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Effects of four years of elevated ozone on microbial biomass and extracellular enzyme activities in a semi-natural grassland

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Abstract

Reduced belowground carbon (C) allocation by plants exposed to ozone may change properties and activities of the microbial community in soils. To investigate how soil microbial biomass and extracellular enzyme activities respond to elevated ozone, we collected soils from a temperate grassland after four years of ozone exposure under fully open-air field conditions. We measured soil microbial biomass, the metabolism of low molecular weight C substrates and hydrolytic extracellular enzyme activities in both bulk soil and isolated aggregates to assess changes in microbial activity and community function. After four years of elevated ozone treatment, soil total organic C was reduced by an average of 20% compared with ambient condition. Elevated ozone resulted in a small but insignificant reduction (4–10%) in microbial biomass in both bulk soil and isolated aggregates. Activities of extracellular enzymes were generally not affected by elevated ozone, except β-glucosidase, whose activity in bulk soil was significantly lower under elevated ozone than ambient condition. Activities of β-glucosidase, leucine aminopeptidase and acid phosphatase were higher in microaggregates (< 0.25 mm) as compared to macroaggregates (> 0.25 mm). Elevated ozone had no effects on mineralization rates of low molecular weight C substrates in both bulk soil and isolated aggregates. We therefore conclude that the size and activity rather than function of the soil microbial community in this semi-natural grassland are altered by elevated ozone.

Keywords: (semi-)natural vegetation, climate change, hydrolytic enzymes, FACE, soil aggregates

1. Introduction

Tropospheric ozone is currently considered to be a key air pollutant because of its negative impact on plant productivity in most parts of the world (Ashmore, 2005; Fuhrer, 2009). During the past three decades, the background concentration of tropospheric ozone over the Northern Hemisphere midlatitudes has increased at a rate of 0.5–2% per year (Vingarzan, 2004). Further increases in the Northern Hemisphere background ozone concentrations may occur over this century if current emission trends continue (Meehl et al., 2007), although this view is being questioned (Oltmans et al., 2013; Ridley et al., 2017). Studies exploring ecosystem responses to elevated ozone have received widespread attention in the last two decades. There is mounting evidence that increasing tropospheric ozone concentration has many direct effects on plants, including lower net primary productivity (Ainsworth, 2008; Feng et al., 2008; Mills et al., 2018; Morgan et al., 2003), changes in plant chemistry (Booker et al., 2005; Kasurinen et al., 2007; Morgan et al., 2003), reduced stomatal conductance of plants (Feng et al., 2008; VanLoocke et al., 2012; Wittig et al., 2007), reduced root growth (Grantz et al., 2006), as well as altered root longevity and turnover (Andersen, 2003).

In contrast to the aboveground part, belowground processes in soils in response to elevated ozone have received less attention, despite its critical roles in biogeochemical cycles (Agathokleous et al., 2016; Andersen, 2003; Fuhrer et al., 2016). Since the penetration of ozone into the soil is limited (Toet et al., 2009), the indirect effects of ozone exposure on belowground communities and ecosystem processes are primarily due to reduced C allocation below ground. The belowground components (e.g. soil microorganisms) responses to elevated

ozone in terrestrial ecosystems occurs indirectly through plant-derived deposits, which has not been well documented. Under fully open-air field conditions or in open-top chambers, for example, how the composition and structure of the soil microbial community respond to elevated ozone has been examined in a soybean field (He et al., 2014), a wheat field (Li et al., 2013), a subarctic forest (Kasurinen et al., 2005), a temperate forest (Phillips et al., 2002) and a hay meadow (Kanerva et al., 2008). However, the results in these studies are conflicting, showing that elevated ozone altered (He et al., 2014; Kanerva et al., 2008; Kasurinen et al., 2005; Phillips et al., 2002) or had no significant effect (Li et al., 2013) on the composition and structure of the soil microbial community. Thus, while the inconsistent findings have often been attributed to the differences in experimental durations and other factors (e.g. fumigation facility, ecosystem type and management regime), this reflects an incomplete understanding of the response of soil microorganisms to elevated ozone.

Soil microorganisms are the main sources of crucial enzymes in the cycling of main nutrients (e.g. C, N and P). Moreover, soil enzyme activities are highly sensitive to environmental changes and could serve as indicators of various changes in the plant-soil system (Burns et al., 2013; Saiya-Cork et al., 2002). Activities of extracellular enzymes are strongly regulated by the presence of plants, and the release of labile substrates by living roots into soil enhances extracellular enzyme activities (Nannipieri et al., 2002). Therefore, the aforementioned changes in belowground plant growth under elevated ozone could have the potential to alter both substrate availability and extracellular enzyme activities (Andersen, 2003). Studies in aspen and aspen-birch forest ecosystems have shown that elevated ozone significantly reduced cellobiohydrolase activity but did not affect N-acetyl-glucosaminidase

78 activity in the forest floor after 2- or 10-year treatment (Edwards and Zak, 2011; Larson et al.,
79 2002). In a lysimeter study with young planted beech, Esperschütz et al. (2009) reported that
80 soil extracellular enzyme activities were generally not affected after 4 years of ozone
81 treatment. In contrast, Williamson et al. (2010) measured the decomposition rates of wetland
82 plants exposed to elevated ozone and showed that the responses of activities of β-glucosidase
83 and N-acetyl-glucosaminidase to elevated ozone were species-dependent. Thus, how soil
84 extracellular enzyme activities respond to elevated ozone remains uncertain.

85 Soil aggregation physically protects certain soil organic matter (SOM) fractions via
86 influencing soil microbial communities and activities. In general, soil aggregates are
87 fractionated by three different approaches: wet-sieving (Six et al., 1998), dry-sieving (Chenu
88 and Cosentino, 2011) and optimal moisture (Dorodnikov et al., 2009; Kristiansen et al.,
89 2006). To link in situ microbial communities and activities with ecological processes, the
90 optimal moisture approach can provide an advantage of minimizing microbial responses to
91 lab processing for a wide-range of biological assays (i.e., microbial biomass and extracellular
92 enzyme activities) (Bach and Hofmockel, 2014). The reported decrease of the available
93 substrates under elevated ozone, through decreased C allocation and fluxes into belowground
94 components, are expected to affect microbial biomass and extracellular enzyme activities
95 (Andersen, 2003). However, less is known about how extracellular enzyme activities respond
96 to elevated ozone in either bulk soil or isolated aggregates.

97 In this study, we aimed (i) to investigate changes in soil properties, microbial biomass
98 and extracellular enzyme activities in bulk soil after four years of elevated ozone treatment,

and (ii) to relate these changes observed in bulk soil to contrasting environment of differently sized aggregates. Given the aforementioned ozone effects on above- and belowground components, we hypothesized that field experimental exposure to elevated ozone in a grassland ecosystem would change soil microbial biomass and extracellular enzyme activities. For verifying this hypothesis, we collected soils from a temperate, semi-natural grassland after four years of ozone treatment under fully open-air field conditions.

2. Material and methods

2.1. Experiment site

Soil samples were taken from the ozone free-air controlled exposure (O_3-FACE) field located at CEH Bangor Air Pollution Facility, Abergwyngregyn, North Wales, UK (13 m asl, 53°15'N, 4°01'W). The study site has a temperate oceanic climate, with a mean annual soil temperature of 11°C at 10 cm depth and a mean annual rainfall of 1250 mm. The soil is classified as Eutric Cambisol (FAO) or Dystric Eutrudepts (US Soil Taxonomy) with a sandy clay loam texture, which is derived from Ordovician postglacial alluvial deposits. Vegetation was classified as *Lolium perenne leys* and related grasslands according to the UK National Vegetation Classification (MG7; Rodwell, 1992), without sheep grazing for more than 15 years prior to this study. No fertilizer was applied at this site throughout the experimental period. Grass was cut 2–3 times during each growing season.

The O_3-FACE system was established in the spring of 2014, consisting of nine rings of 4 m diameter. Three ozone treatments with three replicates, namely low (ambient air), medium

(ambient air + 10 ppb) and high (ambient air + 20 ppb), were randomly assigned to the rings (Table 1), where the latter two treatments are hereafter referred to as elevated ozone. The rings were arranged in a replicated 3 × 3 Latin square with 10 m between the centers of each ring. Ozone was generated by passing oxygen from a SeQual Integra 10 Oxygen Concentrator (SeQual Technologies, Inc., San Diego, CA, USA) through a Pacific Ozone G11 ozone generator (Benicia, California, USA). Small fans (Redring Xpelair Group Ltd, Southampton, UK) were used to push the ozone through the delivery pipe (65 mm, with 3 mm holes every 10 cm). Ozone delivery was achieved via computer controlled (LabView Version 2012, National Instruments) solenoid valves operating using pulse width modulation. Wind speed was monitored continuously (WindSonic, Gill Instruments Ltd, UK) and used to instantaneously adjust solenoid operation and thus ozone delivery. Ozone release was reduced at wind speeds below 16 m s^{-1} and did not occur when wind speeds fell below 2 m s^{-1}. Ozone was sampled adjacent to the plants in each ring at a height of 30 cm for approximately 3.5 min in every 30-min using an ozone analyzer (Thermo-Scientific, Model 49i, Reading, UK). Compared with previous studies using similar free-air systems (Paoletti et al., 2017; Watanabe et al., 2013), at very high wind speeds the ozone concentrations may not be well controlled and thus did not reach the target maximum concentrations. Despite this, we still got elevated ozone with the higher in the 'high' ozone treatment compared to that of the 'medium' treatment as the solenoid valves were <1 m from the O_3-FACE rings, the response time of ozone delivery to track windspeed was fast. Exposure to elevated ozone lasted from 17 July to 13 October in 2014, from 13 May to 11 September in 2015, from 1 June to 30 September in 2016, and from 25 May to 9 October in 2017. Ozone release was 93, 67, 93 and

99% of the time during the fumigation periods in the years 2014, 2015, 2016, and 2017, respectively.

2.2. Aggregate-size fractionation

Soil was collected from the top 10 cm of soil using 6.5 cm-diameter soil cores in November 2017. Three intact soil cores were collected from each ring, placed in CO_2 permeable polythene bags and then transported to the laboratory. Each soil core was gently broken up along natural points of weakness and passed through an 8-mm sieve, removing visible roots and rocks. Replicated soil cores were combined into one composite sample for each ring and then stored at 4 ºC to await further analysis. Prior to aggregate-size fractionation, subsamples of bulk soil were obtained from the cold-dried soils. Similar to previous studies (Bach and Hofmockel, 2014; Kristiansen et al., 2006), the optimal moisture approach was used for aggregate isolation to minimize microbial responses to lab processing for the following biological assays. Briefly, soils were cold dried at 4 ºC to approximately 10% gravimetric water content. Approximately 400 g of cold-dried soil was placed on a stack of sieves including 2 mm- and 0.25 mm-mesh openings. The stack was bolted to a circular sieve shaker intend for soil particle analysis and shaken at 200–250 rpm for 3 min. Soil was gently removed from each sieve and weighed to determine the mass distribution of aggregates into the following fractions: large macroaggregates (>2 mm), small macroaggregates (0.25–2 mm) and microaggregates (<0.25 mm). Subsamples of bulk soil and individual aggregate-size fractions were saved to determine gravimetric water content, total C, microbial biomass and

mineralization rates of low molecular weight C substrates. Subsamples for the enzyme assay detailed below were frozen immediately at –20 ºC until analysis.

2.3. Soil analysis

Bulk density was determined after insertion of 100 cm^3 metal rings into the soil, removal of soil, and drying at 105°C (24 h). Bulk density was calculated by dividing soil mass by core volume. Soil characteristics of both bulk soil and aggregate fractions were determined. Soil water content was determined gravimetrically by drying soil at 105 °C (24 h). Soil pH was measured using standard electrodes in a 1:2.5 (*w*/*v*) soil-to-deionized water mixture. Subsamples of bulk soil and aggregate fractions were directly extracted with 0.5 M K_2SO_4 (1:5 *w*/*v*) for available soil C and N pools measurement. For soil microbial biomass, additional subsamples were fumigated for 24 h with chloroform and similarly extracted with 0.5 M K_2SO_4 (1:5 *w*/*v*) (Vance et al., 1987). The 0.5 M K_2SO_4 extracts of non-fumigation and fumigation samples were quantified using a Multi N/C 2100 TOC analyzer (AnalytikJena, Jena, Germany) to determine soil dissolved organic C (DOC), microbial biomass C and N. Microbial biomass C and N concentrations were corrected using correction factors of 0.45 for C and 0.54 for N (Brookes et al., 1985; Wu et al., 1990). Total C (TC) and N (TN) of oven-dried and ground soils were determined with a TruSpec® elemental analyzer (Leco Corp., St Joseph, MI, USA). Based on the relative weight distribution of aggregates, the total microbial biomass C in different aggregates were recalculated for bulk soil. Net N mineralization and nitrification rates were determined by the aerobic incubation of soil samples for 14 days at

181 10 °C in the dark (Hart et al., 1994), followed by extraction with 0.5 M K_2SO_4 and analyzing
182 for soil mineral N as described above.

183 Carbon mineralization was estimated using a short-term incubation method following
184 Robertson et al. (1999). Briefly, 20 g fresh soils for bulk soil and aggregate fractions was
185 moistened to field moisture content (25%) with deionized water in a 1-L jar. The mason jar
186 was closed with airtight screw-cap lid, fitted with a gas sampling port (butyl rubber septum)
187 at the center, and was incubated at 10 ^{o}C for 21 d. Soil respiration were measured on 1, 3, 5,
188 7, 14 and 21 d after incubation by measuring CO_2 concentration in the headspace air samples
189 of the jar using a portable infrared gas analyzer (EGM-5 Environmental Gas Monitor for CO_2,
190 PP Systems, Hitchin, UK). Carbon mineralization rate was calculated and expressed as mg C
191 kg^{-1} h^{-1}.

192 In addition, the mineralization of glucose, amino acids and peptide were determined to
193 estimate rates of low molecular weight dissolved organic C and N following the method of
194 Hill et al. (2012). Briefly, 1 g fresh weight (equivalent to *c*. 0.87 g dry weight) soil was placed
195 into a 1.5-mL microcentrifuge tube in which a hole had been pierced in bottom. This
196 assembly was placed into another intact microcentrifuge tube. To the surface of the soil, 150
197 μL ^{14}C-labelled glucose (25 μM, 1.85 kBq mL^{-1}), amino acids (10 μM, 1.55 kBq mL^{-1}) and
198 peptide (10 μM of L-trialanine, 1 kBq mL^{-1}) were added. It has been suggested that an
199 incubation period of 3 min can reflect maximum variance between treatments (Hill et al.,
200 2012). Thus, these samples were incubated at 20°C for 3 min and then centrifuged at 4 000 *g*
201 for 1 min to facilitate collection of free soil solution. An aliquot of this solution was then

transferred to a 6-mL scintillation vial to which 4 mL Scintisafe3 Scintillation cocktail (Fisher Scientific, Loughborough, Leicestershire, UK) was added before analysis using a Wallac 1404 liquid scintillation counter (Wallac, EG&G, Milton Keynes, UK). The amino acids consisted of an equimolar mix of 20 different L-amino acids (glycine, isoleucine, arginine, glutamine, phenylalanine, histidine, asparagine, valine, threonine, leucine, alanine, methionine, cysteine, lysine, tryptophan, serine, proline, glutamate, aspartic acid and ornithine).

2.4. Enzyme assays

The potential activities of six extracellular hydrolytic enzymes: β-glucosidase, cellobiohydrolase, β-xylosidase, N-acetyl-glucosaminidase, leucine aminopeptidase and acid phosphatase were measured according to the fluorimetric protocol of Saiya-Cork et al. (2002) with modification by DeForest (2009). Briefly, 1 g of fresh soils was suspended in 125 mL sodium acetate buffer with pH adjusted to mean of soils. Soil suspensions were pipetted into 96-well microplates, and enzyme activities were determined by adding 4-methylumbelliferyl (MUB)- or 7-amino-4-methylcoumarin (AMC)-linked substrates for a final concentration of 40 μM. Assays were incubated in the dark for 2 h, and the reactions were stopped with 10 μL 0.5 M NaOH. The microplates were then scanned on a fluorescence spectrophotometer (Cary Eclipse, Agilent Technologies, Inc., Santa Clara, CA, USA) using the excitation and emission filters at 365 and 450 nm, respectively. Potential enzyme activity for bulk soil and aggregate-size fractions was expressed as MUB or AMC released in nanomol per gram of dry soil or aggregate and hour (nmol g^{-1} soil h^{-1} or nmol g^{-1} aggregate h^{-1}) as described previously

(DeForest, 2009). Specific activities of extracellular enzymes were also calculated as a measure of activity per unit microbial biomass and expressed as MUB or AMC released in nanomol per milligram microbial biomass C and hour (nmol mg^{-1} C_{mic} h^{-1}). The recovery of potential enzyme activity was calculated and expressed as a proportion of the bulk soil based on the weight distribution of aggregates.

2.5. Statistical analysis

All data were checked for assumptions of normality and log-transformed if necessary. A linear mixed effect model (LME, package LME4; Bates et al., 2014) was used to test ozone and/or aggregate-size class effects on investigated parameters with column and row included as random effects. Multiple comparisons between treatment means were conducted using post-hoc Tukey HSD tests (glht package: 'multcomp'). We accepted P values of $P \leq 0.05$ as significant and those with $P > 0.05$, but < 0.1 as marginally significant. All statistical analyses were performed in R version 3.2.2 (R Development Core Team, 2015).

3. Results

3.1. The O_3-FACE system

The semi-natural grassland was exposed to ozone under fully open-air field conditions from July 17, 2014 through to October 9, 2017 during the growing season, with an average of 101 days effective fumigation. Inter-annual variations in ambient ozone concentration (24 h means) showed only a small variation and ranged from 20.6 ppb in 2016 to 28.2 ppb in 2014 (Table 1). Across all years, mean ozone concentrations in medium and high ozone rings were

69 and 116% higher than that in ambient air, respectively. Accumulated exposures above a threshold of 40 ppb (AOT40) averaged 1.3±0.7 ppm h in the ambient rings, 14.0±3.6 ppm h in the medium ozone rings and 26.4±8.0 ppm h in the high ozone rings over the four-year period.

3.2. Soil properties, low molecular weight C substrate mineralization and enzyme activities in bulk soils

After 4 years of ozone treatment, soil total C and N were lower by an average of 20% and 16% under elevated ozone (medium and high ozone rings) than ambient ozone, respectively (both $P < 0.05$; Table 2), while soil bulk density, pH and C-to-N ratio did not differ between treatments. There was an apparent decrease in DOC and microbial biomass C in the elevated ozone treatments, which was not statistically significant when compared with those of the ambient ozone treatment. The ratios of microbial biomass C to total C were higher in the elevated ozone treatments than the ambient treatment ($P = 0.06$). Neither short-term C mineralization nor mineralization of low molecular weight C substrates for bulk soil was affected by elevated ozone.

Averaged over all treatments, higher extracellular enzyme activities in bulk soil were found for β-glucosidase and acid phosphatase (on average 293 and 578 nmol g^{-1} soil h^{-1}, respectively), while the other four enzymes showed lower and similar activities (Table 2). Elevated ozone significantly decreased β-glucosidase activity ($P < 0.05$) but not the activities of cellobiohydrolase, β-xylosidase, N-acetyl-glucosaminidase, leucine aminopeptidase and acid phosphatase in bulk soil.

3.3. Aggregate-size distribution, total C and microbial biomass C content

Elevated ozone did not affect the relative distribution of three aggregate fractions (Table 3). Large and small macroaggregates dominated in this grassland soil, whereas the microaggregate fraction accounted for a very small percentage of total soil mass ($P < 0.001$). The weight distribution among the aggregate-size classes of the bulk soil was as follows: large macroaggregates (>2 mm) contributed 52.1–57.4%, small macroaggregates (0.25–2 mm) 35.6–38.3% and microaggregates (<0.25 mm) 6.5–9.6% of the weight of bulk soil. Total C content were higher in the large macro- and microaggregate fractions than in the small macroaggregate fraction ($P < 0.001$) but did not significantly differ between ozone treatments within each aggregate fraction.

Across aggregate fractions, microbial biomass C showed a marginally significant reduction by an average of 10% under elevated ozone ($P = 0.086$; Table 3). There was no clear relationship between microbial biomass C and aggregate-size classes. Relative to the bulk soil, the total microbial biomass C in different aggregates showed approximately 100% recoveries across ozone treatments (Fig. 1A). The ratios of microbial biomass C to total C were affected by aggregate-size class ($P < 0.01$) and its interaction with ozone ($P = 0.064$; Fig. 1B).

3.4. Low molecular weight C substrate mineralization and enzyme activities in isolated aggregates

283 As with bulk soil, short-term C mineralization in isolated aggregates did not differ between
284 ozone treatments (Fig. 1C), though C mineralization rates in small macroaggregates and
285 microaggregates were lower by 32 and 31%, respectively under elevated ozone as compared
286 to ambient conditions. Neither ozone nor its interaction with aggregate-size class had effects
287 on mineralization rates of low molecular weight C substrates, except that stimulated glucose
288 mineralization was detected in the large macroaggregate from the high ozone treatment (Fig.
289 1D-F). It should be noted that the pronounced effects of aggregate-size class on
290 mineralization rates of low molecular weight C substrates were primarily due to
291 underestimated turnover in the large macroaggregates with a 3-min incubation period.

292 Activities of β-glucosidase, N-acetyl-glucosaminidase, leucine aminopeptidase and acid
293 phosphatase were distributed differently through aggregate-size classes ($P < 0.05–0.01$; Fig.
294 2). Across ozone treatments, activities of β-glucosidase and acid phosphatase were of the
295 order microaggregate > large macroaggregate > small macroaggregate. The lowest activity of
296 leucine aminopeptidase was found both in the high ozone treatment and small
297 macroaggregate fraction. Activities of cellobiohydrolase and β-xylosidase showed similar
298 across all aggregate-size classes irrespective of ozone. Since aggregate-size class had no
299 effect on microbial biomass, the patterns of specific activities of extracellular enzymes are
300 almost identical to patterns as seen above (data not shown). Cumulative proportional enzyme
301 activity in isolated aggregates did not differ from bulk soil, with somewhat larger variation
302 ranged from 89% to 144% across enzymes (data not shown).

303 **4. Discussion**

304 *4.1. Aggregate-size fractionation*

305 According to the concept of aggregate hierarchy (Tisdall and Oades, 1982), the bulk soil has
306 been fractionated into its constituent aggregates using different disruptive techniques (Chenu
307 and Cosentino, 2011; Mendes et al., 1999; Six et al., 1998). In this study, we chose the
308 optimal moisture sieving technique which allows limited mechanical stress to breakdown of
309 macroaggregates along the planes of weakness, releasing the microaggregates located on
310 surfaces of macroaggregates and along their planes of weakness (Dorodnikov et al., 2009;
311 Kristiansen et al., 2006). The small portion of microaggregates isolated in this study (6.5–
312 9.6%) was comparable to those reported in other studies (Bach and Hofmockel, 2016; Kumar
313 et al., 2017). This finding further supports the claim that free microaggregates and the
314 microaggregates adhering on the surface of macroaggregates are isolated. On the other hand,
315 the most distinguishing characteristics of optimal moisture sieving compared to the
316 conventional wet- and dry sievings is to minimize effects on the soil microbial community
317 and biological parameters. This is supported by our results showing that cumulative
318 recoveries of microbial biomass and enzyme activity were 99–102% and 89–144%,
319 respectively, across all treatments and enzymes.

320 The aggregate weight distribution detected here were in the order: large
321 macroaggregates > small macroaggregates > microaggregates (Table 3). This is in agreement
322 with other studies showing that large and small macroaggregates dominated in agricultural
323 soils (Bach and Hofmockel, 2014; Kristiansen et al., 2006; Kumar et al., 2017). The
324 distribution of aggregate-size classes was not altered after four years of ozone treatment,

325 although a significant reduction of root biomass under elevated ozone was detected (ambient
326 ozone: 1176±142 g m^{-2} vs. elevated ozone: 725±87 g m^{-2}; $P = 0.024$). Consistent with this
327 finding, the high plant density resulted in a two-fold increase of root biomass but had no
328 effect on aggregate redistribution in a maize field (Kumar et al., 2017). Consequently, our
329 findings indicate that elevated ozone had no effect on the distribution of soil aggregate-size
330 classes, although there are negative impacts of elevated ozone on root growth and
331 belowground C allocation (Andersen, 2003; Grantz et al., 2006).

332 *4.2. Effects of elevated ozone on microbial biomass in bulk soil and isolated aggregates*

333 Numerous studies have been conducted to assess the effect of elevated ozone on soil
334 microbial biomass, but the results remain controversial. Whereas some studies showed a
335 decrease in microbial biomass (Bao et al., 2015; Kanerva et al., 2008; Phillips et al., 2002),
336 others reported no difference (Cheng et al., 2011; Zhang et al., 2014) or even an increased
337 microbial biomass (Mörsky et al., 2008) from soils under elevated ozone. Our results support
338 those studies that found a negative response of soil microbial biomass to elevated ozone,
339 partly corroborating our initial hypothesis. Ozone exposure is considered to alter C flux to
340 soil via changes in rhizodeposition and litter quality or quantity (Andersen, 2003), and
341 therefore, the decreased microbial biomass in bulk soil is most likely due to reduced root
342 biomass and substrate availability under elevated ozone. Further, this is primarily associated
343 with a significant reduction of microbial biomass in the microaggregate fraction under
344 elevated vs. ambient ozone (Table 3). Since macroaggregates and microaggregates are
345 inhabited predominately by fungal and bacterial communities, respectively, we speculate that

346 bacterial communities in microaggregates might be strongly affected by elevated ozone in this
347 grassland soil. In contrast, some studies have shown that elevated ozone significantly reduced
348 both fungal biomass and the fungal-to-bacterial ratio, suggesting that fungi may be more
349 sensitive to elevated ozone as compared to bacteria (Kanerva et al., 2008; Li et al., 2013;
350 Phillips et al., 2002). This inconsistency could be due to the differences in ecosystem types,
351 experimental duration and methods, as well as environmental conditions. Nonetheless, we are
352 aware that the present study is the first to assess the response of microbial biomass to elevated
353 ozone among different aggregate fractions and further investigations are required.

354 The lack of correlation between soil microbial biomass and aggregate-size class
355 contradicts the findings of others in agricultural soils, where they found soil microbial
356 biomass were positively or negatively correlated with decreasing aggregate size (Dorodnikov
357 et al., 2009; Kumar et al., 2017). Different microbial biomass between microaggregates and
358 macroaggregates are often attributed to the contrasting environment of differently sized
359 aggregates, which in turn contributes to the differential distribution of bacteria and fungi in
360 micro- and macroaggregates (Chenu et al., 2001; Gupta and Germida, 1988; Jastrow et al.,
361 2007). Since the composition and structure of the soil microbial community were not
362 determined in isolated aggregates, we are not sure if the lack of correlation between microbial
363 biomass and aggregate-size class is related to changes in microbial communities. In a recent
364 review, Gupta and Germida (2015) also point out that further studies are warranted to
365 investigate the distribution and temporal dynamics of microbes in distinct aggregates. While
366 total organic C and microbial biomass C did not differ between ozone treatments within each
367 aggregate fraction, the reduced ratio of microbial biomass C to total organic C in

368 microaggregates may have contributed to the decline in total C in bulk soil under elevated
369 ozone (Sparling, 1992). In contrast, the increased ratio of microbial biomass C to total organic
370 C in the bulk soil under elevated ozone may be caused by decreases in total organic C content
371 rather than microbial biomass.

372 *4.3. Effects of elevated ozone on extracellular enzyme activities in bulk soil and isolated*
373 *aggregates*

374 As an overall indicator of microbial activity, the significantly lower activity of β-glucosidase
375 in bulk soil under elevated vs. ambient ozone supports the findings suggesting depressed
376 microbial activity due to reduced C allocation into the belowground ecosystem (Andersen,
377 2003). Further, the significant reduction of the ratio of the natural logarithm of *β*-glucosidase
378 and the sum of N-acetyl-glucosaminidase and leucine aminopeptidase in bulk soil indicates
379 that elevated ozone could stimulate microbes to produce enzymes towards acquisitions of
380 organic N (Sinsabaugh et al., 2008), despite the absence of ozone effect on individual
381 enzymes (Table 2). Chitin is one of the dominant sources of organic N to soil, and N-acetyl-
382 glucosaminidase releases small, N-containing amino sugars from chitin in addition to C
383 (Olander and Vitousek, 2000). In this grassland without fertilizers application and grazing for
384 a long-term period, elevated ozone might have resulted in microbially decomposing
385 recalcitrant organic matter for both energy source and nutrient demand (e.g. N). Thus, these
386 findings support our hypothesis regarding ozone effects on extracellular enzyme activities.
387 Yet, there are very few studies addressing the responses of extracellular enzyme activity to
388 elevated ozone and showing mixed results. For example, studies in aspen and aspen-birch

389 forest ecosystems reported that elevated ozone had no effects on enzyme activities in the
390 second year of treatment (Larson et al., 2002), whereas after 10 years cellobiohydrolase
391 activity was affected in the forest floor but N-acetyl-glucosaminidase remained unaffected
392 (Edwards and Zak, 2011). Further, Williamson et al. (2010) measured the decomposition rates
393 of wetland plants exposed to elevated ozone and concluded that the response of hydrolytic
394 enzyme activity to ozone was species dependent. Collectively, these conflicting results
395 indicates that ozone effects on extracellular enzymes remain poorly understood and further
396 work is needed.

397 Across all enzymes, enzyme activities were somewhat higher in microaggregates than in
398 macroaggregates irrespective of ozone treatment. This is consistent with the previous findings
399 showing that the highest enzyme activities occurred in microaggregates, especially for β-
400 glucosidase (Dorodnikov et al., 2009; Kumar et al., 2017). We found that enzyme activities in
401 isolated aggregates generally equaled or exceeded those in bulk soil and may have been even
402 greater if there were enzyme losses during the aggregate fractionation. This supports the
403 findings by several researchers who reported similar or higher recovery of enzyme activity in
404 isolated aggregates as compared to the bulk soil (Allison and Jastrow, 2006; Bach and
405 Hofmockel, 2014; Dorodnikov et al., 2009). This indicates that a lack of enzyme activity
406 might be not responsible for C accumulation associated with soil aggregation. In addition,
407 elevated ozone affected neither enzyme activities nor low molecular weight C substrate
408 mineralization within each aggregate fraction, suggesting that substrate utilization patterns of
409 soil microbial communities were unchanged.

5. Conclusions

To our knowledge the present study is the first to assess the responses of microbial biomass and extracellular enzyme activities in bulk soil and isolated aggregates to elevated ozone under O_3-FACE conditions. Our results demonstrated that elevated ozone for a period of four years had negative impacts on both soil C sequestration and total microbial biomass activity (i.e., decreased microbial biomass and β-glucosidase activity), which was mainly due to reduced belowground C allocation. Ozone exposure did not affect soil aggregation in this semi-natural grassland, probably contributing to the absence of effects of ozone and its interaction with aggregate-size class on low molecular weight C substrate utilization and extracellular enzyme activities. It should also be noted that the small, statistically insignificant changes (e.g. microbial biomass) could be associated with high variability. Therefore, our results suggest that changes in the quantity and quality of plant C inputs at elevated ozone can contribute to reduce soil total C content but not to alter the function of the soil microbial community in this semi-natural grassland.

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639

Table 1 Mean ozone concentrations (24 h), mean daily maximum ozone concentration and AOT40 in daylight hours (08:00 to 20:00 GMT) measured in the ozone free-air controlled exposure (O_3-FACE) experiment at CEH Bangor Air Pollution Facility during the growing seasons in 2014–2017. Values represent means ± SEM (n = 3)

	July–October 2014			May–September 2015			June–September 2016			May–October 2017		
Ozone level	Mean conc. (ppb)	Daily max. (ppb)	AOT40 (ppm h)	Mean conc. (ppb)	Daily max. (ppb)	AOT40 (ppm h)	Mean conc. (ppb)	Daily max. (ppb)	AOT40 (ppm h)	Mean conc. (ppb)	Daily max. (ppb)	AOT40 (ppm h)
Low	28.2±1.2	39.9±1.4	1.1±0.2	28.1±0.4	40.5±0.5	3.5±0.5	20.6±0.1	31.8±0.8	0.4±0.0	22.9±0.6	32.8±0.5	0.3±0.0
Medium	36.8±4.0	68.1±11.1	7.1±3.1	40.5±5.4	71.4±15.9	8.4±2.2	43.3±3.6	77.4±6.6	20.3±4.8	44.1±2.5	86.1±4.2	20.2±4.7
High	49.5±5.8	99.9±12.5	16.2±5.2	40.4±1.6	67.8±2.9	11.2±1.1	62.6±7.7	101.5±11.2	46.2±10.3	54.9±6.1	106.5±12.3	31.9±10.1

644 **Table 2** Soil characteristics, mineralization rates of low molecular weight C substrates and

645 potential extracellular enzyme activity under different ozone treatments

	Ozone level			
	Low	Medium	High	*P* value
Total C (g C kg^{-1})	39.8±1.7	31.3±0.4	32.6±2.2	*
Total N (g N kg^{-1})	3.5±0.2	2.8±0.1	3.0±0.2	*
C:N ratio	11.6±0.9	11.2±0.3	10.8±0.4	*NS*
Bulk density (g cm^{-3})	0.83±0.01	0.83±0.02	0.87±0.01	*NS*
pH	5.1±0.2	5.3±0.1	5.1±0.1	*NS*
Dissolved organic C (mg C kg^{-1})	215±11	192±12	202±12	*NS*
Microbial biomass C (mg C kg^{-1})	903±37	889±33	849±51	*NS*
Microbial biomass N (mg N kg^{-1})	95±3	107±6	96±10	*NS*
Microbial biomass C-to-N ratio	9.5±0.5	8.4±0.2	8.9±0.4	*NS*
Microbial biomass C-to-total C ratio (%)	2.27±0.08	2.84±0.08	2.61±0.17	•
C mineralization (mg C kg^{-1} h^{-1})	1.13±0.13	0.75±0.08	1.18±0.29	*NS*
Glucose mineralization (mg C kg^{-1} h^{-1})	1.27±0.12	1.32±0.26	1.34±0.10	*NS*
Amino acids mineralization (mg N kg^{-1} h^{-1})	0.15±0.01	0.18±0.02	0.17±0.00	*NS*
Peptide mineralization (mg N kg^{-1} h^{-1})	0.31±0.02	0.30±0.04	0.30±0.02	*NS*
β-glucosidase (nmol g^{-1} soil h^{-1})	332±28	293±20	224±11	*
Cellobiohydrolase (nmol g^{-1} soil h^{-1})	50.5±10.7	74.0±10.7	54.4±15.5	*NS*
N-acetyl-glucosaminidase (nmol g^{-1} soil h^{-1})	40.7±3.1	47.7±9.2	53.0±0.6	*NS*
β-xylosidase (nmol g^{-1} soil h^{-1})	39.0±3.8	47.1±4.3	39.8±8.1	*NS*
Leucine aminopeptidase (nmol g^{-1} soil h^{-1})	19.5±0.9	23.7±3.4	20.1±1.1	*NS*
Acid phosphatase (nmol g^{-1} soil h^{-1})	537±34	535±36	599±108	*NS*

646 Values represent means ± SEM (n = 3). Statistical results from linear mixed effect model with

647 ozone as a fixed factor and column/row as random effects are reported. *NS*, • and * indicate

648 not significant ($P \geq 0.1$), significant difference at $P < 0.1$ and $P < 0.05$, respectively.

649

650 **Table 3** Aggregate-size distribution, organic C content and microbial biomass C in soil aggregates under different ozone treatments

	Weights distribution (%)			C content (g C kg^{-1})			Microbial biomass C (mg C kg^{-1})		
Aggregate-size class	Low	Medium	High	Low	Medium	High	Low	Medium	High
> 2 mm	57.4±3.2	56.6±1.6	52.1±1.4	41.0±2.4	36.8±2.1	35.9±1.0	912±47	829±24	891±43
0.25–2 mm	36.1±2.5	35.6±2.1	38.3±1.9	32.0±2.8	31.8±0.4	28.8±0.2	896±9	935±5	848±86
< 0.25 mm	6.5±0.9	7.8±0.5	9.6±0.4	37.1±1.6	36.5±1.6	36.8±1.3	1160±92	958±65	819±34
Ozone	*NS*			*NS*			•		
Aggregate size	***			***			*NS*		
Interaction	*NS*			*NS*			•		

651 Values represent means ± SEM (n = 3). Statistical results from linear mixed effect model with ozone and aggregate-size class as fixed factors and column/row

652 as random effects are reported. *NS*, • and *** indicate not significant ($P \geq 0.1$), significant difference at $P < 0.1$ and $P < 0.001$, respectively.

Figure captions

Fig. 1 Microbial biomass C recovery, microbial biomass C-to-total C ratio, short-term C mineralization, mineralization rates of low molecular weight C substrates (glucose, amino acids (AAs) and peptide) in three aggregate fractions under different ozone treatments. Values represent means ± SEM (n =3). See text for further explanation on statistical results from linear mixed effect model with ozone and aggregate-size class as fixed factors and column/row as random effects.

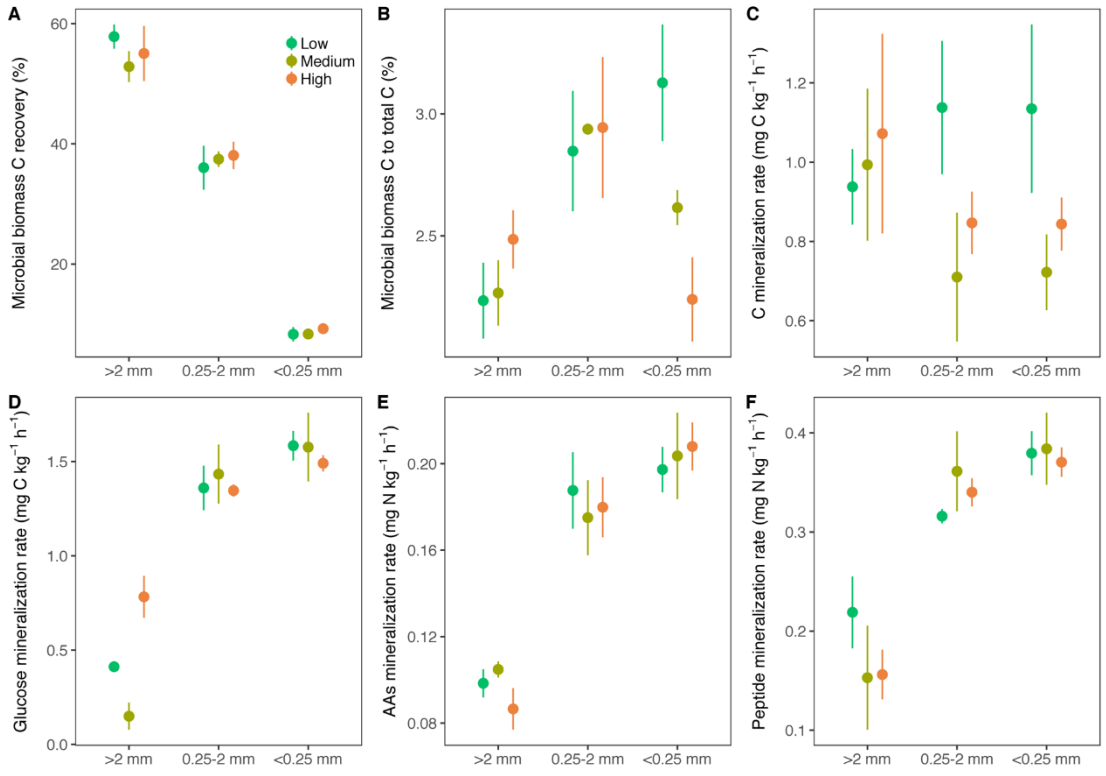

Fig. 2 Potential activities of *β*-glucosidase, cellobiohydrolase, *β*-xylosidase, N-acetyl-glucosaminidase, leucine aminopeptidase and acid phosphatase in three aggregate fractions under different ozone treatments. Values represent means ± SEM (n =3). See text for further explanation on statistical results from linear mixed effect model with ozone and aggregate-size class as fixed factors and column/row as random effects.