (Fig. 1).

Enzyme activities after substrate amendment. Adding glucose significantly increased the activity of all studied enzymes in bulk soil and in the three aggregate-size classes. Moreover, the response of enzymes was always considerably higher under elevated CO_2 (Figs 1 and 2). Thus, after amendment of bulk soil under ambient CO_2, the activity of β-glucosidase, chitinase, phosphatase, and sulfatase increased 1.7, 2.4, 1.8, and 6.0 times, respectively. Simultaneously, the respective increase in bulk soil under elevated CO_2 treatment was 3.2, 3.3, 2.3, and 7.3 times (Figs 1 and 2). A similar trend of greater enzyme response to glucose addition was observed in soil aggregates under elevated compared with ambient CO_2. Furthermore, microaggregates responded more than macroaggregates under both treatments (Fig. 1).

Recovery of enzyme activities in aggregate-size classes. The total enzyme activities in isolated aggregates were recalculated for bulk soil based on aggregate weight distributions (Fig. 2). The contribution of activities in aggregates to overall activity of bulk soil coincided with the weight distribution of aggregate-size classes: the highest activity of the four enzymes occurred in 0.25–2 mm (the heaviest class by weight), the lowest in <0.25 mm (lightest class). Total activities in each aggregate class without glucose amendment were similar under both treatments. However, adding glucose and nutrients to bulk soil and soil aggregates significantly increased total activities of four enzymes, and the increase was especially pronounced under elevated CO_2 treatment (Fig. 2). The sieving approach we used was chosen as less destructive as compared with other conventional sieving approaches. This was proved by the nearly 100% recoveries for microbial biomass in isolated aggregates (Table 1). The variation of recoveries of enzyme activities in soil aggregates was somewhat larger and varied from 80% to 130% (Fig. 2).

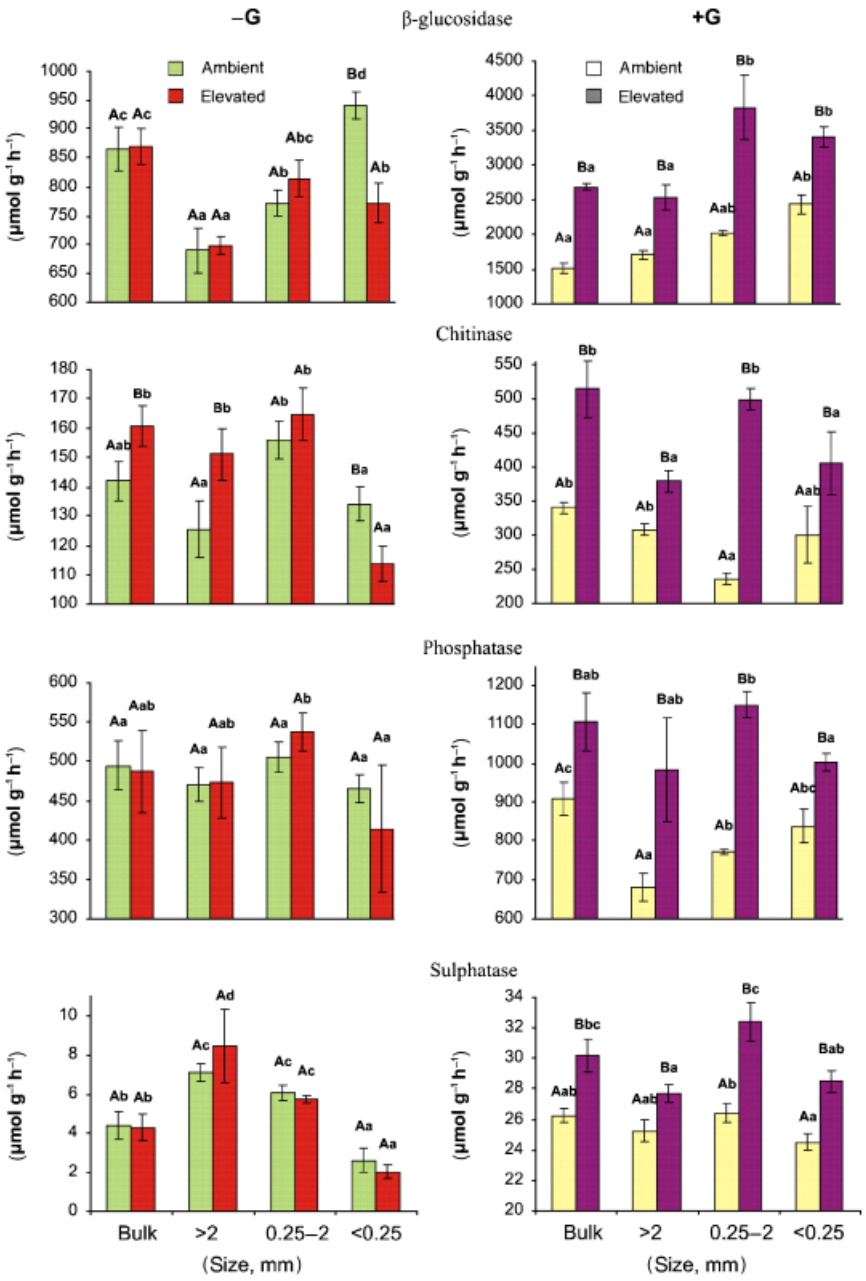

Fig. 1 Activities of *β*-glucosidase, chitinase, phosphatase, and sulfatase in bulk soil and three aggregate-size classes under ambient and elevated CO_2 treatments before (−G) and after (+ G) activation with glucose and nutrients. Values are averages of four replicates (± SE). Treatments followed by the same letters are not significantly different between elevated and ambient CO_2 (uppercase letters) and aggregate size classes (lowercase letters) at $P \leq 0.05$.

Discussion

Aggregate-size fractionation

To study the relationship between soil aggregates and SOM turnover, the bulk soil has to be fractionated into its constituent aggregates using some disruptive technique. The aggregate-size fractionation procedure used in the current study was chosen because it is gentler than conventional wet and dry sieving techniques (Kristiansen *et al*., 2006). Thus, wet-sieving procedure releases water soluble OM and dispersible colloids, causing changes in aggregate composition (Emerson & Greenland, 1990; Watts *et al*., 1996). Furthermore, complete soil watering could change the activity of soil microorganisms through anaerobic conditions (Kieft

et al., 1987; Zhang & Zak, 1998). In turn, prolonged sieving of air-dry soil tends to increase aggregate abrasion rather than fragmentation due to the great tensile strength of dry aggregates (e.g. Munkholm & Kay, 2002). The same as Kristiansen *et al*. (2006), we fragmented the soil into constituent aggregates when a water content of individual clods and peds was near the lower plastic limit, corresponding to the optimum water content of medium textured soils at which soil friability is maximal (Dexter & Bird, 2001). Thereby, we maximized brittle fracturing along natural planes of weakness while minimizing changes in aggregate-size distribution (Kristiansen *et al*., 2006). However, in contrast to Kristiansen and colleagues, we did not isolate silt and clay fraction (<63 μm) because the procedure would not allow to separate such a fraction with moisture optimal for microbial activity. Because the approach used is considerably distinguished from conventional wet- and dry sievings, we propose to determine such aggregate-size fractionation approach as optimal moist sieving.

The aggregate weight distribution detected here – microaggregates <large macroaggregates <small macroaggregates – coincided with fractionation results obtained for other agricultural soils (Fansler *et al*., 2005; Kristiansen *et al*., 2006). The small macroaggregate class (0.25–2 mm) was found to dominate in arable silty soils mostly due to the effect of tillage (Väisänen *et al*., 2005).

Five years of elevated CO_2 did not affect the distribution of the aggregate-size classes in our study. The results of other studies showed both increase (Rillig *et al*., 1999b; van Groenigen *et al*., 2002) and decrease (Del Galdo *et al*., 2006; Niklaus *et al*., 2007) of elevated CO_2 on soil aggregation. The increase of soil aggregation under elevated CO_2 was explained by either the enmeshment with enlarged roots (van Groenigen *et al*., 2002) and/or by increased mycorrhizal production of glomalin, which promotes soil aggregation (Rillig *et al*., 1999a, b). The decrease of soil aggregation was assumed to be due to decrease of total C content because of enhanced microbial activity ((Del Galdo *et al*., 2006) or due to slowed-down soil drying cycles because of increased water use efficiency by plants under elevated CO_2 (Niklaus *et al*., 2007). In contrast to referred studies, the lack of the difference in soil aggregation between ambient and elevated CO_2 in our study is explained by the tillage of experimental plots. Tillage had drastically higher direct effect on aggregate-size formation than the possible indirect effects of elevated atmospheric CO_2.

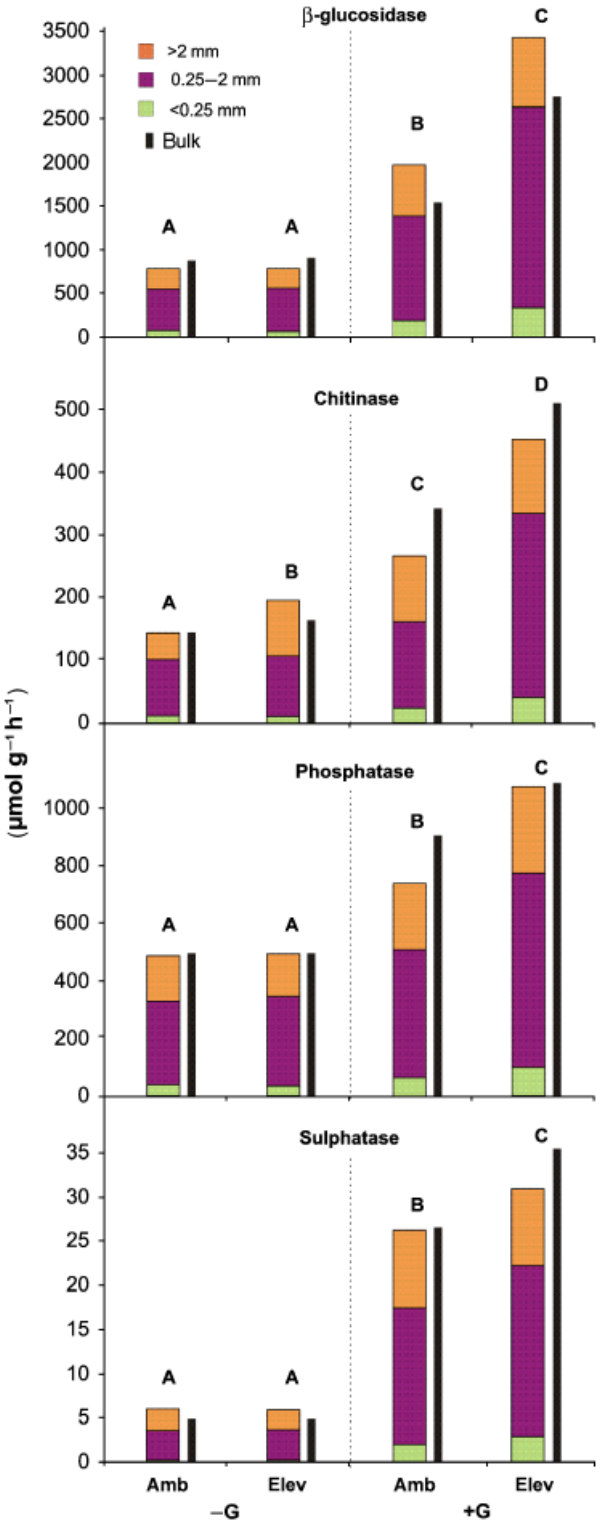

Fig. 2 Cumulated enzyme activities in three aggregate-size classes as compared with enzyme activities of bulk soil under ambient and elevated CO_2. The results of actual (−G, without glucose and nutrients addition) and potential enzyme activities (+ G, after glucose and nutrients addition) are presented. Treatments followed by the same letters are not significantly different between elevated and ambient CO_2 (uppercase letters) at $P \leq 0.05$.

Microbial biomass in soil aggregates under ambient and elevated CO_2

Higher microbial biomass in micro- compared with macroaggregates was found in our study when optimal moist sieving approach was used to separate different aggregate size classes. Some researchers have reported that microbial biomass is greater in macro vs. microaggregates (Chotte *et al*., 1998; Guggenberger *et al*., 1999) but other researchers have reported the opposite (Šantrůčková *et al*., 1993; Schutter & Dick, 2002; Askin & Kizilkaya, 2006). These contrasting results could reflect the differences in aggregate separation by wet and dry sieving. Fractionation is clearly a destructive procedure and some microorganisms are transferred from the surfaces of larger to smaller aggregates (dry sieving) or adhere to aggregates during slaking in water (wet sieving), persisting in macroaggregates (Ashman *et al*., 2003). The optimal moist sieving approach is less destructive (Kristiansen *et al*., 2006), thus it maintained the microbial biomass: cumulative recovery of C_{mic} in aggregates vs. bulk soil was 97.8% under ambient CO_2 and 100.2% under elevated CO_2 (Table 1). Such a high recovery of microbial biomass in separated soil aggregates indicates the appropriateness of the method for soil microbiological studies.

Higher microbial biomass in micro- compared with macroaggregates could be explained by the contrasting environment of differently sized aggregates. The location of microorganisms within the soil matrix is a key factor for their survival (Chenu *et al*., 2001). Small pores dominate within microaggregates, whereas both small and large pores occur in macroaggregates (Jastrow *et al*., 2007). Small pores, however, enhance survival by protecting microorganisms from predation by Protozoa (Vargas & Hattori, 1986; Postma & van Veen, 1990; Wright *et al*., 1993) or from desiccation (Nishiyama *et al*., 1992). Furthermore, the texture (sand–silt–clay content) differs in microaggregates vs. macroaggregates (Tisdall & Oads, 1982; Bronick & Lal, 2005). Clay particles can also directly affect microbial survival in soils (van Gestel *et al*., 1996), because adsorption of microbial cells to the surfaces of clay particles is crucial for microbial biomass accumulation in soils (Amato & Ladd, 1992).

Elevated CO_2 has either increased soil microbial biomass mostly due to greater root growth and C availability (e.g., Zak *et al*., 1993; Mikan *et al*., 2000; Drissner *et al*., 2007) or had no effect on soil microbial biomass because of decreased quality of plant rhizodeposits and N-limitation of microbial growth (e.g., Kampichler *et al*., 1998; Niklaus *et al*., 2003; Blagodatsky *et al*., 2006). Our results support those numerous studies that also failed to find a significant response of microbial biomass to elevated CO_2. In this study, the lack of the response of microbial biomass to elevated CO_2 can not be explained by N-limitation, because the N fertilizers were applied in equal amounts to each plot under ambient and elevated CO_2 treatments (Erbs & Fangmeier, 2006). Probably, in spite of higher plant biomass under elevated CO_2 reported for the same FACE experiment (Marhan *et al*., 2008) the harvest of spring wheat induced lower plant residue inputs to the soil, thus leveling the indirect effect of elevated atmospheric CO_2 on the increase of microbial biomass. Moreover, we observed no changes in microbial biomass under elevated vs. ambient CO_2 in isolated soil aggregates. This indicates that the effect of atmospheric CO_2 enrichment on allocation of microorganisms in soil macro- and microaggregates is negligible, at least when agroecosystems with high levels of manipulation are considered. We, therefore, conclude that total microbial biomass is an insensitive parameter to evaluate small changes caused by elevated CO_2.

Extracellular enzyme activities in bulk soil and isolated aggregates under ambient and elevated CO_2

Enzyme activities in bulk soil without substrate amendment. As reviewed by Freeman *et al*. (2004), many experiments found no difference in soil enzyme activities. In our experiment, *N*-acetylglucosaminidase (chitinase) was the only enzyme that showed higher activity in bulk, nonamended soil under elevated vs. ambient CO_2 (Fig. 1). The results of another research group working on the same experimental site (FACE–Hohenheim) support our findings. They measured the activities of α- and β-glucosidase, *N*-acetyl-glucosaminidase, xylosidase, cellobiosidase, phosphatase, L-leucin-, and L-tyrosin-peptidase in bulk soil sampled in 2002–2005 from plots under both treatments. All showed no significant treatment-related differences, except a trend to higher chitinase activity under elevated CO_2 (S. Marhan, personal communication), which we found to be significant in our measurements. The increased chitinase activity in soil mostly reflects higher fungal abundance (Chung *et al*., 2007). Miller *et al*. (1998) documented a significant positive relationship between chitinase activity and fungal biomass. In their study, *N*-acetylglucosaminidase was produced by a diverse group of fungi, but not by any of bacterial species. Our findings support those authors, suggesting an increased role of fungi compared with bacteria in SOM turnover under elevated CO_2 (Rillig *et al*., 1999a, b; Treseder & Allen, 2000).

Enzyme activation in bulk soil after substrate amendment. Because microbial activity in soil is controlled by the availability of easily decomposable organic substrates (Friedel *et al*., 1996; Klose *et al*., 1999), glucose amendment activates microbial growth (Anderson &

Domsch, 1973; Lin & Brookes, 2000). In turn, activation of microbial biomass boosted soil respiration and enzyme activities (Dilly & Nannipieri, 2001; Vong *et al*., 2003; Renella *et al*., 2007).

As we expected, glucose amendment revealed the potential differences in SOM turnover under elevated vs. ambient CO_2: activities were higher in the former. This conclusion is novel because little information is available on the effect of atmospheric CO_2 enrichment on enzyme activities in soil amended with easily available substrate and nutrients. The strong increase of enzyme production by activated microbial biomass under elevated CO_2 explains why C stock and microbial biomass content often change only insignificantly in the soil under CO_2 enrichment (Freeman *et al*., 2004; van Kessel *et al*., 2006) despite significant increase of roots (Paterson *et al*., 1997; Rogers *et al*., 1998) and rhizodeposition (van Veen *et al*., 1991; Cheng, 1999). Higher belowground translocation of C by plants under these conditions (Rogers *et al*., 1994), and especially rhizodeposition (Cheng, 1999), increases microbial activity, which we evaluated based on activity of extracellular enzymes. Accordingly, the contribution of potentially active microorganisms to enzyme production increases under elevated CO_2. This enables the microbial community to utilize and decompose available substrates faster. Thus, the higher input of root-C will be compensated and occasionally overcompensated (Zak *et al*., 1993) by higher decomposition rates and probably lower use efficiency. This fine balance between higher C input and its faster decomposition explains the varying results (no or low changes of C_{org} and C_{mic}) in soil under elevated atmospheric CO_2.

Enzyme activities in soil aggregates. Higher enzyme activities in macro- vs. microaggregates in our study support the findings suggesting quantitative and qualitative SOM differences between the two sizes classes (reviewed in Jastrow *et al*., 2007). Thus, more labile SOM is concentrated in macroaggregates, more recalcitrant SOM in microaggregates (Jastrow *et al*., 2007). Gupta & Germida (1988) as well as Miller & Dick (1995) concluded that macroaggregate structures provide habitat for soil microorganisms and enzyme activity.

The insignificant increase of enzyme activities in soil aggregates under elevated CO_2 was fully revealed after activation by glucose. Microbial activity in soil aggregates under elevated CO_2 is very poorly studied: no studies are directly comparable with our results. Nonetheless:

(i) Enzyme activity in macroaggregates (0.25–2 mm and >2 mm) was higher under elevated vs. ambient CO_2 and the glucose amendment increased this effect. Extra C transferred by plants belowground under elevated CO_2 (van Veen *et al*., 1991; Cheng, 1999; Phillips *et al*., 2006) is preferentially allocated in macroaggregates (Jastrow *et al*., 2007), increasing microbial activity and hence enzyme activity. Especially pronounced increase was detected for chitinase. A similar increase of chitinase activity in macro- vs. microaggregates was reported in experiments not devoted to elevated CO_2 (Guggenberger *et al*., 1999; Väisänen *et al*., 2005). The authors explained the higher chitinase activity by the preference of fungi for macroaggregates as more appropriate living space in comparison to microaggregates. Furthermore, the significantly higher chitinase activity in macroaggregates under elevated CO_2 detected in our study indicates the increasing role of fungi in decomposition of SOM under elevated CO_2 conditions. Because lignin and cellulose content increase in plants grown under elevated compared with ambient CO_2 (Cotrufo *et al*., 1994) and decomposition of those compounds is mediated mainly by fungi (Kirk & Farrel, 1987), the increase of chitinase activity in our study confirms an altered functional structure of the soil microbial community under elevated CO_2.

(ii) The most pronounced changes in enzyme activities after substrate amendment were observed in microaggregates (<0.25 mm): being lower before substrate amendment, after amendment the activities of four studied enzymes significantly increased under elevated vs. ambient CO_2 treatment. We explain this by the shift of the microbial community structure to fast-growing microorganisms rather in soil micro- vs. macroaggregates under elevated CO_2. Such microorganisms benefit from the increased labile rhizodeposits, yet when removed from the ecosystem in a soil sample they lose this supply and become dormant (lower enzyme activities in microaggregates under elevated vs. ambient CO_2 before substrate amendment). Adding glucose and nutrients to microaggregates stimulated fast-growing microorganisms, boosting enzyme activities under elevated vs. ambient CO_2. This hypothesis should be tested using other methods to determine microbial growth strategies.

Conclusions

Estimation of enzyme activities in bulk soil and aggregate fractions after 5 years of elevated CO_2 lead to the following conclusions:

- The pools of C_{org} and microbial biomass in soil were not affected by 5 years of elevated CO_2. However,

increased enzyme production after stimulating the microorganisms confirm accelerated C turnover under elevated CO_2. This effect was pronounced in aggregates of different size.

- Aggregate-size fractionation using optimal moist sieving satisfactorily separated the bulk soil into micro- and macroaggregates with different properties. This fractionation technique had minimal mechanical impact on the biological properties of the separates, as demonstrated by a high recovery of microbial biomass and extracellular enzyme activities.
- Macroaggregates had higher specific and total enzyme activities indicating a potentially more intensive SOM turnover in comparison with microaggregates;
- Elevated atmospheric CO_2 stimulated enzyme activity especially in macroaggregates. The effect was particularly pronounced for chitinase, indicating a higher contribution of fungi to litter decomposition under elevated CO_2.
- Glucose and nutrient amendment to bulk soil and isolated aggregates revealed the potential differences in microbial activity under elevated vs. ambient CO_2. Increased enzyme activities in the former due to substrate amendment represented a shift in microbial community function rather than in its total content.

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