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Elevated CO_2 and Tree Species Affect Microbial Activity and Associated Aggregate Stability in Soil Amended with Litter

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Academic Editors: Robert Jandl and Mirco Rodeghiero
Received: 31 January 2017; Accepted: 28 February 2017; Published: 3 March 2017

Abstract: (1) Elevated atmospheric CO_2 (eCO_2) may affect organic inputs to woodland soils with potential consequences for C dynamics and associated aggregation; (2) The Bangor Free Air Concentration Enrichment experiment compared ambient (330 ppmv) and elevated (550 ppmv) CO_2 regimes over four growing seasons (2005–2008) under *Alnus glutinosa*, *Betula pendula* and *Fagus sylvatica*. Litter from the experiment (autumn 2008) and *Lumbricus terrestris* were added to mesocosm soils. Microbial properties and aggregate stability were investigated in soil and earthworm casts. Soils taken from the field experiment in spring 2009 were also investigated; (3) eCO_2 litter had lower N and higher C:N ratios. *F. sylvatica* and *B. pendula* litter had lower N and P than *A. glutinosa*; *F. sylvatica* had higher cellulose. In mesocosms, eCO_2 litter decreased respiration, mineralization constant (respired C:total organic C) and soluble carbon in soil but not earthworm casts; microbial-C and fungal hyphal length differed by species (*A. glutinosa* = *B. pendula* > *F. sylvatica*) not CO_2 regime. eCO_2 increased respiration in field aggregates but increased stability only under *F. sylvatica*; (4) Lower litter quality under eCO_2 may restrict its initial decomposition, affecting C stabilization in aggregates. Later resistant materials may support microbial activity and increase aggregate stability. In woodland, C and soil aggregation dynamics may alter under eCO_2, but outcomes may be influenced by tree species and earthworm activity.

Keywords: FACE; litter quality; respiration; carbon; microbial biomass; fungal hyphae

1. Introduction

Atmospheric CO_2 concentrations have increased significantly over recent decades [1]. Atmospheric CO_2 taken up by plants and incorporated into soil may be partly protected from decomposition within stable aggregates [2], a key mechanism facilitating soil carbon gain under elevated CO_2 [3]. Consequently, changes in soil aggregation may influence soil respiration responses to atmospheric CO_2 increases.

Leaf litter is a major C input to woodland soils and thus plays key roles in nutrient cycling and organic matter dynamics [4]. Litters vary in their nutrient, cellulose and lignin contents, factors which largely determine decomposition rates [5]. Litter quality is affected by intrinsic species characteristics. Thus, the nitrogen concentration of *A. glutinosa* leaves may be twice that of western red cedar and

western hemlock [6]. Concentrations of N are low and C:N ratios are high in beech (*Fagus sylvatica*) compared to oak (*Quercus robur*) leaves [7]. Elevated atmospheric CO_2 (eCO_2) may affect litter quality. In litter from three *Populus* spp., eCO_2 caused a general decrease in nitrogen concentrations [8]. From a meta-analysis [9], it was estimated that nitrogen concentrations in leaf litter under eCO_2 were reduced by approximately 7% compared to ambient CO_2, with lignin concentration about 6.5% higher. However, these changes were not linked to any consistent effect on litter decomposition.

Tree species influence soil microbial communities, although these effects are often indirect and complex; leaf litter diversity increased microbial abundance at the soil surface but nutrients were the primary drivers at depth [10]. There are also some inconsistencies in reported responses of soil microbiota to varying CO_2 regimes. Thus, eCO_2 over six growing seasons did not significantly alter soil microbial biomass carbon, metabolic quotient and basal respiration in nutrient-poor grassland [11]; these findings were attributed to nitrogen limitations. However, eCO_2 over five years did not affect microbial biomass, even where nitrogen fertilizer was added [12], suggesting that microbial biomass is insensitive to changes in atmospheric CO_2. In contrast, under chickpea, eCO_2 increased labile soil C, microbial biomass and respiration [13]. In a review based mainly on forest ecosystems [14], evidence of eCO_2 effects on soil microbial composition was considered weak, whereas that for increased physiological activity was more consistent.

Interactions between soil aggregation and atmospheric CO_2 are poorly understood with contrasting findings arising from different studies and limited information on woodland soils. A Californian chaparral ecosystem exposed for six years to CO_2 concentrations ranging from 250 to 750 $\mu L \cdot L^{-1}$ CO_2 exhibited decreased macro-aggregate and micro-aggregate stability at the highest CO_2 levels [15]; the same study also found reduced protection of soil organic matter and faster turnover of carbon at high CO_2 levels. No effect of elevated CO_2 on aggregate stability was found after four years of CO_2 exposure in a grassland mesocosm experiment, confounding expectations that increased labile carbon and root biomass under eCO_2 would promote aggregation [16]. However, increased aggregate stability under eCO_2 was found in grassland soils and attributed to higher glomalin production by fungi [17]. In poplar plantations, eCO_2 did not affect macro-aggregation, but increased micro-aggregation, although these changes were small relative to those between poplar genotypes [18]. Earthworms at the ORNL-FACE temperate woodland site [3] directly contributed to the formation of soil aggregates, stabilizing increased carbon inputs resulting from atmospheric CO_2 enrichment.

In general, aggregate stability increases rapidly following addition of more labile organic matter but effects are transient. Thus, initial increases in aggregate stability were lost four weeks after glucose application but were more persistent with cellulose [19]. Organic materials have been classified into four groups [20]: (i) simple labile compounds which increased aggregate stability in less than month; (ii) more complex residues which maximized aggregate stability between one and three months; (iii) stable compounds which increased aggregate stability only after three months; (iv) ligneous residues which might have some benefit in the longer-term.

The objectives of this study were to evaluate the effects of eCO_2 on C dynamics and associated aggregate stability in response to litter inputs (mainly) from three contrasting tree species. Working with materials from a plantation experiment allowed for the evaluation of species-specific responses to eCO_2 under both mesocosm and field conditions. It was hypothesized that elevated CO_2 would reduce litter quality, thus slowing its decomposition, and that this would reduce initial aggregate stabilization; aggregate formation was expected to mitigate this effect. Elevated CO_2 effects were expected to be more pronounced in species producing higher quality litter and these litters were expected to have more pronounced early effects than those of low quality litter. Ingestion of soil and litter by earthworms was expected to mitigate these effects.

2. Materials and Methods

For practical reasons, the main investigation was based on a mesocosm experiment with a single visit to the experimental site providing the opportunity to corroborate mesocosm findings under field conditions.

2.1. Experimental Set-Up

The investigation was undertaken in field and, in greater detail, greenhouse experiments. The field experiment was the Bangor FACE site, at Henfaes experimental farm (UK 53°14′N; 4°01′W). The soil at Henfaes is a fine loamy brown earth over gravel (Rheidol series) classified as a Dystric Cambisol in the Food Agriculture Organisation system [21]. CO_2 levels were manipulated in eight site rings which included four ambient and four elevated treatments. Within each ring, groups of three tree species (*Alnus glutinosa* L. Gaertn., *Betula pendula* Roth and *Fagus sylvatica* L.) were planted either individually or in mixtures; in this study, only single species plots were investigated to avoid complex responses to heterogenous litter inputs.

The FACE rings operated at CO_2 330 ppmv for ambient and at 550 ppmv for eCO_2. For fuller details of the experimental design and its operation, see [22]. In the field, litter was collected on a weekly basis in baskets located within the plots, then air-dried and stored; amounts collected were highest for *A. glutinosa*, intermediate for *B. pendula*, but markedly lower for *F. sylvatica*. There was a similar species ranking for woody tissue, which increased significantly at eCO_2 [22]. Litters collected in autumn 2008 were used as inputs in the mesocosm experiment.

In spring 2009, field soil samples (24 in total) were taken from the 0–5 cm depth after removal of any litter layer, from each of the CO_2 regime X tree species combinations for subsequent measurement of field soil responses to treatments and as microbial inocula for mesocosm soils. For *A. glutinosa* and *B. pendula*, little litter remained at sampling, but some *F. sylvatica* litter was still apparent. This depth was chosen as being most affected by differences in litter quality. Sub-samples were either stored at 2 °C prior to microbial analyses or their use as inocula, or air-dried over 5 days at room temperature for subsequent organic C and aggregate stability determinations.

The experimental design of the mesocosm trial was based on that of the field experiment, with litters from each plot representing all species and CO_2 regime combinations being tested (four replicates per treatment combination). A soil similar to that on the experimental site (Rheidol series—Dystric Cambisol) was taken from 10–20 cm depth to avoid initial differences arising from experimental treatments. The percentage of sand, silt and clay was 44%, 35% and 20% respectively; initial aggregate stability (see Section 2.2 for method) was 30%. Bulk soil was mixed thoroughly with 5 g of soil, sampled as described above from the Bangor FACE site, as a microbial inoculum for mesocosms (2 liter pots with 10 cm diameter to which 2 kg of soil was added; $n = 24$). Soil inocula were matched to the litters subsequently applied as amendments, to take account of field treatment adaptations in microbial populations. Litters were ground to <1 mm and added to the soil surface (2 g) three times a week. Soil moisture was maintained at approximately 20% w/w during this experiment by addition of water and the shaded glasshouse had a temperature range of 10–21 °C over the 30-day duration of the experiment. The experiment was terminated at this point because supplies of litter from *F. sylvatica* plots had been exhausted.

Lumbricus terrestris L. was the predominant anecic earthworm on the field site [23] and has been shown to promote aggregation [24]. As supplies of litter were limited, the inclusion of a no earthworm treatment would have compromised the experiment. Thus, in order to assess the effects of earthworm processes on measured parameters, their casts were sampled and considered to represent recently formed potential aggregates. Three adult earthworms were added to each mesocosm (Neptune Ecology, Ipswich).

In the mesocosm experiment, the first collection of surface casts was two weeks after commencement of litter additions; very little casting occurred prior to 2 days before this collection. These materials were collected three times a week and bulked after air-drying for the entire experimental period (ambient temperatures) for total C and stability measurements. The final collection

(28–30 days) was retained fresh for cast microbial measurements, with sub-samples incubated for 22 days (respiration had stabilized by this time) at 20 °C and moisture content similar to that at sampling; this investigation aimed to assess temporal trends in microbial activity and C turnover in fresh cast aggregates. Soil from the mesocosms was sampled at the end of the experiment, 30 days after amendments commenced, for assessment of soil aggregate properties. Sub-samples of these stable aggregates were then incubated for 37 and 67 days at 20% moisture content and 20 °C during which time respiration was measured in order to monitor medium-term trends in aggregate C dynamics.

2.2. Measurements

Litter quality was assessed by measuring lignin, cellulose, nutrients (N and P) and the C:N ratio using standard methods [25]. Briefly, acid detergent fibre was determined, then lignin in residues from this analysis was dissolved using a saturated potassium permanganate/buffer solution; lignin was determined by weight loss and cellulose by weight loss from these residues after ignition at 500 °C. C and N were measured using a LECO CHN analyzer (LECO Corp., St Joseph, MI, USA); P was determined colorimetrically.

Microbial biomass carbon (MBC) was estimated by the fumigation-extraction method [26] using 0.5 M K_2SO_4 to extract organic C from chloroform-fumigated and non-fumigated samples. Extracts from the fumigated and non-fumigated samples were analyzed using a Total Organic Carbon analyzer (Shimadzu TOC-5050, Shimadzu UK Ltd., Milton Keynes, UK). MBC was calculated by subtracting the extracted organic carbon in the non-fumigated samples from that in the fumigated samples and using a standard conversion factor. Fungal hyphal length was measured using the membrane filtration method [27]. Soils (0.5 g) were placed in 30 mL of distilled water and shaken (Griffin & George Ltd., London, UK) for 1 h. The suspensions were allowed to settle, the supernatant was decanted, mixed with Calcofluor white M2R (Sigma-Aldrich, Gillingham, UK; final concentration 0.1%) and then passed through 2 µm filters using a vacuum pump. Hyphal lengths were estimated using a grid intersect method (20 µm grid) at ×100 magnification [28].

Soil respiration (20 °C and moisture content at sampling) was measured by two methods. The first, used for casts, where material available was limited, was based on the MicroResp procedure [29], using a BioTek ELx808 plate reader (BioTek Instruments, Inc, Swindon, UK) to monitor pH change (phenol red indicator) due to increasing dissolved CO_2. The second method for soil aggregates was based on a static alkali trap procedure [30].

Organic carbon in mesocosm aggregates and casts, and in field aggregates, was estimated by loss on ignition at 400 °C for 16 h in a muffle furnace [31] using a conversion factor of 1.724. Extractable (K_2SO_4) carbon (KSE-C) in mesocosm aggregates and casts was estimated [32] from the C concentrations of the non-fumigated extracts used for the microbial biomass C determinations. Mineralization constant [33] was determined as a means of discounting variations in respiration due to differing amounts of organic matter in samples; it was calculated as the ratio of respired C to total organic C.

Aggregate stability was measured on both cast and bulk soil samples using an adaptation of a standard method [34]. Air-dry cast and bulk soil samples (10 and 50 g respectively) were gently passed through an 8 mm sieve and recovered on a 4 mm sieve. Wet macro-aggregate (>2 mm) stability was then measured by wet sieving (Russell Finex, Feltham, UK, model 85521) on a 2-mm sieve for 2 min under a flow rate of 6.8 liters per minute; soil remaining on the sieve was counted as stable aggregates >2 mm (SA). The %SA was then calculated as the proportion of dry (8–4 mm) aggregates stable >2 mm (both weights corrected for moisture and gravel contents).

2.3. Statistical Analyses

Data were analyzed using Minitab 14 (Minitab Inc., State College, PA, USA, two-way ANOVA) following checks for normality and equality of variance. The analyses tested two factors; species including (*A. glutinosa*, *B. pendula* and *F. sylvatica*) and CO_2 regime (ambient and elevated); interaction effects were also tested. Incubation time or differences between soil and casts were not assessed as

experimental factors; in the cast incubation experiment, the former was of lesser interest than the differences between treatments on each sampling occasion whilst differences in sampling protocols rendered soil and cast samples non-comparable other than in a general sense. Differences between individual species' means were assessed where appropriate using the TUKEY multiple range test, with a significance level of $p < 0.05$. Relationships between various parameters were assessed by linear correlation analyses.

3. Results

3.1. Chemical Composition of Litter

There were marked differences in chemical composition between litter types (Table 1a). Cellulose, C:N ratios, and lignin:nitrogen ratios were lower ($p = 0.009$) for *B. pendula* and particularly *A. glutinosa* litter compared with *F. sylvatica*. Nitrogen and phosphorous concentrations were higher for *A. glutinosa* and *B. pendula* than for *F. sylvatica.* Litter from the ambient CO_2 regime ($p = 0.037$) had a higher N concentration and lower C:N ratio ($p = 0.041$) than that from the eCO_2 treatment (Table 1b). There were no other significant CO_2 regime effects; although interaction effects were non-significant ($p = 0.072$ and 0.064 respectively), the CO_2 effects described above tended to be most pronounced for *A. glutinosa* and least pronounced for *F. sylvatica*.

Table 1. Effect of (**a**) litter species and (**b**) CO_2 regime on the chemical composition of the litter used in the mesocosm experiment.

	N g·kg^{-1}	C:N Ratio	P g·kg^{-1}	Cellulose g·kg^{-1}	Lignin g·kg^{-1}	Lignin:N Ratio
(a)						
Alnus	27.95 ^{a}	16.8 ^{b}	1.95 ^{a}	71.1 ^{b}	300.9 ^{a}	10.76 ^{c}
Betula	18.83 ^{b}	24.9 ^{b}	1.65 ^{a}	67.6 ^{b}	281.1 ^{a}	14.92 ^{b}
Fagus	9.95 ^{c}	46.8 ^{a}	0.81 ^{b}	139.9 ^{a}	290.3 ^{a}	29.17 ^{a}
(b)						
Ambient	20.2 ^{a}	27.6 ^{b}	1.52 ^{a}	86.0 ^{a}	283.5 ^{a}	14.03 ^{a}
Elevated	17.8 ^{b}	32.1 ^{a}	1.41 ^{a}	99.8 ^{a}	302.5 ^{a}	17.00 ^{a}

Litter species and CO_2 regime means with a common letter superscript do not differ significantly ($p < 0.05$).

3.2. Microbial Indices

In casts (Table 2), *B. pendula* litter produced significantly greater ($p = 0.019$) hyphal length than *A. glutinosa* and *F. sylvatica.* MBC, in contrast, was markedly higher ($p = 0.017$) in *A. glutinosa* compared to the other species; CO_2 regime had no significant effects although MBC was substantially lower for eCO_2. In mesocosm soil aggregates (Table 2), although MBC and fungal hyphal length tended to be higher in *B. pendula* compared to *A. glutinosa* and *F. sylvatica*, and in elevated compared with ambient CO_2, these differences were non-significant.

Table 2. Effect of (**a**) litter species and (**b**) CO_2 regime on microbial biomass carbon and fungal hyphal length in the soil and cast aggregates—mesocosm experiment.

	Microbial Biomass C (mg·kg^{-1})		Fungal Hyphal Length (m·g^{-1})	
	Soil	Cast	Soil	Cast
(a)				
Alnus	133.9 ± 28.4 ^{a}	2798 ± 265 ^{a}	2.71 ± 0.59 ^{a}	12.74 ± 1.86 ^{a,b}
Betula	157.5 ± 15.1 ^{a}	1773 ± 210 ^{b}	3.98 ± 1.59 ^{a}	19.16 ± 4.01 ^{a}
Fagus	129.9 ± 19.0 ^{a}	2198 ± 179 ^{a,b}	0.67 ± 0.16 ^{a}	6.75 ± 1.53 ^{b}
(b)				
Ambient	124.0 ± 14.0 ^{a}	2428 ± 250 ^{a}	2.20 ± 0.49 ^{a}	13.44 ± 2.97 ^{a}
Elevated	156.9 ± 19.3 ^{a}	2085 ± 162 ^{a}	2.71 ± 1.15 ^{a}	12.33 ± 2.25 ^{a}

Mean ± standard error: litter species and CO_2 regime means with a common letter superscript do not differ significantly ($p < 0.05$).

Respiration in incubated earthworm casts (Table 3) was generally higher for *A. glutinosa* compared to *B. pendula* and *F. sylvatica* litter. Differences between *A. glutinosa* and *F. sylvatica* were significant ($p < 0.05$ or $p < 0.01$) up to the final incubation period, but those with *B. pendula* were significant ($p = 0.031$) at day 18 only. By 22 days, respiration had stabilized across all treatments at low rates. Initially respiration for ambient and eCO_2 litter did not differ, however in later stages of the incubations, eCO_2 increased respiration ($p < 0.001$). There was a CO_2 litter X interaction ($p = 0.036$) at 18 days (Figure 1). Although respiration under eCO_2 was higher for all species, this effect was more pronounced for *A. glutinosa.*

Table 3. Effect of (**a**) litter species and (**b**) CO_2 regime on respiration in earthworm casts at four incubation times—mesocosm experiment.

	Respiration (mg CO_2-C $kg^{-1} \cdot h^{-1}$)			
	Time (Days)			
	2	**6**	**18**	**22**
(a)				
Alnus	5.2 ± 0.32^{a}	3.6 ± 0.33^{a}	2.5 ± 0.52^{a}	0.16 ± 0.12^{a}
Betula	$4.1 \pm 0.33^{a,b}$	$2.7 \pm 0.32^{a,b}$	1.5 ± 0.25^{b}	0.09 ± 0.04^{a}
Fagus	3.0 ± 0.47^{b}	1.8 ± 0.27^{b}	0.7 ± 0.26^{c}	0.13 ± 0.07^{a}
(b)				
Ambient	4.1 ± 0.34^{a}	2.8 ± 0.29^{a}	0.8 ± 0.17^{b}	0.02 ± 0.03^{b}
Elevated	4.0 ± 0.46^{a}	2.6 ± 0.36^{a}	2.3 ± 0.37^{a}	0.24 ± 0.07^{a}

Mean ± standard error: litter species and CO_2 regime means with a common letter superscript do not differ significantly ($p < 0.05$).

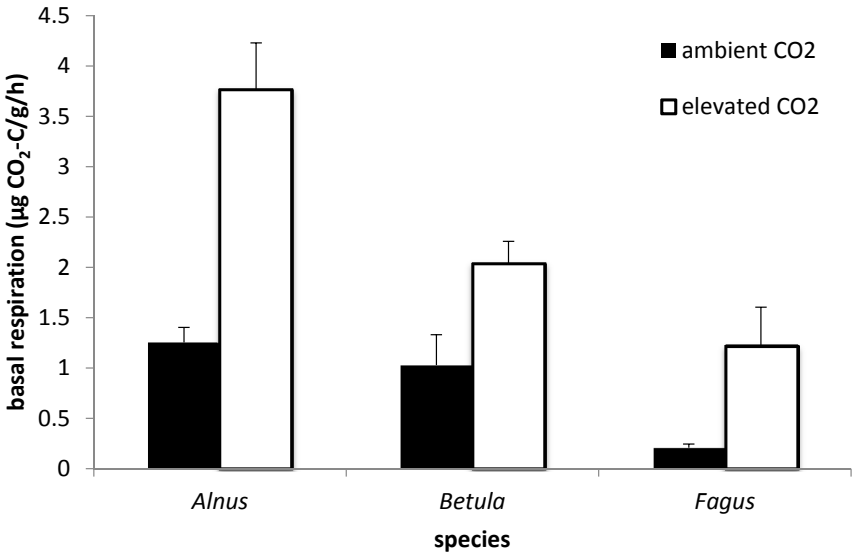

Figure 1. Interaction ($p = 0.036$) plot for CO_2 regime and litter species (*A. glutinosa*, *F. sylvatica* and *B. pendula*) for respiration in earthworm casts at day 18 of incubation. Error bars represent standard errors ($n = 4$).

Respiration in mesocosm soil (30 days) and in field aggregates (Table 4) was not significantly affected by species. In mesocosm soil aggregates, respiration 30 days after inputs commenced was reduced by eCO_2 ($p = 0.019$). At day 30, aggregate stability in mesocosm soil was negatively correlated ($n = 24$; $r = 0.64$; $p < 0.001$) with respiration; no similar relationships were found in mesocosm cast or in field aggregates. In field aggregates, eCO_2 increased respiration significantly compared to ambient CO_2.

Table 4. Effects of (**a**) litter species and (**b**) CO_2 regime on respiration in soil aggregates from mesocosm and field experiments.

	Respiration (mg CO_2-C $kg^{-1} \cdot h^{-1}$)			
	Mesocosm			Field
	Soil Day 30	Soil 30–67 Days	Soil 67–97 Days	Soil
(a)				
Alnus	0.32 ± 0.08 ^{a}	1.09 ± 0.13 ^{a,b}	0.62 ± 0.09 ^{a,b}	1.16 ± 0.16 ^{a}
Betula	0.45 ± 0.11 ^{a}	0.61 ± 0.08 ^{b}	0.41 ± 0.04 ^{b}	1.42 ± 0.19 ^{a}
Fagus	0.39 ± 0.09 ^{a}	3.23 ± 0.42 ^{a}	0.86 ± 0.17 ^{a}	1.37 ± 0.15 ^{a}
(b)				
Ambient	0.52 ± 0.08 ^{a}	1.65 ± 0.43 ^{a}	0.63 ± 0.12 ^{a}	1.08 ± 0.08 ^{b}
Elevated	0.25 ± 0.05 ^{b}	1.64 ± 0.36 ^{a}	0.62 ± 0.09 ^{a}	1.55 ± 0.15 ^{a}

Mean ± standard error: litter species and CO_2 regime means with a common letter superscript do not differ significantly ($p < 0.05$).

When mesocosm aggregates were incubated for 37 and 67 days after the end (days 67–97) of the experiment (Table 4), cumulative respiration was significantly higher ($p < 0.001$) in *F. sylvatica* compared to *A. glutinosa* and *B. pendula* (30–67 days), but later (67–97 days) only the difference between *F. sylvatica* and *B. pendula* remained significant ($p = 0.038$). Respiration was not significantly affected by CO_2 regime nor were there any significant interaction effects.

Mineralization constant did not significantly differ between species in mesocosm or field aggregates (Table 5). The mineralization constant in recently formed casts was significantly higher ($p = 0.028$) for *A. glutinosa* compared to *F. sylvatica* but not *B. pendula.* Elevated CO_2 significantly decreased ($p = 0.035$) the mineralization constant in mesocosm aggregates, but increased it significantly ($p = 0.022$) in field aggregates; CO_2 regime had no effect on cast aggregates from the mesocosm experiment.

Table 5. Effect of (**a**) litter species and (**b**) CO_2 regime on mineralization constant in aggregates from the mesocosm and field experiments.

	Mineralization Constant (mg CO_2-C g^{-1}C h^{-1})		
	Mesocosm		Field
	Soil	Cast	Soil
(a)			
Alnus	0.0058 ± 0.0015 ^{a}	0.06172 ± 0.0033 ^{a}	0.0246 ± 0.0042 ^{a}
Betula	0.0099 ± 0.0028 ^{a}	0.04750 ± 0.0057 ^{a,b}	0.0297 ± 0.0045 ^{a}
Fagus	0.0073 ± 0.0018 ^{a}	0.03687 ± 0.0073 ^{b}	0.0322 ± 0.0031 ^{a}
(b)			
Ambient	0.0103 ± 0.0020 ^{a}	0.04906 ± 0.0049 ^{a}	0.0235 ± 0.0021 ^{b}
Elevated	0.0051 ± 0.0010 ^{b}	0.04833 ± 0.0059 ^{a}	0.0341 ± 0.0035 ^{a}

Mean ± standard error: litter species and CO_2 regime means with a common letter superscript do not differ significantly ($p < 0.05$).

3.3. Total Carbon and K_2SO_4 Extractable Carbon (KSE-C)

At the end of the mesocosm experiment (day 30), aggregates had significantly higher organic carbon ($p = 0.004$) and KSE-C ($p < 0.001$) for *B. pendula* than for *F. sylvatica* (Table 6); this effect arose in part from the observed lesser incorporation of *F. sylvatica* litter into soil. In mesocosm casts (day 30), there were no significant species effects. Elevated CO_2 litter decreased ($p = 0.010$) KSE-C in mesocosm soil aggregates. In the field, eCO_2 had no effect on either organic carbon index. Mesocosm soil (day 30) aggregate stability was positively correlated with organic carbon ($n = 24$; $r = 0.58$; $p = 0.002$) and

KSE-C (n = 24; r = 0.57; p = 0.003), but neither of these parameters correlated with the stability of cast aggregates.

Table 6. Effect of (**a**) litter species and (**b**) CO_2 regime on organic carbon and K_2SO_4 extractable carbon (KSE-C) in aggregates from the mesocosm and field experiments.

	Organic Carbon%			KSE-C mg kg^{-1}	
	Mesocosm		Field	Mesocosm	
	Soil	Cast	Soil	Soil	Cast
(a)					
Alnus	4.17 ± 0.04 ^{a,b}	8.49 ± 0.24 ^{a}	4.91 ± 0.30 ^{a}	155.05 ± 5.04 ^{b}	413.4 ± 21.6 ^{a}
Betula	4.23 ± 0.05 ^{a}	8.97 ± 0.44 ^{a}	4.99 ± 0.30 ^{a}	174.61 ± 4.15 ^{a}	358.3 ± 34.0 ^{a}
Fagus	3.93 ± 0.08 ^{b}	8.66 ± 0.10 ^{a}	4.22 ± 0.12 ^{a}	144.67 ± 3.36 ^{b}	366.3 ± 24.8 ^{a}
(b)					
Ambient	4.16 ± 0.07 ^{a}	8.79 ± 0.32 ^{a}	4.77 ± 0.23 ^{a}	163.80 ± 5.82 ^{a}	358.0 ± 25.9 ^{a}
Elevated	4.07 ± 0.06 ^{a}	8.63 ± 0.36 ^{a}	4.64 ± 0.22 ^{a}	152.42 ± 3.34 ^{b}	400.6 ± 17.4 ^{a}

Mean ± standard error: litter species and CO_2 regime means with a common letter superscript do not differ significantly ($p < 0.05$).

3.4. Aggregate Stability

In both soils and casts, *B. pendula* litter had the highest aggregate stability, with *F. sylvatica* lowest and *A. glutinosa* intermediate (Table 7). These trends were statistically significant ($p < 0.001$) for the mesocosm soils only. In casts, aggregate stability differences between species were close to significance (p = 0.068).

There were no direct CO_2 regime effects on aggregate stability (Table 7). However, there was a significant CO_2 regime X litter species interaction (p = 0.046) in the field soils (Figure 2). Elevated CO_2, increased aggregate stability under *F. sylvatica* but decreased stability under the other two species; these effects were small relative to background stability. Although not statistically significant (p = 0.09), a broadly similar interaction was found for cast aggregate stability in the mesocosm study, with only *F. sylvatica* litter showing a positive response to eCO_2.

Table 7. Effect of (**a**) litter species and (**b**) CO_2 regime on % stable aggregates in soil and casts from mesocosm and field experiments.

	% Stability		
	Mesocosm		Field
	Soil	Cast	Soil
(a)			
Alnus	35.0 ± 2.16 ^{b}	86.9 ± 1.49 ^{a}	90.1 ± 2.20 ^{a}
Betula	44.9 ± 4.43 ^{a}	87.5 ± 1.43 ^{a}	92.2 ± 1.40 ^{a}
Fagus	25.8 ± 0.89 ^{c}	79.1 ± 4.48 ^{a}	88.8 ± 1.98 ^{a}
(b)			
Ambient	36.9 ± 3.45 ^{a}	82.4 ± 3.35 ^{a}	91.3 ± 1.71 ^{a}
Elevated	33.6 ± 3.04 ^{a}	86.6 ± 0.99 ^{a}	89.4 ± 1.34 ^{a}

Mean ± standard error: litter species and CO_2 regime means with a common letter superscript do not differ significantly ($p < 0.05$).

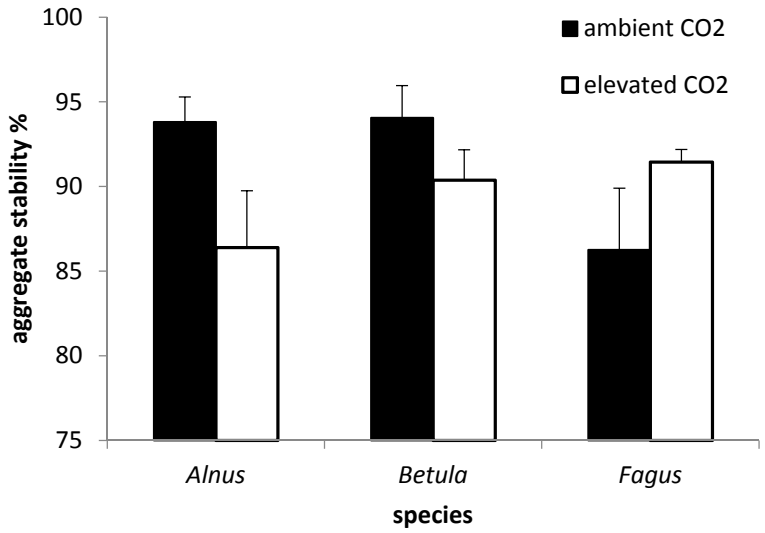

Figure 2. Interaction (p = 0.046) plot for CO_2 regime and litter species *(A. glutinosa*, *B. pendula* and *F. sylvatica)* for stable aggregation—field experiment. Error bars represent standard errors (n = 4).

4. Discussion

For most indices, litter quality was ranked *A. glutinosa* > *B. pendula* > *F. sylvatica* although in some cases differences between *A. glutinosa* and *B. pendula* were small. Evidence from other studies has indicated similar rankings of litter quality by species [6,7]. Elevated CO_2 tended to reduce litter quality in general, although this effect was marked for N and C:N only. Again, these findings are broadly in line with those of other similar studies [9]. For most quality parameters, species differences were larger than those for CO_2 regime.

Treatment effects on microbial biomass C (MBC) and fungal hyphal length were found in cast aggregates only and then only between species, not CO_2 regimes. Both indices tended to be greater for *B. pendula* and *A. glutinosa* compared to *F. sylvatica* litter; higher nutrient contents in these litters may explain the enhanced microbial responses observed. Litter quality effects on MBC in casts may vary with time; thus, high quality litter (lucerne) gave higher MBC at 14 and 28 days compared to wheat straw, but these amendments did not differ at 7 and 56 days [35]; data here for very recent casts showed higher respiration with better quality litter but in later soil aggregates these differences were inconsistent. Given the effects of eCO_2 on N concentrations, a reduced microbial community might have been expected; other studies [11] have attributed the absence of any eCO_2 effect on MBC in grassland soils to N limitation. If N is a key determinant of MBC responses to eCO_2 [14], in our study, some variation between species in microbial eCO_2 responses might have been expected given their very different litter N contents; only species' differences were observed and then only in casts.

The effects of treatments on respiration were complex and time-dependent. Higher quality litters (*A. glutinosa* and *B. pendula*) had higher respiration in recently formed earthworm casts up to 18 days. The generally low rates of cast respiration at 22 days were consistent with trends in other studies [35], with respiration and microbial biomass decreasing steadily over 100 days. In contrast, for incubated soil aggregates (37 and 67 days after inputs ceased) the quality effect was reversed with respiration being higher in poor quality *F. sylvatica* litter, due probably to delayed mineralization of this litter. As with MBC, litter quality X time interactions have been shown for respiration [36]; here, for casts and incubated aggregates, the ranking of litters by respiration varied with time.

When casts were incubated, respiration was unaffected by CO_2 regime initially, but was significantly higher for eCO_2 litter in the later stages of cast incubation, a trend also seen in soil aggregates; there was also evidence of CO_2 X litter species treatment interactions, with the later stimulatory effect of eCO_2 being more pronounced in *A. glutinosa* litter. Again, eCO_2-induced

reductions in litter quality may have delayed decomposition by some days, a response more pronounced in otherwise rapidly decomposing litter. Elevated CO_2 decreased respiration and mineralization constant in soil aggregates from the mesocosm trial, but the reverse was the case in field aggregates sampled some months after litterfall. Other studies [14,37] have found that eCO_2 stimulates respiration in field soils; findings reported here suggest a more complex and time-dependent response in aggregates. Differences in mineralization constant suggest that respiration responses were influenced by the composition of organic materials present in aggregates. Findings concur with the view [38] that litter quality influences aggregate C dynamics but only in the short-term. It is likely that a higher C:N ratio litter at eCO_2 initially restricted microbial decomposition but that this led to increased materials available for mineralization later. Slower litter decay rates have been linked to increases in lignin:N ratios [39].

Organic carbon and KSE-C in earthworm casts were not affected by treatments. In mesocosm soil aggregates, organic carbon and KSE-C were high for *A. glutinosa* and *B. pendula* compared to *F. sylvatica*. Lower soil aggregate KSE-C for *F. sylvatica* and under elevated CO_2 may in part be explained by limited mineralization of this litter; evidence of higher respiration associated with *F. sylvatica* litter occurred at a later stage to that when KSE-C data were obtained. There were no significant treatment effects on these parameters in the field, as found in a previous investigation [22] on the same experimental site.

Elevated CO_2 can affect soil aggregation, a dynamic property that responds to environmental changes, feeding back to other ecosystem functions [39]. Interactions between micro-organisms, organic residues and mineral particles affect the extent and temporal dynamics of aggregation. There is evidence that high quality residues improve aggregation quickly, whereas lower quality residues are more effective in the longer-term [20]. Species litter was ranked as *A. glutinosa* > *B. pendula* > *F. sylvatica* in terms of decomposability; the effects of eCO_2 were less pronounced and more complex.

In the short term, more easily decomposable *A. glutinosa* and *B. pendula* litters produced higher aggregate stability than *F. sylvatica* litter. Compared to low and high C:N ratio litter, intermediate quality *B. pendula* litter may have provided a better balance between stimulation of microbial activity to promote early aggregate stabilization and greater persistence of stabilizing agents. In this context, the markedly higher KSE-C contents in soil aggregates with added *B. pendula* litter at the end of the mesocosm experiment may indicate mineralization of stabilizing compounds; soluble carbon is considered a key source of energy for microorganisms [40]. Additionally, organic carbon, MBC, fungal hyphae and microbial activity tended to be higher in aggregates of *B. pendula* compared to the other litters. Some months after litterfall, *F. sylvatica* had similar aggregate stability to the other species in the field. *F. sylvatica* litter may sustain microbial activity, generating stabilizing compounds over a longer time period after inputs compared with more labile litters. There is some evidence supporting this interpretation in the low respiration rates (Table 3) in casts for *F. sylvatica*, but high rates in aggregates incubated to 67 days (Table 4) after amendments to the mesocosm experiment had ceased. Tree root and associated fungal responses, in addition to litter effects, may have influenced aggregate stability in the field.

Species effects on aggregate stability, at least in the mesocosm experiment, were larger than those of the CO_2 regime; this was expected given that CO_2 differences in litter quality were generally smaller, as found previously on this experimental site [22]. There was, however, evidence of species X CO_2 interactions, with elevated CO_2 having a positive effect on aggregate stability only with *F. sylvatica*. These findings suggest that atmospheric CO_2 effects on aggregation in woodland soils may be species-specific. The aggregation interaction may be explained in part by associated, though non-significant interactions for respiration. Microbial activity enhances aggregate stability soon after aggregate formation or organic inputs [41], but in the mesocosm study was negatively correlated with stability at day 30, suggesting that, by this stage, respiration was associated with decomposition of aggregate stabilizing agents. Field soils sampled some months after litterfall may have entered a microbial 'degradative' phase in aggregates receiving the more easily decomposable litter of *A. glutinosa* and *B. pendula*, whereas delayed microbial decomposition of lower quality *F. sylvatica* litter may not

have reached this phase. This interpretation is consistent with evidence that aggregates formed with higher quality litter have a faster turnover than those with lower quality litter [42]. Findings relating to differential eCO_2 effects are broadly similar to other studies [15]; thus, the composition of the vegetation cover and its response to eCO_2 will have a significant influence on outcomes for aggregation and associated parameters.

The effects of earthworm activity were not directly addressed in this study as soil and cast materials in the mesocosm study were not directly comparable. Nevertheless, it is clear that most microbial indices were markedly higher in casts, as was the case for C and aggregate stability indices. For most measured indices, though not respiration, treatment effects observed in soil aggregates were absent or reduced in casts. This may indicate that earthworm comminution of litter and its thorough mixing with mineral fractions mitigated at least some of the differences in C inputs.

5. Conclusions

Exposure to elevated CO_2 may alter microbial populations and their activity, in addition to aggregate stability. Where there are marked differences in litter quality between tree species, species effects are likely to be more pronounced. Earthworms may mitigate some of these CO_2 and species effects. At least for the contrasting species investigated, significant shifts in their relative proportion in response to atmospheric and climatic changes may have more impact on forest C and aggregate dynamics than intra-specific respiration responses to eCO_2; any such changes may have implications for the seasonal dynamics of aggregation and nutrient cycling. Resolving the complex and often species-specific responses to eCO_2 as they affect C inputs to soils, with associated impacts on soil characteristics, remains an important challenge [14].

Acknowledgments: S. Al-Maliki acknowledges financial support from the Ministry of Higher Education and Scientific Research, Iraq. This study was undertaken as part of CIRRE under the Bangor–Aberystwyth University initiative.

Author Contributions: S.M.J.A. and J.S. respectively carried out and supervised the research. D.L.G. and D.L.J. designed the field experiment. D.G.-J. and J.S. had the major role in revising an initial draft by S.M.J.A. D.L.G. and D.L.J. commented on the final draft.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Intergovernmental Panel on Climate Change; Core Writing Team; Pachauri, R.K.; Meyer, L.A. (Eds.) *Climate Change 2014: Synthesis Report, Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*; Intergovernmental Panel on Climate Change: Geneva, Switzerland, 2014; 151p.
2. Bronick, C.; Lal, R. Soil structure and management: A review. *Geoderma* **2005**, *124*, 3–22. [CrossRef]
3. Sánchez-de León, Y.; Lugo-Pérez, J.; Wise, D.H.; Jastrow, J.D.; González-Meler, M.A. Aggregate formation and carbon sequestration by earthworms in soil from a temperate forest exposed to elevated atmospheric CO_2: A microcosm experiment. *Soil Biol. Biochem.* **2014**, *68*, 223–230. [CrossRef]
4. Berg, B.; McClaugherty, C. *Plant Litter: Decomposition, Humus Formation, Carbon Sequestration*, 2nd ed.; Springer: Berlin/Heidelberg, Germany, 2008; p. 338.
5. Le Guillou, C.; Angers, D.A.; Maron, P.A.; Leterme, P.; Menasseri-Aubry, S. Linking microbial community to soil water-stable aggregation during crop residue decomposition. *Soil Biol. Biochem.* **2012**, *50*, 126–133. [CrossRef]
6. Richardson, J.S.; Shaughnessy, C.R.; Harrison, P.G. Litter breakdown and invertebrate association with three types of leaves in a temperate rainforest stream. *Arch. Hydrobiol.* **2004**, *159*, 309–325. [CrossRef]
7. Loranger-Merciris, G.; Laossi, K.R.; Bernhard-Reversat, F. Soil aggregation in a laboratory experiment: Interactions between earthworms, woodlice and litter palatability. *Pedobiologia* **2008**, *51*, 439–443. [CrossRef]
8. Cotrufo, M.F.; de Angelis, P.; Polle, A. Leaf litter production and decomposition in a poplar short rotation coppice exposed to free air CO_2 enrichment (POPFACE). *Glob. Chang. Biol.* **2005**, *11*, 971–982. [CrossRef]

9. Norby, R.J.; Cotrufo, M.F.; Ineson, P.; O'Neill, E.G.; Canadell, J.G. Elevated CO_2, litter chemistry, and decomposition: A synthesis. *Oecologia* **2001**, *127*, 153–165. [CrossRef] [PubMed]
10. Thoms, C.; Gattinger, A.; Jacob, C.; Thomas, F.M.; Gleixner, G. Direct and indirect effects of tree diversity drive soil microbial diversity in temperate deciduous forest. *Soil Biol. Biochem.* **2010**, *42*, 1558–1565. [CrossRef]
11. Niklaus, P.; Alphei, J.; Ebersberger, D.; Kampichler, C.; Kandeler, E.; Tscherko, D. Six years of in situ CO_2 enrichment evoke changes in soil structure and soil biota of nutrient poor grassland. *Glob. Chang. Biol.* **2003**, *9*, 585–600. [CrossRef]
12. Dorodnikov, M.; Blagodatskaya, E.; Blagodatsky, S.; Kuzyakov, Y.; Marhan, S. Stimulation of microbial extracellular enzyme activities by elevated CO_2 depends on soil aggregate size. *Glob. Chang. Biol.* **2009**, *15*, 1603–1614. [CrossRef]
13. Saurav Sahaa, D.; Chakrabortya, V.K.; Sehgal, L.N.; Madan, P. Long-term atmospheric CO_2 enrichment impact on soil biophysical properties and root nodule biophysics in chickpea (*Cicer arietinum* L.). *Eur. J. Agron.* **2016**, *75*, 1–11. [CrossRef]
14. Norby, R.J.; Zak, D.R. Ecological lessons from Free-Air CO_2 Enrichment (FACE) experiments. *Ann. Rev. Ecol. Evol. Syst.* **2011**, *42*, 181–203. [CrossRef]
15. Del Galdo, I.; Oechel, W.C.; Cotrufo, M.F. Effects of past, present and future atmospheric CO_2 concentrations on soil organic matter dynamics in a chaparral ecosystem. *Soil Biol. Biochem.* **2006**, *38*, 3235–3244. [CrossRef]
16. Eviner, V.T.; Stuart Chapin, F. The influence of plant species, fertilization and elevated CO_2 on soil aggregate stability. *Plant Soil* **2002**, *246*, 211–219. [CrossRef]
17. Rillig, M.C.; Wright, S.F.; Allen, M.F.; Field, C.B. Rise in carbon dioxide changes soil structure. *Nature* **1999**, *400*, 628. [CrossRef]
18. Hoosbeek, M.; Vos, J.M.; Bakker, E.J.; Scarascia-Mugnozza, G.E. Effects of free atmospheric CO_2 enrichment (FACE), N fertilization and poplar genotype on the physical protection of carbon in the mineral soil of a polar plantation after five years. *Biogeosciences* **2006**, *3*, 479–487. [CrossRef]
19. Tisdall, J.M.; Oades, J.M. Organic matter and water-stable aggregates in soils. *J. Soil Sci.* **1982**, *33*, 141–163. [CrossRef]
20. Abiven, S.; Menasseri, S.; Chenu, C. The effects of organic inputs over time on soil aggregate stability—A literature analysis. *Soil Biol. Biochem.* **2009**, *41*, 1–12. [CrossRef]
21. Teklehaimanot, Z.; Jones, M.; Sinclair, F. Tree and livestock productivity in relation to tree planting configuration in a silvopastoral system in North Wales, UK. *Agrofor. Syst.* **2002**, *56*, 47–55. [CrossRef]
22. Hoosbeek, M.; Lukac, M.; Velthorst, E.; Smith, A.; Godbold, D. Free atmospheric CO_2 enrichment increased above ground biomass but did not affect symbiotic N_2-fixation and soil carbon dynamics in a mixed deciduous stand in Wales. *Biogeosciences* **2011**, *8*, 353–364. [CrossRef]
23. Scullion, J.; Smith, A.R.; Gwynn-Jones, D.; Jones, D.L.; Godbold, D. Deciduous woodland exposed to elevated atmospheric CO_2 has species-specific impacts on anecic earthworms. *Appl. Soil Ecol.* **2014**, *80*, 84–92. [CrossRef]
24. Scullion, J.; Malik, A. Earthworm activity affecting organic matter, aggregation and microbial activity in soils restored after opencast mining for coal. *Soil Biol. Biochem.* **2000**, *32*, 119–126. [CrossRef]
25. Harborne, J.B.; Harborne, A.J. *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*; Taylor and Francis Group: London, UK, 1998; p. 302.
26. Vance, E.; Brookes, P.; Jenkinson, D. Microbial biomass measurements in forest soils: The use of the chloroform fumigation-incubation method in strongly acid soils. *Soil Biol. Biochem.* **1987**, *19*, 697–702. [CrossRef]
27. Hanssen, J.F.; Thingstad, T.F.; Goksøyr, J. Evaluation of hyphal lengths and fungal biomass in soil by a membrane filter technique. *Oikos* **1974**, *25*, 102–107. [CrossRef]
28. Olson, F. Quantitative estimates of filamentous algae. *Trans. Am. Microsc. Soc.* **1950**, *69*, 272–279. [CrossRef]
29. Campbell, C.D.; Chapman, S.J.; Cameron, C.M.; Davidson, M.S.; Potts, J.M. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. *Appl. Environ. Microbiol.* **2003**, *69*, 3593–3599. [CrossRef] [PubMed]
30. Rowell, D.L. *Soil Science: Methods and Applications*; Addison Wesley Longman: Harlow, UK, 1994; p. 117.
31. Ball, D. Loss-on-ignition as an estimate of organic matter and organic carbon in non-calcareous soils. *Eur. J. Soil Sci.* **1964**, *15*, 84–92. [CrossRef]

32. Milne, R.; Haynes, R. Soil organic matter, microbial properties, and aggregate stability under annual and perennial pastures. *Biol. Fertil. Soils* **2004**, *39*, 172–178. [CrossRef]
33. Gil-Sotres, F.; Trasar-Cepeda, M.; Ciardi, C.; Ceccanti, B.; Leirós, M. Biochemical characterization of biological activity in very young mine soils. *Biol. Fertil. Soils* **1992**, *13*, 25–30. [CrossRef]
34. Yoder, R. A direct method of aggregate analysis of soils and a study of the physical nature of erosion losses. *J. Am. Soc. Agron.* **1936**, *28*, 337–351. [CrossRef]
35. Haynes, R.; Fraser, P. A comparison of aggregate stability and biological activity in earthworm casts and uningested soil as affected by amendment with wheat or lucerne straw. *Eur. J. Soil Sci.* **1998**, *49*, 629–636. [CrossRef]
36. Tiunov, A.V.; Scheu, S. Microbial biomass, biovolume and respiration in *Lumbricus terrestris* L. cast material of different age. *Soil Biol. Biochem.* **2000**, *32*, 265–275. [CrossRef]
37. Taneva, L.; Gonzalez-Meler, M.A. Decomposition kinetics of soil carbon of different age from a forest exposed to 8 years of elevated atmospheric CO_2 concentration. *Soil Biol. Biochem.* **2008**, *40*, 2670–2677. [CrossRef]
38. Gentile, R.; Vanlauwe, B.; Six, J. Litter quality impacts short- but not long-term soil carbon dynamics in soil aggregate fractions. *Ecol. Appl.* **2011**, *21*, 695–703. [CrossRef] [PubMed]
39. Niklaus, P.A.; Alphei, J.; Kampichler, C.; Kandeler, E.; Korner, C.; Tscherko, D.; Wohlfender, M. Slowed-down soil drying cycles resulted in lower soil aggregation under elevated CO_2. *Ecology* **2007**, *88*, 3153–3163. [CrossRef] [PubMed]
40. Haynes, R. Labile organic matter as an indicator of organic matter quality in arable and pastoral soils in New Zealand. *Soil Biol. Biochem.* **2000**, *32*, 211–219. [CrossRef]
41. Abiven, S.; Menasseri, S.; Angers, D.; Leterme, P. Dynamics of aggregate stability and biological binding agents during decomposition of organic materials. *Eur. J. Soil Sci.* **2007**, *58*, 239–247. [CrossRef]
42. Six, J.; Carpentier, A.; van Kessel, C.; Merckx, R.; Harris, D.; Horwath, W.R.; Lüscher, A. Impact of elevated CO_2 on soil organic matter dynamics as related to changes in aggregate turnover and residue quality. *Plant Soil* **2001**, *234*, 27–36. [CrossRef]