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Warming and ozone exposure effects on silver birch (*Betula pendula* Roth) leaf litter quality, microbial growth and decomposition

ANNE KASURINEN[1*], TARJA SILFVER[2], MATTI ROUSI[3] & JUHA MIKOLA[2]

1) Department of Environmental and Biological Sciences, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland. Email: Anne.Kasurinen@uef.fi

2) Department of Environmental Sciences, University of Helsinki, Niemenkatu 73, FI-15140 Lahti, Finland. Emails: Tarja.Silfver@helsinki.fi, Juha.Mikola@helsinki.fi

3) Natural Resources Institute Finland, Vantaa Research Unit, FI-01301 Vantaa, Finland. Email: Matti.Rousi@luke.fi

***Anne Kasurinen, phone: +358-40-355 3997; e-mail: Anne.Kasurinen@uef.fi

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Abstract

Background and aims Climate warming is expected to accelerate decomposition in boreal forests, but the concomitant effects of tropospheric ozone (O_3), a phytotoxic greenhouse gas, alone and in combination with warming, are poorly understood. We studied how these two climatic factors affect leaf litter decomposition of two silver birch genotypes.

Methods We used field exposure for growing saplings and native and reciprocal transplant experiments for litter incubation to disentangle environmental and litter quality mediated effects of temperature and O_3 on litter mass loss. We analysed litter C% and N% and microbial biomass (using qPCR) in fresh litter and after 217 and 257 days of incubation.

Results Warming decreased fresh litter C% and N% and bacterial DNA, whereas elevated O_3 increased N% and bacterial and fungal DNA, equally for both genotypes. In contrast, most effects on microbes during litter incubation varied between the two genotypes. Warming effects on microbes were mainly environmental, but despite having effects on litter quality and microbial growth, warming and O_3 both had only weak or no effects on litter mass loss.

Conclusions Litter quality and microbial growth in northern birch stands are likely to change due to warming and O_3 exposures, but effects on litter decomposition rate may remain weak.

Introduction

Boreal forests are already experiencing the climate change. Global surface temperatures will continue to rise and the projected temperature change by the end of the 21st century ranges from 2 to 5 °C relative to the preindustrial values (IPCC 2013). In Finland, especially the wintertime temperatures are expected to rise, but also the growing seasons will become warmer and longer (Jylhä et al. 2009; Ruosteenoja et al. 2011). Besides causing warming, human activities change the chemical composition of the troposphere, and for instance the ozone (O_3) levels have significantly increased in the Northern Hemisphere (Vingarzan et al. 2004, The Royal Society 2008, Zeng et al. 2008, Young et al. 2013). At the moment, the Northern Hemisphere O_3 levels range from 30 to 40 ppb (Cooper et al. 2014), but it seems that O_3 production is levelling off in some parts of Europe, and estimates of surface O_3 changes in the near future vary from slight decrease to no change, or increases in the most polluted cases only (Oltmans 2013, IPCC 2013). However, according to Wittig et al. (2009), even the current O_3 levels are sufficient to cause harmful effects on forest trees, including e.g., reduced gas exchange rates and tree growth.

The proportion of silver birch (*Betula pendula* Roth) has been predicted to increase in Finnish forests by the end of this century (Kellomäki et al. 2008). This is likely to affect litter decomposition rates as the soil beneath silver birch trees has higher soil microbial biomass and C mineralization rates than the soil in the vicinity of coniferous trees (Saertre et al. 1999, Priha et al. 2001). In future, direct effects of climate warming on decomposition rates are also likely in the northern areas. For instance, Cornelissen et al. (2007) showed that litter decomposition was on average 42 % faster when the soil temperature was increased by 3.7 °C. This effect may not be universal, however, and in a recent infrared heater study, the decomposition rates in heated plots were only slightly faster than in the non-heated plots (Gong et al. 2015). The effects of warming on leaf litter decomposition process can also be

mediated by changes in leaf litter quality and the responses of decomposer organisms (Aerts 2006, Berg and Laskowski 2006). Leaf N concentrations seem to increase due to experimental warming (Bai et al. 2013), but there are also exceptions: e.g. no clear change was reported by Hudson et al. (2011) and a decrease by Sandvik and Eide (2011). Warming can also increase C concentrations in the leaves, but the magnitude of this response is likely to be plant species-specific (Sandvik and Eide 2011). Microbial responses to warming have been measured in a few field experiments only, and so far the results have been mixed. Zhang et al. (2005) observed a shift towards fungal dominance after three years of warming of tall grass prairie soil and air (2 °C increase), whereas fifteen years of warming of subarctic heath soil (1–2 °C increase) led to a significant reduction in the relative abundance of fungi (Rinnan et al. 2007). In another long-term experiment, soil warming (5°C increase for 15 years) decreased microbial activity and biomass and especially, the total quantity of fungal biomarkers (Frey et al. 2008).

In contrast to warming, tropospheric O_3 effects on litter decomposition are considered to be mainly driven by litter quality changes (Andersen 2003). So far, the field studies have revealed complex litter quality and decomposition responses to the O_3 exposure (Liu et al. 2005, Kasurinen et al. 2007, Parsons et al. 2008). For instance, Kasurinen et al. (2006) observed minor O_3 effects on the chemical quality of leaf litter, but nonetheless reduced decomposition rates during an 11-month-incubation period. Similarly, Parsons et al. (2008) reported that elevated O_3 decreased leaf litter decomposition in aspen (*Populus tremuloides* Michaux) despite small changes in litter C:N-ratios and N concentrations. In contrast to the aspen response, however, decomposition of paper birch (*Betula papyrifera* Marsh.) litter was accelerated and litter C:N-ratio decreased under elevated O_3 (Parsons et al. 2008). Based on current evidence, the O_3 effects on soil microbial biomass and enzyme activities under the trees are equally unpredictable. Larson et al. (2002) did not observe any

clear effects on soil microbial biomass in response to elevated O_3 beneath three temperate tree species, whereas Pritsch et al. (2009) observed that a three-year-long O_3 exposure decreased soil microbial activity and the abundance of bacterial biomarkers, but not the fungal biomarkers, in the top soil of lysimeters with young beech (*Fagus sylvatica* L.) saplings. Opposite to Pritsch et al. (2009), Esperschütz et al. (2009) observed that young beech saplings, when planted outside the lysimeters, had increased soil microbial biomass under increased O_3 at the end of the growing season, the response likely being due to earlier senescence of plants exposed to O_3.

Earlier studies suggest that silver birch sensitivity to O_3 exposure (Oksanen et al. 2007, 2009) and warming (Kasurinen et al. 2012) vary among genotypes. In addition, the intrapopulation genotypic variation of silver birch litter decomposition is known to be substantial (Silfver et al. 2007). Here we exposed young birch saplings to warming and O_3 treatments in an open-air exposure system (Hartikainen et al. 2009; Kasurinen et al. 2012). To shed light on the magnitude of genotypic variation in such effects, we used two silver birch genotypes, whose carbon allocation patterns have earlier been found to respond differently to warming and ozone treatments (Kasurinen et al. 2012). The decomposition of naturally senesced leaf litter was followed in an experimental-setup, which allowed us to disentangle the environmental and litter-mediated effects of warming and ozone on litter mass loss and microbes growing on the litter during the incubation period. We also examined the relationship between the initial litter quality, microbes and mass loss in the two genotypes over the litter incubation period. In short, our experimental set-up consisted of three litterbag experiments, where we followed decomposition and microbial responses (i) in litters that were produced under warming and ozone treatments and incubated either at their original growth environment (Native Replacement experiment) or ambient control conditions (Common Garden experiment) and (ii) in litter that was produced in ambient conditions and

incubated in different temperature and O_3 regimes (Common Substrate experiment). We hypothesized that 1) warming will affect microbial abundance and decomposition rates directly (environmental effect) or indirectly by improving litter quality (litter-mediated effect), whereas-2) the ozone effects on litter quality and microbial growth will be minor and the effects on decomposition indirect. In addition, we hypothesized that 3) in treatment combinations, the ozone effects may cancel out the warming effects and both warming and ozone effects will depend on silver birch genotype (Kasurinen et al. 2012).

Materials and methods

Open-air exposure experiment: tree material, experimental design and exposures

The intrapopulation genetic variation of silver birch has been studied since 1997, when 30 different clones were selected from a naturally regenerated mixed birch stand to represent the magnitude of genetic variation in local birch populations (Laitinen et al. 2000). These clones or different subsets of these clones have since been used in several experiments, including those that have studied the genotypic variation in leaf litter decomposition (Silfver et al. 2007, 2015). For this experiment, we used a subset of two silver birch clones (gt14 and gt15), which have been previously found to have a contrasting carbon allocation response to warming and ozone treatments (Kasurinen et al. 2012). Our clones were micropropagated in the Natural Resources Institute Finland, Luke, Haapastensyrjä using material collected from the original mother trees in March 2009 and transferred to the Research Garden, UEF, Kuopio in April 2009. Plantlets were hardened for outdoor conditions in May, and altogether 192 birches were planted into plots of mull:sand mixture (2:1, v:v) at the Ruohoniemi open-air exposure site (62º53'N, 27º37'E, 80 masl) in the beginning of June. The mull:sand mixture (hereafter called soil) was not sterilised, neither was any microbial inoculation added

before the planting.

The experimental birches were planted into four ambient and four elevated O_3 open-air exposure plots (each Ø 10m), each plot being further divided into two infrared-heated and two ambient temperature subplots (Hartikainen et al. 2009; Kasurinen et al. 2012). This arrangement resulted in four different treatment combinations: 1) control (C) = ambient O_3 and air temperature, 2) elevated temperature (T), 3) elevated O_3 (O) and 4) elevated temperature and elevated O_3 (TO), each with four replicates. Each subplot (1.9 m x 1.4 m) had six plantlets of each genotype, planted in two adjacent rows circa 0.2 m apart. Additional birches were planted around the experimental saplings to protect them from edge effects. All the birches were watered when soil moisture in subplots and pots fell below 10 vol%, but fertilized only in the beginning of the experiment with a N dose of 5 kg/m^3 soil (Peatcare Slow Release, N:P:K, 9:3.5:5, Yara).

The warming and ozone exposures were run from June 9 to September 30, 2009, and from May 5 to September 2, 2010. In both years, the O_3 levels in elevated O_3 plots were 1.4 x times the ambient levels (Table 1). Ozone was generated from pure oxygen using an ozone generator (Ozone Generator G21, Pacific Ozone Technology Inc., Brentwood, CA, USA) and released into the treatment plots through vertical perforated tubes from the upwind direction as described in Karnosky et al. (2007). Ozone concentrations were constantly monitored at 1.5 m height in the centre of each plot using UV photometric ozone analyzers (Model 1008-RS, Dasibi Environmental Corp., Glendale, CA; Model $O_3$42 Module, Environnement S.A., Poissy, France). Each plot was also continuously monitored for wind speed and direction (Anemometer A100; Windvane W200, Vector Instruments, UK). Ozone fumigation was on 14 h day^{-1} (8:00 - 22:00), seven days a week, except during rain, very high or low wind velocities or if the ambient ozone concentration was below 10 ppb. The monthly

O_3 ppb and AOT40 values are given in Table 1 till the end of the leaf litter incubation period (July 2010).

The warming treatment (24 h day^{-1}) was realised by installing one elongated infrared heater (Model Comfortintra CIR 105–220, 230–400 V, Frico AB, Sweden) circa 70 cm above the canopy in the middle of each heated subplot. In the ambient temperature subplots, a wooden bar of the same size, shape and colour was used to mimic the shading effect of the heater. The heaters and wooden bars were lifted during the growing season to keep the distance between the heater/bar and the canopy constant. The increase in air temperature measured with T-type thermocouples at top canopy level was ca. 0.9 °C in 2009 and 0.5 °C in 2010 (Table 1), but a previous birch experiment, using the same exposure system, have showed that leaf temperatures are approximately 0.5 °C higher than air temperatures (Riikonen et al. 2009). Soil temperature was measured with two T-type thermocouple sensors installed at a soil depth of 5 cm, data showing that soil temperature was increased on average by 4.2 °C in 2009 and 2.1 °C in 2010 (Table 1). In both years, the warmed subplots had lower air humidity than the ambient temperature subplots (Table 1). During the litter bag incubation period (from mid-November till the end of July) the total rainfall was 379 mm. The winter and especially December and January were cold (mean temperature -15.9 and -12.1°C, respectively), whereas July was warm and dry (mean temperature 21.6°C and cumulative rainfall 15 mm).

Litter material, reciprocal litter bag experiments and chemical analyses

Leaf litter was collected from 64 birch saplings (two saplings per genotype per subplot per plot) using mesh bags (mesh size 1 mm) in the autumn 2009 (the saplings were covered from the middle of September till the middle of November). Within each subplot, the litter of the two saplings of the same genotype was pooled, giving four replicate litter sets for

each treatment combination (C, T, O and TO) for both genotypes. From each litter set, the litter was packed into two types of litterbags: 5 intact leaves of fresh litter were used for producing 'mass loss bags' (litter bag size 10 cm × 10 cm, mesh size 1 mm), while 40-50 leaves were used for producing 'microbial bags' (20 cm × 20 cm, mesh size 1 mm). Four mass loss bags and two microbial bags were established from each replicate T, O and TO litter sets, and six mass loss bags and three microbial bags from each replicate C litter sets. Fresh leaf litter, instead of air-dried litter, was used in all litterbags as drying is known to change the subsequent microbial growth and litter mass loss (e.g. Clein & Schimel 1994; Taylor 1998; Schimel et al. 1999). To estimate the initial dry mass of litter in the bags, two leaves from each litter set were weighed before and after oven-drying (4 d in 60 °C). At the same time, leaves were collected from each litter set for analysing the initial microbial abundance and C and N concentrations of the litter. The C and N samples were oven-dried as above, ground in liquid nitrogen and analysed at the Luke laboratory using a CHN-1000 Analyzer (Leco Co., Joseph, MI, USA).

The litter bags (160 mass loss bags, 80 microbial bags) were transferred to the Ruohoniemi exposure field in the middle of November, and sampled twice in the summer 2010 (middle of June and end of July, i.e. after 217 and 257 days of incubation). The incubation period and harvest dates were chosen to resemble those used in our earlier silver birch decomposition study (Kasurinen et al. 2006). On both occasions, 80 mass loss bags and 80 microbial samples (10-15 leaves from each microbial bag) were harvested. The litter incubation experiments followed the design first introduced by Parsons et al. (2004): in the Native Replacement experiment, the litter bags were incubated in the subplots of their origin, while in the Common Garden experiment, the litter bags originating from the four exposure treatments were all incubated in the control subplots. Finally, in the Common Substrate experiment, the litter bags that originated from control subplots were incubated in all four

different exposure treatments. All litter bags were randomly placed on the surface of plot soils.

DNA isolation and quantitative real-time PCR

The abundances of bacteria and fungi growing on the litter were determined using the quantitative real-time PCR (qPCR) analysis. Litter samples were ground in liquid nitrogen, stored at -80 °C and a 25-125 mg subsample of each sample was used to extract the DNA using the FastDNA@Spin Kit for Soil (Obiogene, USA). Another subsample of 50-100 mg was dried (24 h at 60 °C) and used to determine the dry mass content of the extracted subsample. The same extraction method was used for the pure cultures of bacteria (*Escherichia coli*, own collection) and fungi (*Saccharomyces cerevisiae*, commercially available yeast), which served as positive controls in the qPCR analysis.

LightCycler Quantitative real-time PCR machine (Roche Diagnostics) was used for the amplifications. Two sets of primers specific for bacteria and fungi were applied in qPCR to estimate the amount of bacterial and fungal DNA in our samples. The primers pE (5'-AAA CTC AAA GGA ATT GAC GG-3') and pF' (5'-ACG AGC TGA CGA CAG CCA TG-3') were used for the bacteria (Edwards et al. 1989) and the primers ITS3 (5'-GCA TCG ATG AAG AAC GCA GC-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') for the fungi (Manerkar et al. 2008). The total reaction volume was 20 µl, which included 2 µl of diluted template (the dilution for bacteria 1:100 and for fungi 1:1000), 0.5 µl of each of the bacterial or 0.25 µl of each of the fungal primers, 10 µl of reaction mix (Dynamo HS SYBR Green qPCR Kit) and 7 µl or 7.5 µl of water (for bacterial and fungal analysis, respectively). The qPCR temperature program for bacteria (after 10 min initial denaturation at 94 °C) was 30 cycles of 10 s at 94 °C followed by annealing for 20 s at 57 °C and extensions for 30 s at 72 °C and for 1 s at 81 °C. For the fungi, the program (after 15 min initial denaturation at 95

°C) was 41 cycles of 60 s at 95 °C followed by annealing for 60 s at 58 °C and extensions for 60 s at 72 °C and for 1 s at 77 °C. For both microbial groups, the melting curve analysis for the amplicon was done at 60-95 °C with measurements of the fluorescence signal every 0.2 °C for 1 s. The amount of DNA copies in the original template was calculated using a standard curve. This value was then divided by the dry weight of the leaf litter subsample that was used for DNA extraction.

Statistical analyses

The effects of the treatments on fresh litter quality (C%, N% and C:N-ratio) and abundance of microbes (bacteria and fungi) were tested using Linear Mixed Models (LMM) ANOVA, where the genotype and the temperature and ozone treatments were treated as fixed factors and the subplot, nested within plot, as a random factor. During litter incubation, the effects on litter mass loss (expressed as % mass loss of the initial litter mass) and microbial abundance (expressed as the amount of DNA copies per g of litter dry weight) were tested using similar LMM ANOVA models, except that harvest time, treated as a repeated variable, was also included. To fulfil ANOVA assumptions, the data were log- or arcsin-transformed when necessary. Relationships between litter quality, microbial growth and mass loss were tested separately for both harvests and genotypes using non-parametric Spearman's correlation. P-values ≤ 0.05 were considered as statistically significant, but due to the low replicate number, typical to open-air exposure experiments, we also present and discuss results with P-values ≤ 0.1. All statistical analyses were performed using IBM SPSS Statistics version 21.

Results

Quality and microbial abundance of freshly fallen litter

Birches grown under warming treatment produced litter of higher C:N-ratio than those grown under ambient temperature ($p<0.0005$), whereas elevated O_3 decreased the ratio ($p=0.012$) (Table 2). This was because warming decreased litter C% by 4 % and N% by 17 % ($p<0.0005$ for both) and elevated ozone increased litter N% by 7 % ($p=0.026$). The O_3 effect was stronger in gt14 than in gt15 (genotype x ozone interaction, $p = 0.076$), but even for gt14 the effect was weak (1.4 % reduction). The amount of bacterial DNA in the freshly fallen litter was 39 % higher in gt15 than gt14 ($p=0.070$), 53 % lower under warming than ambient temperature ($p = 0.038$, Fig. 1a) and 196 % higher under elevated than ambient ozone ($p = 0.002$, Fig. 1b). Elevated ozone increased the amount of fungal DNA by 61%, but this effect was only marginally significant ($p = 0.095$, Fig. 1c).

Microbial abundances and litter mass loss during the incubation experiments

In the Native Replacement experiment (litter returned to their original treatment subplots), warming reduced the amount of bacterial DNA on average by 24 % (Table 3, Fig 2a). However, while in gt14 this effect was constant, in gt15 the warming and ozone effects varied over time (Table 3, Figs. 2b-c): warming, elevated ozone and their combination all decreased bacterial DNA at the first harvest, but at the second harvest, only the combination decreased and the single exposures increased the bacterial DNA (Fig. 2c). Warming decreased the amount of fungal DNA by 40 % in gt14 litter (Table 3). Litter mass loss varied from 23 to 44 % in June and from 22 to 46 % in July, but was not significantly affected by the treatments on either harvest.

Bacterial DNA had clear treatment responses in the freshly fallen litter, but these trends were no more visible during the Common Garden experiment (litter produced under different treatments and incubated in ambient conditions). In contrast, the amount of fungal DNA was on average 27 % higher in gt14 than gt15 litter (Table 3, Fig. 3a) and there was a marginally significant harvest×genotype×ozone interaction effect on fungi (Table 3, Figs. 3b-c). The gt14 litter that was produced under elevated O_3 had less fungal DNA than the litter produced under ambient O_3 at both harvest (Fig. 3b), whereas for gt15, this effect was found at the first harvest only (Fig. 3c). There was also a marginally significant genotype×temperature interaction effect on mass loss ($p=0.06$) in the Common Garden experiment: for gt14, the litter that originated from warmed plots decomposed slower than the litter produced in ambient temperature, while for gt15, the trend was opposite (Fig. 4).

In the Common Substrate experiment (litter produced in ambient conditions and incubated in different treatment combinations), most treatment effects varied temporally and were genotype-specific. For instance, warming decreased the amount of bacterial DNA by 19-25 %, but for gt15 this effect was found at the first harvest only (Table 4, Figs. 5a-b). Similarly, warming decreased the fungal DNA in gt14 litter at the first harvest by 56%, and in the litter of both genotypes at the second harvest (13-18 % reduction), but increased the fungal DNA by 38 % in gt15 litter at the first harvest (Table 4, Figs. 6b-c). The responses of microbes to O_3 were equally complex: elevated O_3 reduced bacterial DNA by 14 % in gt15 litter at the first harvest, but increased the DNA by 36% at the second harvest, and in the gt14 litter the pattern was reversed (first 3 % increase, later 11 % decrease; Table 4, Figs. 5c-d). The fungal DNA was reduced in the O_3 treatment by 24 % in gt14, but increased by 44 % in gt15 (Table 4, Fig. 6a). Litter mass loss varied from 28 to 49% in June and from 26 to 47% in July but was not affected by the treatments on either harvest (data not shown).

Correlations between initial leaf litter quality, microbes and the mass loss

In the freshly fallen litter of gt14, the amount of bacterial and fungal DNA correlated positively with litter N% (r=0.497 and r=0.565, respectively, p≤0.05 and n=16 for both) and negatively with litter C:N-ratio (r= -0.479, p≤0.05 and r= -0.600, p≤ 0.1, n=16). In the gt15 litter, fungal DNA correlated negatively with litter C% (r= -0.533, p=0.041, n=15). These trends remained during litter incubation in the Native Replacement experiment: i.e. bacterial and fungal DNA correlated positively with initial litter N% (r=0.424 and r=0.499, respectively, p≤0.05 and n=28 for both) and negatively with initial C:N-ratio (r= -0.398 and r= -0.543, respectively, p≤0.05 and n=28 for both) in gt14, and the fungal DNA negatively with litter C% in gt15 (r= -0.522, p≤0.05, n=29). In the Common Substrate experiment, bacterial DNA correlated positively with litter N% (r=0.503, p≤0.05, n=32) and negatively with C:N-ratio (r= -0.466, p≤0.05, n=32) in gt14, and the fungal DNA negatively with litter C% in both gt14 (r= -0.415, p≤0.05, n=32) and gt15 (r= -0.342, p≤ 0.1, n=31).

Correlations of litter mass loss with litter quality and microbial abundances were genotype specific and found in gt15 only: in the Native Replacement experiment, mass loss was negatively associated with litter C%, litter N% and bacterial DNA (r= -0.524, r= -0.484 and r= -0.420, respectively, p≤0.05 and n=29 for each), and positively with litter C:N-ratio (r=0.459, p≤0.05, n=29). In the Common Garden experiment, mass loss correlated negatively with litter C% (r= -0.462, p≤0.05, n=28) and positively with fungal DNA (r=0.413, p≤0.05, n=28).

Discussion

Warming and ozone altered litter quality

We hypothesized that while warming will improve the quality of litter produced by *B. pendula* saplings, the effects of elevated ozone on litter quality will be minor. This hypothesis was, however, rejected as warming decreased litter N concentration and increased the C:N-ratio, and ozone exposure improved litter quality by increasing litter N concentration and decreasing the C:N-ratio. Warming effects on litter C and N concentrations have earlier been reported to range from neutral to positive (Norby et al. 2000, Hudson et al. 2011, Sandvike and Eide 2011, Bai et al. 2013, Volder et al. 2015), which is in contrast with our results. Increasing specific leaf area (SLA) has been suggested to cause a dilution of leaf nutrients as the quantity of leaf cell wall material increases (Lambers et al. 2008). However, as there was no clear effect of warming on SLA in our plants (Kasurinen et al., unpublished manuscript), changes in leaf growth may not explain the observed N decrease. Furthermore, since we did not measure green foliage N levels before leaf abscission, we cannot state whether the responses in our study were due to altered N resorption process or just mirroring the responses of green foliage N concentrations before leaf abscission. Earlier studies of N resorption neither provides help since warming (Norby et al. 2000, Estiarte and Peñuelas 2015) and ozone (Uddling et al. 2005, Kasurinen et al. 2007) effects on tree N resorption have been found to be highly variable and complex. In summer 2009, when the leaf litter used in the litter bags was produced, the average soil temperature for warmed subplots was ca 4°C higher than for ambient subplots and it is possible that soil moisture was lower in warmed than in non-warmed subplots. However, long-term or severe drought periods were unlikely as all saplings were regularly watered (at least 2-3 times a week if there was no rain) and the cumulative rainfall from June to mid-November was 258 mm, almost a half of total

rainfall (500 – 650 mm) in this region. Hence, it is not likely that drought could explain the lower litter quality in warmed subplots in our experiment.

Nitrogen resorption is considered to be complete in woody plants when litter N concentrations fall below 0.7% of leaf dry weight (Killingbeck 1996). Our values are far above that – ranging from 2.5 to 3.5% - which suggests that N resorption was inefficient in our saplings. This is probably due to N-rich growing conditions (5 kg N m^{-3} added to the plots in the beginning of the experiment) since Silfver et al. (2015) reported litter N% of 1.3 for three-year old, cloned *B. pendula* saplings growing in nutrient deficient forest ground. Due to the high N concentrations, the C:N-ratios were in our study remarkably low (15-19) in comparison to earlier silver birch studies, where the C:N-ratios have varied between 40 and 70 (Kasurinen et al. 2006; Silfver et al. 2015). Despite this, the treatments had an influence and the birches in warmed plots had lower and the ozone-exposed trees higher litter N% than birches grown under ambient conditions.

Litter N% varies among genotypes in silver birch stands (Silfver et al. 2015) and warming responses in growth and soil processes can be genotype-dependent (Kasurinen et al. 2012), but in the current study, the warming effects on litter quality did not depend on the genotype. Neither was there interaction between warming and ozone exposure, but instead, the positive ozone effect on litter quality, earlier also reported by Parsons et al. (2008), was consistent across the treatments. The only genotype-dependent effect was the slight O_3-induced decrease in litter C% that we observed in gt14. This effect was opposite to previous studies, where ozone has been found to have either neutral (Parsons et al. 2008) or positive effects on the concentrations of carbon compounds (Liu et al. 2005, Häikiö et al. 2009, Meehan et al. 2010).

Environmental and litter-mediated effects on microbes were genotype-dependent

The amount of bacterial DNA, but not that of fungal DNA, was reduced in the fresh litter produced under warming. On the other hand, both bacterial and fungal DNA were increased in the fresh litter originating from the O_3-treated saplings. Remarkably, both of these effects were similar for the two genotypes, and only during litter incubation many warming and ozone effects on microbes became genotype-dependent. For instance, the reduced abundance of bacteria and fungi due to warming in the Native Replacement experiment and the reduced abundance of fungi due to elevated ozone in the Common Garden experiment were temporally more consistent for gt14 than gt15. Similarly, in the Common Substrate experiment, warming reduced bacterial and fungal DNA more consistently for gt14 and at the last sampling, ozone increased bacteria and fungi in gt15 only. Our results also suggest that warming effects on bacterial and fungal abundance were mainly environmental: similar warming effects on microbes were found when the litters of different origin were incubated under higher temperature (Native Replacement and Common Substrate experiments), whereas no warming effects were found when litters from different treatments (including warming) were incubated in ambient conditions (Common Garden). It further appeared that microbes growing on gt14 litter were more responsive to warming than those growing on gt15 litter. This finding is in good agreement with an earlier field study (Kasurinen et al. 2012), where foliage growth and soil respiration rates of gt14 were more responsive to warming than those of gt15.

Although warming effects on microbes during litter incubation were mainly environmental, some legacy effects of the initial positive correlation between litter quality and microbial growth (i.e. good quality litter promoting microbial growth) could also be detected after incubations. Surprisingly, however, the legacy effects appeared in the Common Substrate experiment, where all litter originated from the gt14 and gt15 control birches, but

not in the Common Garden experiment where the litters originated from different treatments. Based on the Common Garden correlations, it seems that the temperature- and ozone-induced relationships between litter-quality and microbial growth can quickly disappear during litter decomposition, whereas the genetically controlled relationships, observed in the Common Substrate experiment, can last longer and withstand the environmental variation. On the other hand, the initial positive association of higher quality litter with higher microbial abundance was also detected in the Native Replacement experiment, suggesting that the relationship can remain through decomposition, but only if the litter is returned to its original growth environment and continuously exposed to the warming and ozone exposures. We found that bacterial abundances followed changes in litter N concentrations, whereas fungal abundances were more related to changes in C concentrations. This is in accordance with the findings by Bray et al. (2012), who compared litter decomposition and microbial communities among ten plant species and observed that higher quality litter (higher N, lower C:N-ratio) supported bacterial dominance among the decomposers.

Environmental or litter-mediated effects on mass loss were weak

The winter 2009-2010 was long and harsh and shortened the active decomposition period, but nonetheless, litter mass loss was 22-49 % at our final harvest. This range is close to the mass loss rates of 39-48 % found in an earlier study, where the litter of CO_2 and O_3 exposed birches were incubated at ambient conditions in Central Finland for 11 months (Kasurinen et al. 2006). Decomposition rates of birch leaf litter in less fertile forest sites can be much slower as shown by Silfver et al. (2015) in their study in Southern Finland, where the mass loss rates ranged from 2 to 20 % after a 9-month incubation trial. In our study, the incubation treatments affected microbial growth, but we did not find evidence of litter mass loss being directly affected by warming or ozone treatments. The lack of clear

mass loss response to warming may partly be due to the short exposure period as litter bags were exposed to warming less than 1.5 - 3 months during the growing season 2010, whereas with a longer exposure, responses might have appeared. July 2010 was also a particularly warm and dry month and under such conditions the effectiveness of warming treatment may have been reduced. On the other hand, our result is consistent with earlier findings that the environmental effects of warming on litter mass loss are weak when studied within experimental sites, not across sites or terrestrial biomes. For instance, Gong et al. (2015) reported only a slight increase in decomposition rates of grass litter in their infrared heater exposure system. In another grassland study (Walter et al 2013), wintertime warming reduced snow cover, but even this change did not significantly affect grass litter decomposition. The only litter quality-mediated effect in our study was genotype-dependent warming effect on litter mass loss as the litter produced under warming had lower mass loss in gt14, but higher mass loss in gt15. The reason for such response is unclear as litter quality did not show genotype-dependent warming responses.

Cornwell et al. (2008) have stated that within a biome, litter traits affect decomposition rates more than the prevailing climate. Recently, Bradford et al. (2016) also suggested that climatic effects have been overemphasized as other, local-scale factors might actually account for the majority of variation in litter decomposition rates. Across plant species, litter C:N-ratio correlates strongly negatively with litter decomposition rate (Enríquez et al. 1993), but it seems that this negative association may not hold in within-species comparisons. Silfver et al. (2015) did not find intrapopulation genotypic correlations between litter C:N ratio and mass loss in silver birch and in our study, no correlation was found in gt14 and the correlation found in gt15 was positive. It is thus likely that the observed litter quality-mediated effects of warming on mass loss in our study were mainly due to some other chemical or physical factors that we did not measure. For instance, secondary

compounds, such as condensed tannins are known to restrict litter decomposition (Schweitzer et al. 2004), but on the hand, a recent silver birch study did not find a strong correlation between the genotypic variation of secondary compounds and litter decomposition rates (Silfver et al., unpublished manuscript). Of the physical factors, leaf toughness has been shown to affect the decomposition rate of leaf litter in stream (e.g., Graça and Cressa 2010, Ferreira et al. 2012) and terrestrial environments (e.g. Pérez-Harguindeguy et al. 2000). A recent warming experiment in a stream system further shows how the structure of the decomposer food web also matters. Ferreira et al. (2014) found that depending on the species of plant, macrofauna either enhanced or diminished warming effects on litter decomposition. Our study excluded macrofauna as the litterbag mesh size was only 1 mm. All in all, it appears that warming effects on litter decomposition are governed by multiple factors and wide-ranging predictions may be difficult to achieve.

Although the O_3 exposure affected litter quality, the O_3 effects were not manifested in the subsequent litter mass loss rates in the Native Replacement or Common Garden experiments. In addition, exposure of litter to elevated ozone environment did not significantly change the mass loss rates in the Common Substrate experiment. Previously Kasurinen et al. (2006) have found reduced mass loss rates in silver birch litter produced under elevated O_3 exposure and incubated at ambient conditions, but only when the litter was collected after the third O_3 exposure season and incubated for 11 months. It is thus possible that our experiments were too short to detect possible indirect or direct O_3 effects on mass loss rates.

Conclusions

Our results show that warming and elevated ozone can modify leaf litter quality and the abundance of bacteria and fungi growing on the litter during decomposition.

However, nearly all warming and ozone effects on microbial growth during decomposition varied between the two genotypes, which means that in a genetically diverse population, such effects are hard to predict and the sum effect across the whole population may be negligible. Our results further demonstrate how disentangling environmental and litter quality-mediated effects on decomposer microbes may be difficult as the initial litter quality can have legacy effects on both bacteria and fungi several months after leaf abscission. In general, our results suggest that the effects of warming and ozone on litter decomposition are likely to be limited in northern birch stands.

Compliance with Ethical Standards

Funding

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Table 1. Average O_3 concentrations (14 h d^{-1}) and AOT40 (accumulated over a threshold of 40 ppb) values in ambient and elevated O_3 plots, and average air and soil temperatures (24h d^{-1}) and relative air humidity in ambient and elevated temperature subplots in 2009 and 2010. Values are means or means ± SD (n = 4 for O_3 plot data and n =7- 8 for temperature subplot data).

	O_3 (ppb)		**AOT40 (ppm.h)**		**Air temperature (ºC)**		**Soil temperature (ºC)**		**Relative air humidity (%)**	
2009	**Amb O_3**	**Elev O_3**	**Amb O_3**	**Elev O_3**	**Amb T**	**Elev T**	**Amb T**	**Elev T**	**Amb T**	**Elev T**
June	24.0±0.7	32.2±1.2	0.02	0.77	15.7±0.3	16.8±0.5	15.1±0.9	19.7±2.0	73.0±1.5	68.9±2.8
July	25.0±0.4	36.4±0.5	0.02	2.6	17.0±0.3	18.1±0.3	15.8±1.5	20.5±2.5	73.3±1.7	70.3±3.1
August	23.5±0.2	33.7±0.5	0.02	3.6	15.7±0.3	16.6±0.6	14.4±2.1	18.3±3.4	77.8±1.3	75.2±2.9
September	24.5±0.7	31.8±1.0	0.14	4.7	11.8±0.2	12.5±0.4	10.2±2.2	13.9±2.8	81.7±1.4	80.2±3.1
Whole season	24.3±0.4	33.6±0.7	0.14	4.7	15.1±0.3	16.0±0.3	14.0±1.5	18.2±2.6	76.6±1.4	74.0±2.9
2010										
May	33.6±0.4	45.4±2.9	0.75	4.1	12.6±0.6	13.0±0.4	11.2±1.4	13.3±1.6	70.1±0.9	67.8±0.8
June	29.1±0.2	41.9±1.0	0.82	6.9	14.1±0.4	14.8±0.5	12.9±0.6	14.9±1.9	66.2±1.1	63.4±1.1
July	29.6±0.1	44.0±0.8	1.1	10.7	22.3±0.3	23.0±0.4	20.0±0.5	21.8±1.9	63.9±1.0	61.2±1.3
Whole season	30.7±0.1	43.7±1.4	1.1	10.7	16.4±0.3	16.9±0.4	14.5±0.4	16.6±1.8	66.7±0.9	64.1±1.1

AOT40 values are calculated by summing the hourly values above 40 ppb to achieve ppm.h

Table 2. Elevated temperature and ozone effects on the C and N concentrations and C:N-ratio of litter produced under these treatments (means ± SE, n=4); gt14 = genotype 14, gt15 = genotype 15, C = control (ambient temperature and ozone), T = elevated temperature alone, O = elevated ozone and TO = elevated temperature and elevated ozone in combination.

Treatment	**C mg g^{-1} litter**	**N mg g^{-1} litter**	**C:N-ratio**
gt14			
C	532.5±0.7	34.5±0.20	15.4±0.1
T	509.3±2.3	27.9±1.06	18.4±0.7
O	520.8±5.9	36.1±1.32	14.5±0.5
TO	506.0±3.2	30.4±1.04	16.7±0.7
gt15			
C	532.5±2.0	33.0±1.03	16.2±0.5
T	503.3±4.0	27.2±0.74	18.6±0.4
O	529.3±4.3	35.1±1.61	15.2±0.7
TO	507.3±4.1	29.7±1.39	17.2±0.7

Table 3. Statistics of Mixed Linear Repeated Measures Anova for litter bacterial and fungal DNA in the Native Replacement and Common Garden (fungi only) experiments (data log(X+1)-transformed). In the models, harvest time, genotype, warming and ozone were considered as fixed, and the original subplot nested within original plot (Osubplot(Oplot)), Placement subplot within Placement plot (Psubplot(Pplot) and PCR-run as random factors; effects with P-values <0.1 are in bold (n = 2-4 for the four-way interaction). Missing Wald Z- and P-values for Osubplot(Oplot) indicate that this random factor was redundant.

Native Replacement	**Bacteria**		**Fungi**		**Common Garden**	**Fungi**	
Between-subjects effects	**F**	**P**	**F**	**P**	**Between-subjects effects**	**F**	**P**
Genotype (G)	0.029	0.866	1.552	0.223	Genotype (G)	**6.243**	**0.017**
Temperature (T)	**6.226**	**0.028**	3.069	0.134	Temperature (T)	0.005	0.946
Ozone (O)	0.559	0.469	1.184	0.321	Ozone (O)	1.209	0.279
G x T	2.136	0.155	**2.947**	**0.097**	G x T	0.643	0.428
G x O	1.743	0.197	0.077	0.783	G x O	0.621	0.436
T x O	1.447	0.252	0.446	0.530	T x O	0.131	0.720
G x T x O	1.669	0.207	2.599	0.118	G x T x O	1.345	0.254
Within-subjects effects	**F**	**P**	**F**	**P**	**Within-subjects effects**	**F**	**P**
Harvest time (H)	0.770	0.413	0.115	0.747	Harvest time (H)	0.022	0.892
H x G	1.672	0.206	0.826	0.371	H x G	**3.123**	**0.086**
H x T	0.011	0.916	0.196	0.661	H x T	0.134	0.716
H x O	0.011	0.917	0.557	0.461	H x O	0.066	0.799
H x G x T	0.064	0.802	0.028	0.869	H x G x T	1.035	0.316
H x G x O	0.056	0.814	0.469	0.499	H x G x O	**3.283**	**0.078**
H x T x O	**3.540**	**0.070**	0.539	0.469	H x T x O	0.793	0.379
H x G x T x O	**4.141**	**0.051**	0.399	0.532	H x G x T x O	0.065	0.801
Random effects	**Wald Z**	**P**	**Wald Z**	**P**	**Random factors**	**Wald Z**	**P**
PCR-run	1.581	0.114	1.366	0.172	PCR-run	1.021	0.307
Osubplot(Oplot)	0.387	0.699	0.422	0.673	Osubplot(Oplot)	-	-
					Psublot(Pplot)	0.692	0.489

Table 4. Statistics of Mixed Linear Repeated Measures Anova for litter bacterial and fungal DNA in the Common Substrate experiment (data log(X+1)-transformed). In the models, harvest time, genotype, warming and ozone were considered as fixed and PCR-run, original subplot nested within original plot (Osubplot(Oplot)) and placement subplot nested within placement plot (Psubplot(Pplot)) as random factors; effects with P-values <0.1 are in bold (n = 3-4 for the four-way interaction). Missing Wald Z- and P-values for Osubplot(Oplot) indicate that this random factor was redundant.

Common Substrate	**Bacteria**		**Fungi**	
Between-subjects effects	**F**	**P**	**F**	**P**
Genotype (G)	1.287	0.266	0.712	0.405
Temperature (T)	0.497	0.496	3.219	0.111
Ozone (O)	0.001	0.971	1.440	0.265
G x T	1.375	0.250	1.825	0.186
G x O	1.101	0.303	**6.534**	**0.015**
T x O	0.752	0.405	0.326	0.584
G x T x O	2.116	0.156	0.104	0.749
Within-subjects effects	**F**	**P**	**F**	**P**
Harvest time (H)	0.319	0.593	0.112	0.760
H x G	0.141	0.710	0.086	0.771
H x T	0.845	0.365	0.209	0.650
H x O	**3.698**	**0.064**	1.387	0.248
H x G x T	**3.477**	**0.072**	**4.454**	**0.043**
H x G x O	**6.879**	**0.014**	0.829	0.369
H x T x O	0.178	0.677	1.424	0.241
H x G x T x O	0.229	0.636	0.016	0.901
Random effects	**Wald Z**	**P**	**Wald Z**	**P**
PCR-run	1.624	0.104	1.021	0.307
Osubplot(Oplot)	-	-	0.106	0.915
Psublot(Pplot)	1.019	0.308	0.005	0.996

Figure legends

Fig. 1. The main effects of **a** temperature on bacterial (n = 16), **b** ozone on bacterial (n =16) and **c** ozone on fungal (n =15) DNA (means ± SE) in the freshly fallen leaf litter in November 2009.

Fig. 2. The **a** temperature main effect (n = 28-29) and **b-c** harvest×genotype×temperature×ozone interaction effect (n = 3-4) on bacterial DNA copies (means ± SE) in the litters returned to their original treatment combinations in the Native Replacement experiment.

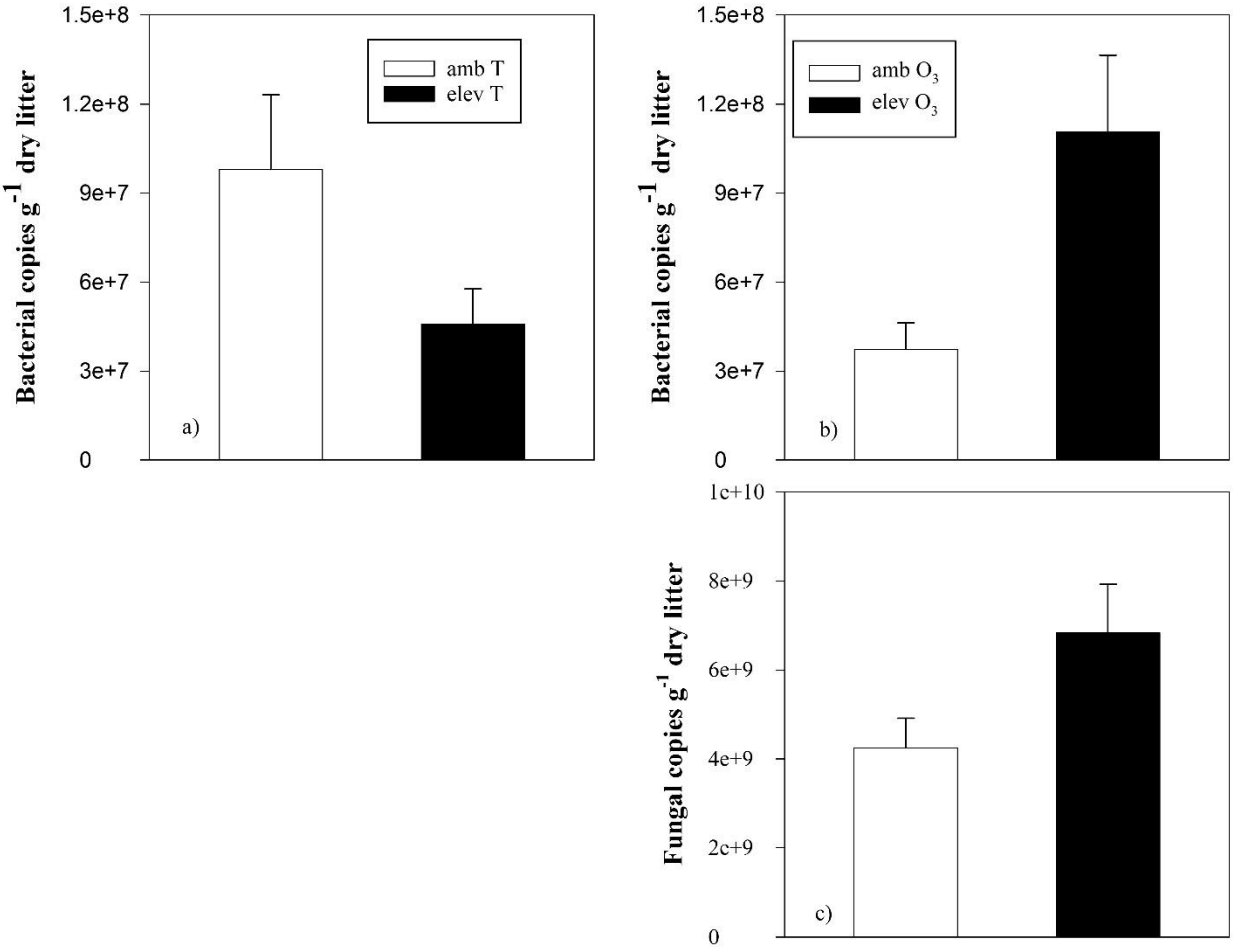
Fig. 3. The **a** genotype main effect (n = 28-32) and **b-c** harvest×genotype×ozone interaction effect (n = 5-8) on fungal DNA copies (means ± SE) in litters incubated at ambient condition (control plots) in the Common Garden experiment.

Fig. 4. The genotype×temperature interaction effect (n = 13-15) on mass loss (means ± SE) in litter bags incubated at ambient conditions (control plots) in the Common Garden experiment.

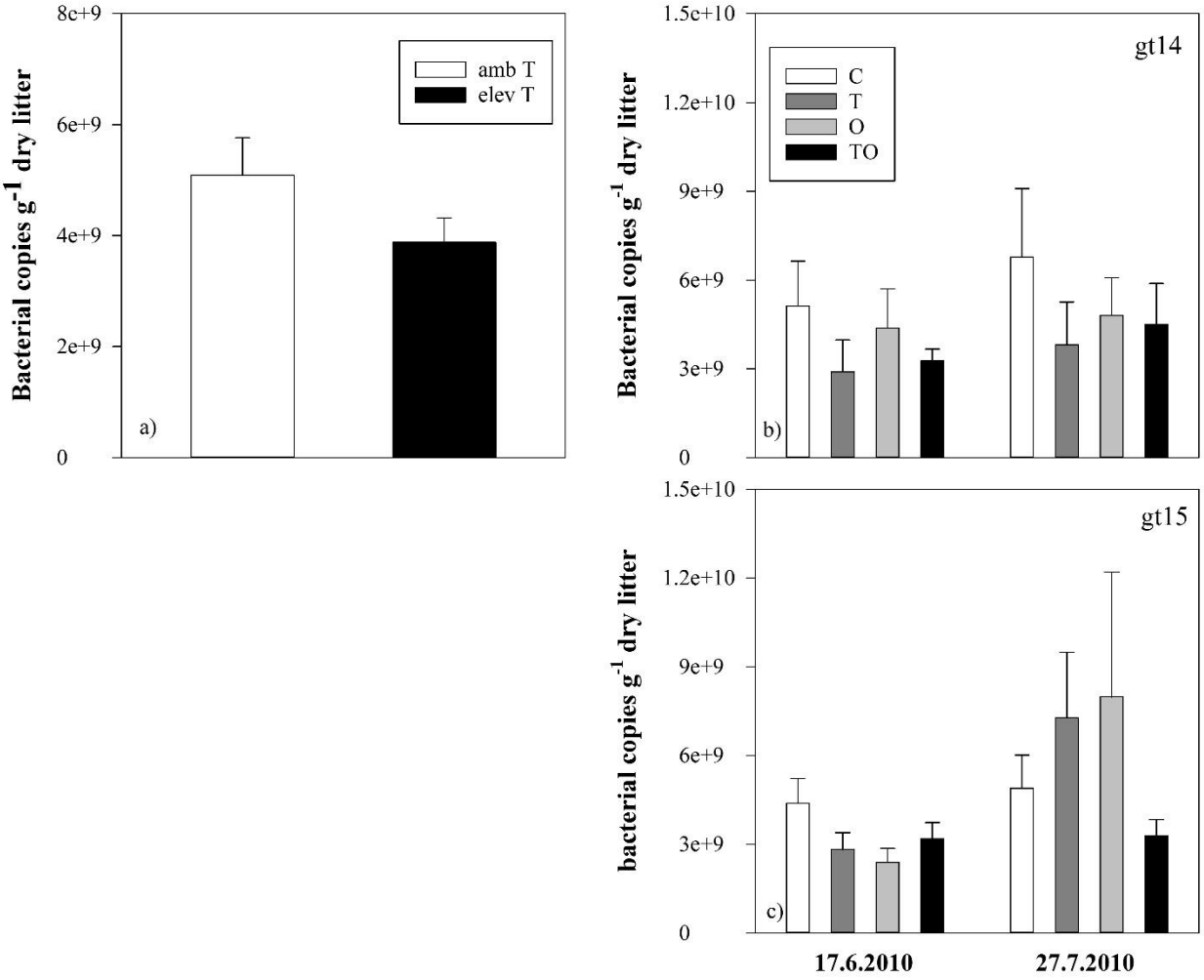
Fig. 5. The **a-b** harvest×genotype×temperature interaction effect (n = 8) and **c-d** harvest×genotype×ozone interaction effect (n = 8) on the amount of bacterial DNA (means ± SE) in the litter produced under ambient conditions and incubated at different temperature and ozone exposures in the Common Substrate experiment.

Fig. 6. The **a** genotype×ozone interaction effect (n = 15-16) and **b-c** harvest×genotype×temperature interaction effect (n = 7-8) on the amount of fungal DNA (means ± SE) in the litter produced under ambient conditions and incubated at different temperature and ozone exposures in the Common Substrate experiment.

line figure



Figs. 1a-c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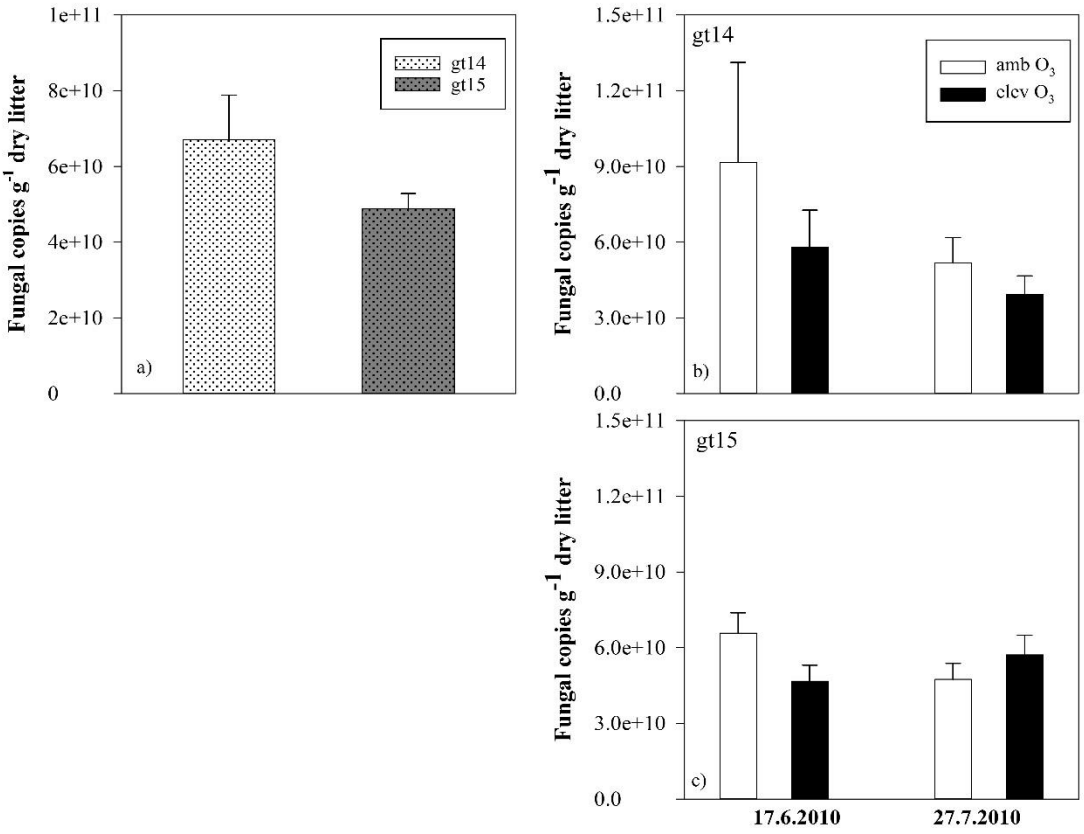
6e+9
4e+9
2e+9
0
Bacterial copies g-1 dry litter
amb T
elev T
a)
1.5e+10
1.2e+10
9e+9
6e+9
3e+9
0
Bacterial copies g-1 dry litter
C
T
O
TO
gt14
b)
1.5e+10
1.2e+10
9e+9
6e+9
3e+9
0
bacterial copies g-1 dry litter
gt15
c)
17.6.2010
27.7.2010

Figs. 2a-c.


1e+11
8e+10
6e+10
4e+10
2e+10
0
Fungal copies g-1 dry litter
gt14
gt15
a)
1.5e+11
1.2e+11
9.0e+10
6.0e+10
3.0e+10
0.0
gt14
amb O3
elev O3
b)
gt15
c)
17.6.2010
27.7.2010

Figs. 3a-c

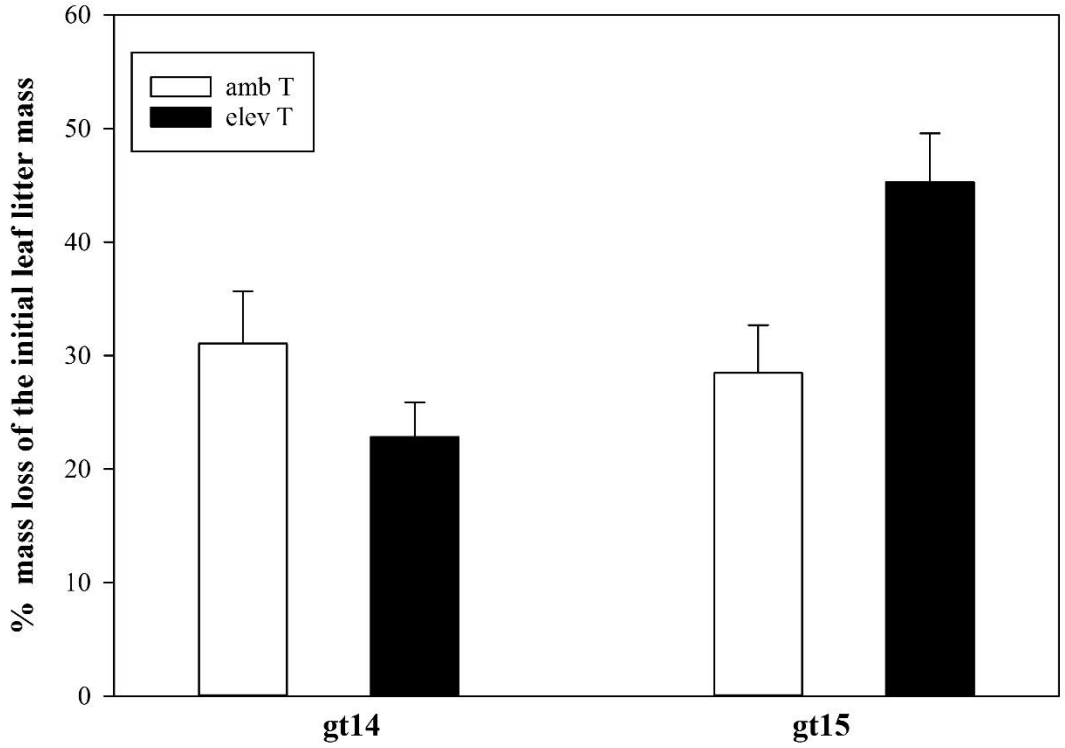
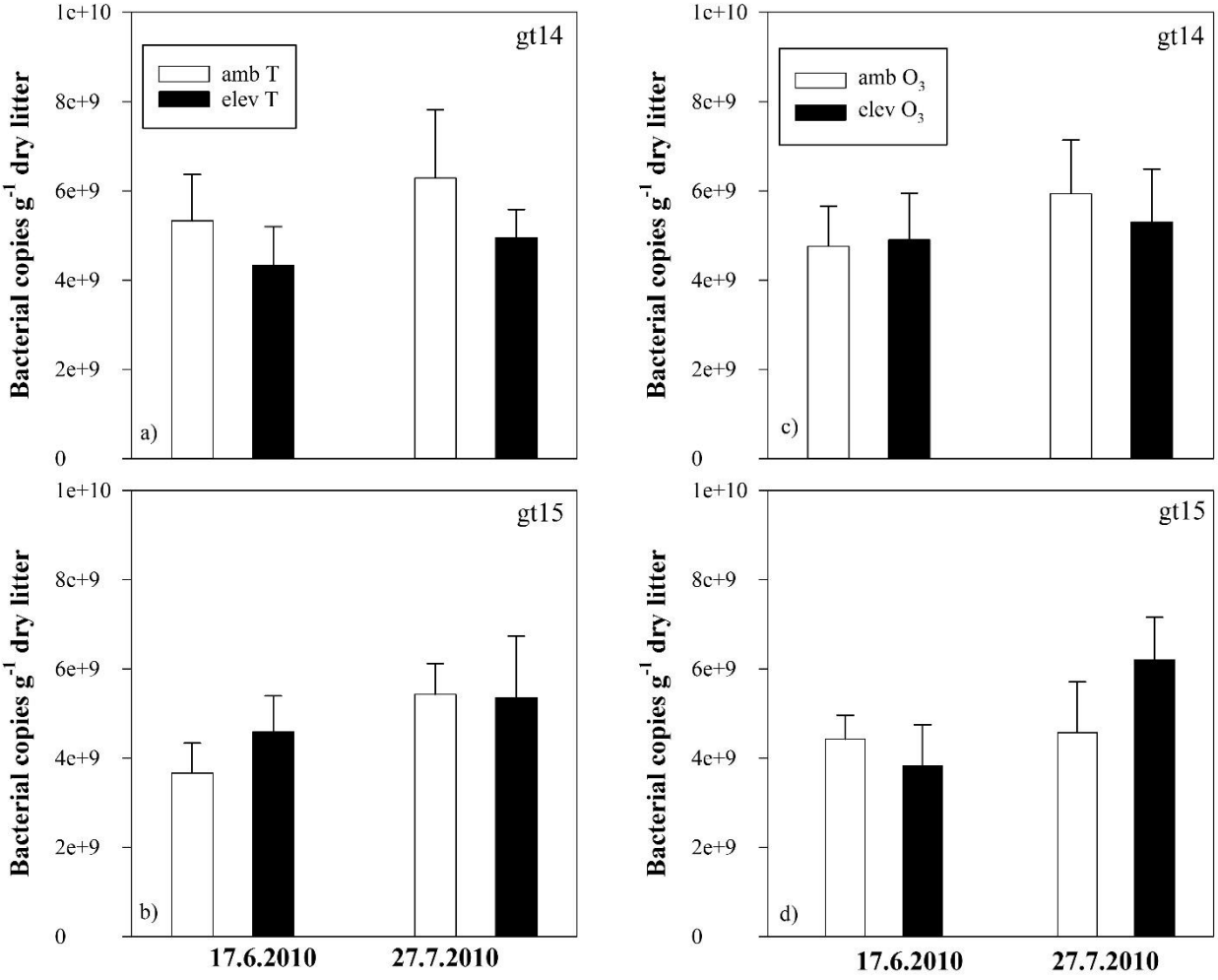
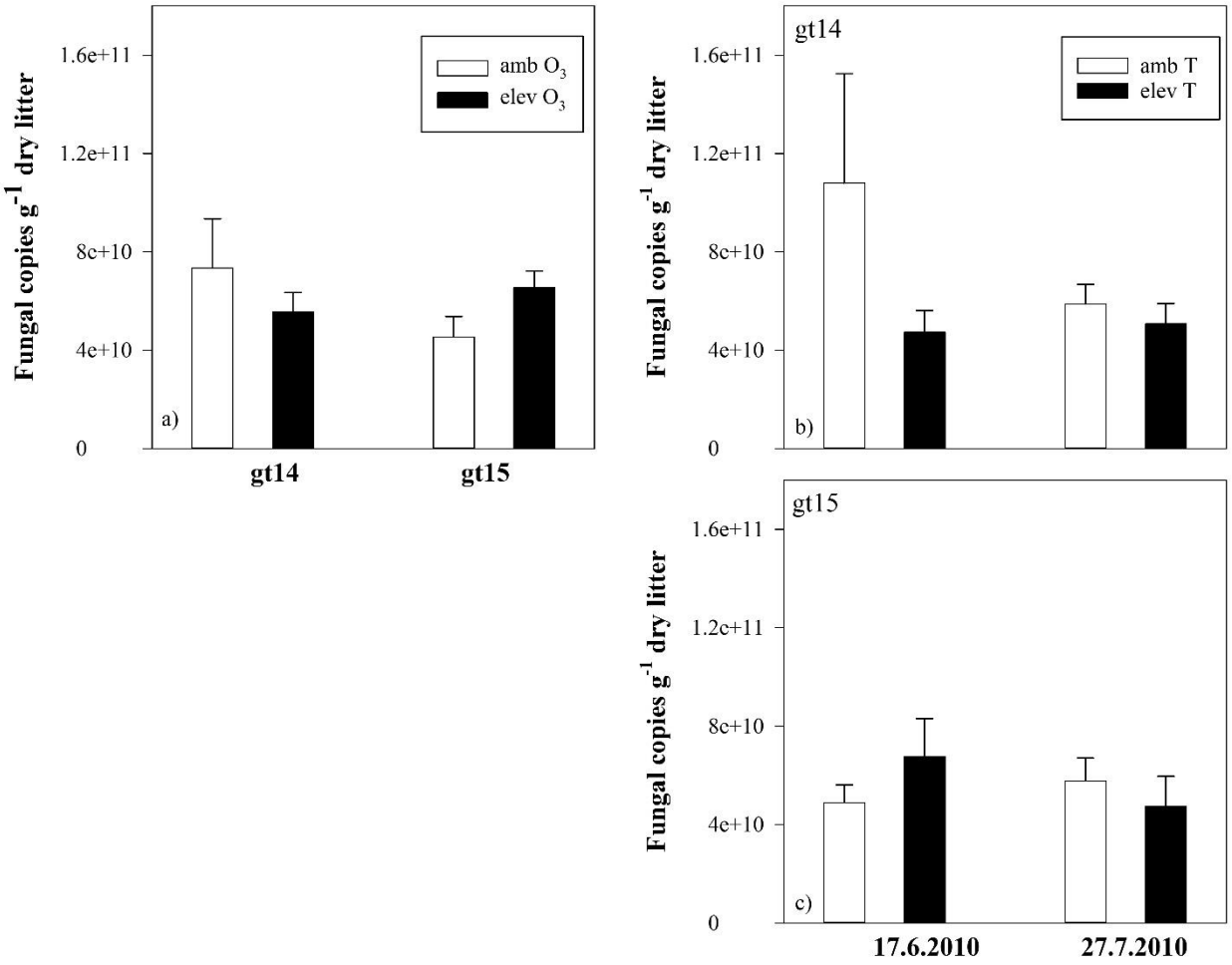

60
50
40
30
20
10
0
% mass loss of the initial leaf litter mass
amb T
elev T
gt14
gt15

Fig. 4.


gt14
amb T
elev T
a)
gt15
b)
gt14
amb O3
elev O3
c)
gt15
d)
Bacterial copies g-1 dry litter
1c+10
8c+9
6e+9
4e+9
2e+9
0
1e+10
17.6.2010
27.7.2010

Figs. 5a-d.


1.6e+11
1.2e+11
8c+10
4e+10
0
Fungal copies g-1 dry litter
amb O3
elev O3
a)
gt14
gt15
gt14
amb T
elev T
b)
gt15
c)
17.6.2010
27.7.2010

Figs. 6a-c.