Published in final edited form as:
Environ Pollut. 2011 February ; 159(2): 401–407. doi:10.1016/j.envpol.2010.10.033.

Soil nitrogen transformations under elevated atmospheric CO_2 and O_3 during the soybean growing season

Engil Isadora Pujol Pereira[a,*], **Haegeun Chung**[b], **Kate Scow**[c], **Michael J. Sadowsky**[d], **Chris van Kessel**[a], and **Johan Six**[a]

Department of Plant Sciences, University of California - Davis, One Shields Avenue, Davis, CA 95616, USA

Division of Environmental Science and Ecological Engineering, Korea University, Anam-dong, Seongbuk-gu, Seoul 136-701, South Korea

Department of Land, Air, and Water Resources, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

Department of Soil, Water, and Climate; and BioTechnology Institute, University of Minnesota, St. Paul, MN 55108, USA

Abstract

We investigated the influence of elevated CO_2 and O_3 on soil N cycling within the soybean growing season and across soil environments (i.e., rhizosphere and bulk soil) at the Soybean Free Air Concentration Enrichment (SoyFACE) experiment in Illinois, USA. Elevated O_3 decreased soil mineral N likely through a reduction in plant material input and increased denitrification, which was evidenced by the greater abundance of the denitrifier gene *nosZ*. Elevated CO_2 did not alter the parameters evaluated and both elevated CO_2 and O_3 showed no interactive effects on nitrifier and denitrifier abundance, nor on total and mineral N concentrations. These results indicate that elevated CO_2 may have limited effects on N transformations in soybean agroecosystems. However, elevated O_3 can lead to a decrease in soil N availability in both bulk and rhizosphere soils, and this likely also affects ecosystem productivity by reducing the mineralization rates of plant-derived residues.

Keywords

Nitrification; Denitrification; Real-time quantitative PCR; FACE; Soil N cycling

1. Introduction

Crop productivity is in part limited by soil nitrogen (N) (Vitousek and Howarth, 1991), which influences plant growth, quality, and yield. Many ecosystem processes, including C and nutrient cycling, are driven by soil microorganisms, which depend on N availability to carry out their activities (Hallin et al., 2009). One of the natural sources of N in the soil is via N_2 fixation, whereby atmospheric N_2 is converted into organic forms. Furthermore, soil

---

[*] Corresponding author. isapereira@ucdavis.edu (E.I.P. Pereira). .

Our findings indicate that although elevated CO_2 increases plant biomass, N transformations were minimally affected. In contrast, elevated O_3 decreased soil mineral N likely through a reduction in plant material input and increased denitrification as indicated by the greater abundance of the denitrifier gene nosZ.

N levels depend on plant N uptake, quality and quantity of the plant residue input, and soil moisture content (Alcoz et al., 1993).

The current and predicted increases of atmospheric CO_2 concentrations (IPCC, 2007) are likely to modify the factors affecting the transformations of soil N (Kanerva et al., 2006). Elevated CO_2 generally enhances above- and belowground plant productivity by increasing photosynthetic rates (Ainsworth and Long, 2005; de Graaff et al., 2006a; Zak et al., 1993) and water use efficiency (Tyree and Alexander, 1993). Both of these factors have the potential to modify N dynamics at the ecosystem level. Several studies have focused on understanding the influence of elevated atmospheric CO_2 on the acquisition, accumulation, and losses of N by measuring changes in plant N uptake, N mineralization, microbial N immobilization, nitrification, and denitrification (de Graaff et al., 2006b; Hungate et al., 1997; Luo et al., 2006; Zak et al., 1993). Previous studies have mainly concentrated in grass and forestland systems, with only limited data collected from croplands.

In contrast to CO_2, the elevation of tropospheric O_3 concentration inhibits plant productivity (Morgan et al., 2003). The atmospheric concentration of O_3 is predicted to increase 20% by 2050 (Prather et al., 2001), reaching levels that can reduce photosynthetic rates of sensitive plant species (Morgan et al., 2003). In soybean (Glycine max L. Merr), elevated O_3 has been shown to reduce plant growth and seed yield (Morgan et al., 2006), and accelerate leaf senescence (Dermody et al., 2006). However, little is known about the effects of elevated O_3 on soil N dynamics, and most studies have focused on forest species (Holmes et al., 2003, 2006). Since elevated CO_2 and O_3 alter plant growth and development in opposing ways (Ainsworth and Long, 2005; Morgan et al., 2006), an understanding of how the concomitant increase in concentration of both these atmospheric gases will affect N transformations in the soil beneath annual plants needs further investigation. The effects of elevated CO_2 and O_3 on belowground N dynamics have been reported to be mediated, indirectly, through altered plant processes and C allocation (Andersen, 2003; Zak et al., 2000a). Therefore, we propose that the effects of elevated CO_2 and O_3 may alter ecosystem N balances by leading to changes in soil N availability, through the increase or decrease of plant N uptake, microbial N immobilization and/or denitrification.

Elevated CO_2 and O_3 may affect soil N dynamics differently at different plant phenological stages in distinct soil environments (rhizosphere vs. bulk soil) through changes in substrate plant input. The plant rhizosphere is a unique environment because of direct inputs of substrate through the sloughing off of root cells and root exudation (Lynch and Whipps, 1990). Because there is greater substrate for decomposition in the rhizosphere than bulk soil (Cheng et al., 2003), there will be more mineral N cycling in the rhizosphere than bulk soil. Therefore, we hypothesized that the rhizosphere would be a microenvironment where the effects of elevated CO_2 and O_3 would be most pronounced.

In this study, we investigated the effects of elevated CO_2 and O_3 on soil N availability and N-transforming microorganisms (nitrifiers and denitrifiers) across different soil environments (i.e., rhizosphere and bulk soil) during the soybean growing season at the SoyFACE (Soybean Free Air Concentration Enrichment) experiment.

2. Materials and methods

2.1. Site description and sampling

This study was conducted at the Soybean Free Air Concentration Enrichment (SoyFACE) facility, located in Champaign, IL, USA at the South Farms, University of Illinois at Urbana-Champaign; 40° 03′21.3″N 88° 12′3.4″W (http://soyface.illinois.edu). The 32-ha facility is located on farmland that is cultivated with an annual rotation of soybean (*Glycine* *max* (L.) Merr.) and corn (*Zea mays* L.) for more than 25 years. The soil at the site is a Drummer fine-silty, mixed, mesic Typic Endoaquoll, and is typical of wet, dark-colored "prairie soils" in northern and central Illinois.

The target concentration of elevated CO_2 treatment was 550 μL L^{-1} while the elevated O_3 treatment was +20% ambient, and these concentrations are based on the Intergovernmental Panel on Climate Change estimates for the year 2050. Fumigation with CO_2 started in 2001 in the 16-ha half of the western side of the field. In 2002, the FACE treatments were resumed on the eastern side of the field, with 12 treatment rings established for the ambient, elevated CO_2, and elevated O_3 treatments. The combined elevated CO_2 and O_3 treatment was started in 2003 (Ort et al., 2006). Since then, the crops within the rings have been fumigated only during the growing seasons. Nitrogen fertilization has been used only for the cultivation of corn, while phosphorus and potassium were applied as needed based on soil test for both crops.

The study reported here was performed during the growing season of 2008 on the 16 ha – eastern side of the field, where soybeans were exposed to factorial treatments of elevated CO_2 and O_3 in a randomized complete block design ($n = 4$). In 2008, the eastern side of the field had been under elevated CO_2 and elevated O_3 treatments for four growing seasons, and under combined elevated CO_2 and O_3 treatment for three growing seasons.

Soil samples were collected at different phenological stages of soybean: the fourth trifoliolate leaf (V4), full pod (R4), and full maturity (R8) stages, following the phenological system of Ritchie et al. (1997). Soil samples (6 cm dia. × 15 cm depth) were collected from the rhizosphere and bulk soil. To collect rhizosphere soils, the soybean aboveground biomass was removed, and a soil core was inserted over the root crown to obtain the root system and associated soil. Bulk soil samples were collected from soils between soybean rows. Four soil cores were taken per ring, two cores from the rhizosphere and two from the bulk soils; and each soil type was pooled into a single sample. Soil samples were cooled to 4 °C and transported overnight on ice to the University of California at Davis, where all analyses were carried out.

2.2. Soil properties

Soil samples were homogenized through an 8-mm sieve and analyzed for their gravimetric soil moisture content. Approximately 100 g aliquots of soil were frozen for molecular analyses of the microorganisms and the remaining soil was air-dried. Rhizosphere soil and bulk soil were ground and analyzed for total N and C concentrations using a PDZ Europa 20–20 Stable Isotope Analyzer (Europa Scientific, Crewe, UK) at the University of California-Davis Stable Isotope Facility (http://stableisotopefacility.ucdavis.edu/). Since no carbonates were present in these soils, carbon associated with the samples was considered to be entirely soil organic carbon (SOC).

2.3. Determination of soil ammonium and nitrate

For soil NH_4^+ and NO_3^- analyses, 10 g aliquots of soil were shaken for 1 h with 50 ml of 2 M KCl, and filtered through Whatman, No. 42, ashless, filters. The concentration of N in samples were determined colorimetrically, by using the Berthelot reaction for NH_4^+ (Forster, 1995) and the vanadium(III) chloride reduction method for NO_3^- (Doane and Horwath, 2003).

2.4. Bacterial community abundance

DNA was extracted from 0.5 g aliquots of soil using the FastDNA Spin Kit for Soil (MP Biomedicals, Illkirch, France). Final DNA extracts were stored at −20 °C before analyzing the extracts using real-time (RT) PCR. The concentration of DNA in the extracts was determined using Qubit with Quant-iT dsDNA HS Assay Kits (Invitrogen, Carlsbad, CA, USA).

The abundance of total eubacterial DNA in rhizosphere and bulk soils was quantified by using a real-time TaqMan qPCR assay targeting the universal bacterial 16S rRNA gene (Suzuki et al., 2000). The quantification of the 16S rRNA gene was performed using 4 μl of template DNA, 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, NJ, USA), 4 μl of H_2O, 0.8 μl each of forward (BACT1369F: 5′-CGG TGA ATA CGT TCY CGG-3′; 800 nM) and reverse primers (PROK1492R: 5′-GGW TAC CTT GTT ACG ACTT-3′; 800 nM), and 0.4 μl of the probe (TM1389: 5′-CTT GTA CAC ACC GCC CGTC-3′; 200 nM) (Suzuki et al., 2000). The PCR conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles consisting of 15 s at 95 °C, and 1 min at 56 °C.

Real-time quantitative PCR of the ammonia monooxygenase gene *amoA* was used to quantify the abundance of nitrifier populations. RT-PCR was performed using 5 μl of template DNA, 1.2 μl of the A189 forward (5′-GNG ACT GGG ACT TCT GG-3′; 0.3 μM) and 3.6 μl of the amoA-2R′ reverse (5′-CCC CTC KGS AAA GCC TTC TTC-3′; 0.9 μM) primers. The PCR conditions were as follows: 15 s at 95 °C, and then 40 cycles consisting of 15 s at 95 °C, 30 s at 55 °C, and 31 s at 72 °C, followed by a dissociation stage of 15 s at 95 °C, 30 s at 60 °C, and 15 s at 95 °C (Okano et al., 2004).

The nitrous oxide reductase gene (*nosZ*) was used to quantify the abundance of denitrifier populations. The *nosZ* gene abundance has been shown to correlate well with the denitrifying activity (Ruyters et al., 2010) and other denitrifier genes, such the nitrite reductase gene *nirS* (Morales et al., 2010), and thus it can be used as a marker for denitrifying bacteria (Rich et al., 2003). RT-PCR reactions contained 5 μl of template DNA, 10 μl of 2× ABI Power SYBR Green PCR Master Mix, and 0.8 μl each of forward (nosZ2F: 5′-CGC RAC GGC AAS AAG GTS MSS GT-3′; 0.3 μM) and reverse (nosZ2R: 5′-CAK RTG CAK SGC RTG GCA GAA-3′, 0.3 μM) primers. The PCR conditions were as follows: an initial cycle of 95 °C for 10 min, followed by 6 cycles of 95 °C for 15 s, 65 °C for 30 s, 72 °C for 30 s, then 40 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 30 s, and 83 °C for 30 s (data acquisition step). Reactions were completed with one cycle at 95 °C for 15 s and 60 °C for 30 s, to 95 °C for 15 s (Henry et al., 2006).

The *amoA*, *nosZ*, and 16S rDNA gene abundance was quantified with Applied Biosystems 7300 Real-Time PCR system (Foster City, CA, USA), using triplicate samples. Standard curves were generated for each gene by using serial dilutions of a standard containing a known number of the target sequences. DNA was extracted with a Plasmid Mini Kit (Qiagen) from three plasmids containing *amoA* (GenBank: Z97833), *nosZ* (GenBank: AF197468), and 16S rRNA gene fragments amplified from *Nitrosomonas europaea* (ATCC 19718), *Bradyrhizobium japonicum* (strain USDA 110), and *Escherichia coli* (strain K-12), respectively. The concentration of plasmid DNA was quantified spectrofluorometrically using the Quant-iT fluorescent dye method (Molecular Probes, Invitrogen, Paisley, UK). Standard curves were linear over six orders of magnitude and the detection limit was approximately 100 copies for the *amoA* and *nosZ* real-time qPCRs and 1000 copies for the 16S rRNA real-time qPCR (data not shown). The number of copies of *amoA*, *nosZ*, and 16S rRNA in soil extracts were calculated from the respective concentrations of extracted plasmid DNA.

2.5. Statistical analysis

The analysis of variance (ANOVA) for a randomized block design was performed for each variable. The analyses were performed using the mixed procedures in the SAS statistical package (SAS, 2002), and the blocks were considered as a random factor. Pairwise comparisons were performed using the Tukey–Kramer method, and significance was accepted at $\alpha = 0.05$.

3. Results

3.1. General soil properties

The greatest soil moisture content was measured at V4, followed by R8, and R4 and soil moisture content was higher in the rhizosphere than in the bulk soil at all plant stages studied (data not shown). No changes in soil moisture were associated with elevated CO_2 or O_3 across the three plant phenological stages.

In contrast, total soil N was 11% lower under ambient O_3 than under elevated O_3 conditions and did not differ over the growing season (Tables 2 and 3). Similarly, SOC was significantly higher under elevated O_3 compared to ambient O_3. Elevated CO_2 had no effect on soil N. SOC concentrations were not affected by elevated CO_2, soil environment, or plant phenological stages.

Soil NH_4^+ concentration was significantly greater in the rhizosphere than in bulk soil. With the exception of the R8 stage in the rhizosphere, NH_4^+ increased significantly over the growing season in both rhizosphere and bulk soil (Fig. 1). Elevated O_3 significantly decreased soil NH_4^+ concentrations by the end of the season, relative to that observed under ambient O_3 (Fig. 1). In contrast, elevated CO_2 did not alter soil NH_4^+ content (Fig. 1). On average, the soil NO_3^- concentration decreased by 59% at R4 compared to the V4 and R8 plant stages. The soil NO_3^- content was similar at the V4 and R8 stages and ranged from 5.26 to 9.29 μg N g^{-1} soil (Fig. 1). Soil NO_3^- concentration did not differ between the rhizosphere and the bulk soil. In addition, the elevated CO_2 or O_3 treatments had no significant effect on the soil NO_3^- concentration.

3.2. Quantification of the 16S rRNA, amoA, and nosZ gene abundance

The 16S rRNA gene abundance ranged from 1.54×10^8 to 3.77×10^8 copies g^{-1} soil (Table 3). Elevated CO_2 increased bacterial populations at the R8 stage, but not V4 and R4 (Table 3). At the V4 stage, elevated O_3 increased the soil bacterial abundance when compared to that under ambient O_3. The 16S rRNA gene abundance under both elevated CO_2 and O_3 was similar to that under elevated O_3.

The *amoA* gene abundance ranged from 6.5×10^6 to 1.5×10^7 copies g^{-1} soil (Table 4). Elevated CO_2 and O_3 had no significant effects on *amoA* abundance. Furthermore, *amoA* abundance was not different across plant developmental stages, nor in bulk and rhizosphere soil environments (Table 2).

The abundance of the *nosZ* gene in soils ranged from 2.87×10^5 to 4.18×10^6 copies g^{-1} soil, and this was less than that found for *amoA* (Table 4). The *nosZ* gene was significantly more abundant in the rhizosphere than the bulk soil, and the rhizosphere effect was much more pronounced at the V4 stage of plant growth. Elevated O_3 tended to increase the abundance of *nosZ* gene, and this was marginally significant. On the other hand, elevated CO_2 had no impact on the abundance of *nosZ* (Table 4).

The flows of N between the plants and soil under elevated CO_2 and O_3 observed in this study are shown in the conceptual diagram in the Fig. 2.

4. Discussion

4.1. The effect of elevated CO_2 and O_3 on total N, SOC, and mineral N

Our results indicated that elevated CO_2 had no effects, whereas elevated O_3 increased total N and SOC in soil. One explanation for this finding is that decomposition processes were reduced under elevated O_3 due to the lower amount of plant material input compared to the ambient and elevated CO_2 plots, and this, in turn, may have reduced microbial activities (Singh and Gupta, 1977).

The concentrations of NH_4^+ and NO_3^- at three plant phenological stages in the rhizosphere and bulk soil were quantified to better understand the influence of elevated atmospheric CO_2 and O_3 on plant- and microbial-available N. Since the deposition of plant-derived-material to soils increases during the growing season, we hypothesized that elevated CO_2 increases the abundance of soil mineral N in the later stages and that these effects are more prominent in rhizosphere, due to higher root inputs, than bulk soil. Since elevated O_3 has been observed to decrease the amount of plant residue added to soils in the SoyFACE experiment (Morgan et al., 2006), we expected the opposite effects under elevated O_3 conditions with respect to interactions with plant phenological stages and soil environments. Surprisingly, no interactions were observed between the FACE treatments and plant phenological stage or soil environment (Table 1), which indicates that the effects of elevated CO_2 and O_3 were uniform across plant phenological stages and soil environments.

The lack of observed interactions between the FACE treatments and plant phenological stage or soil environment might be due to simultaneously occurring, but counterbalancing, changes in N transformations and plant N uptake across the season and soil environments. For example, while there is an increase in plant material input under elevated CO_2, compared to ambient CO_2 conditions, there is also an increasing demand for N by plants and microorganisms across the growing season, and within the rhizosphere (Zak et al., 2000b). In contrast, such interactions may not have been observed under elevated O_3 because plant-derived inputs for N mineralization are lower than in other treatments (Kanerva, 2006). At the same time, the demand for N by the plant is lower in the rhizosphere and across the growing season. Hence, the separation of soil environments across the growing season were likely not of sufficient sensitivity to be able to detect the effect of elevated CO_2 and O_3 on soil N dynamics.

With exception of the V4 stage, soil NH_4^+ decreased in both rhizosphere and bulk soil under elevated compared to ambient O_3 (Fig.1a and b), possibly explained by lower substrate inputs. Kanerva et al. (2006) also observed that elevated O_3 decreased soil NH_4^+ concentration in meadow soil, which was associated with a 34 and 40% reduction in aboveground and root biomass, respectively. Elevated O_3 decreased shoot and root dry biomass by about 21% in the SoyFACE experiment (Morgan et al., 2003), and it is possible that the decrease in NH_4^+ in our study was similarly due to reductions in residue inputs under elevated O_3. Another possible reason for decrease in soil NH_4^+ under elevated O_3 may be related to the decrease in symbiotic N_2 fixation by the soybean plants. Although the N concentration in soybean organs and tissues were not affected by elevated O_3, N_2 fixation has been shown to decrease due to the reduced photosynthate translocation to nodules (Pausch et al., 1996). With less N being supplied via symbiosis under elevated O_3, soybean plants would use the soil mineral N available.

Since plant biomass production, N_2 fixation, and microbial decomposition generally increase under elevated CO_2 (Tarnawski and Aragno, 2006), it was expected that soil mineral N content would also increase. However, no significant differences in soil NH_4^+ and NO_3^- in the elevated CO_2 treatments were found relative to the control plots (Table 1). A lack of response in soil mineral N following an increase in CO_2 has similarly been observed in other systems (Barnard et al., 2004; Kanerva et al., 2006; Niklaus et al., 1998). Specific mechanisms that may have counterbalanced the expected increase in N include increased plant N uptake (King et al., 2003) and increased microbial N immobilization (de Graaff et al., 2006a). Increased shortgrass biomass production under elevated CO_2 conditions resulted in increases in plant N uptake in northeastern Colorado steppe ecosystem (King et al., 2003). Elevated CO_2 also was reported to enhance soil N mineralization and consequently increase plant N uptake (Hungate et al., 1997). Greater N immobilization under elevated CO_2 may also have no measurable effect on soil mineral N (Holmes et al., 2006; Mosier et al., 2002). Overall, however, a meta-analysis of 117 studies indicated that elevated CO_2 increased gross N immobilization and microbial N content by 22% and 5.8% respectively (de Graaff et al., 2006a).

Plants often exhibit increased water use efficiency under elevated CO_2, which reduces plant water loss, but might also increase water loss through the soil profile. Johnson et al. (2001) measured a reduction of soil N under elevated CO_2 in a scrub-oak ecosystem and attributed this effect to increased leaching of mineral N. However, in our studies we did not observe higher soil moisture content under elevated CO_2 (data not shown) and thus, increased leaching of mineral N was unlikely. Therefore, we attribute the lack of differences in soil mineral N content between the elevated and ambient CO_2 plots to the enhanced plant N uptake and microbial immobilization. These processes can counterbalance the increased N input in the elevated CO_2 plots to the stimulated output of N caused by elevated CO_2, thereby we concluded that elevated CO_2 conditions do not lead to an accumulation of mineral N at the SoyFACE.

We detected no apparent interactive effect between elevated CO_2 and O_3 on mineral N content. Soil NH_4^+ and NO_3^- content under elevated CO_2 + O_3 were similar to values measured under elevated O_3. This suggests that any amelioration of elevated CO_2 on the inhibitory effect of O_3 on plant photosynthesis (Fiscus et al., 1997) did not offset the O_3 effects on the mineralization of organic N. Thus, elevated O_3 may lead to changes in the chemical composition of plant material returned to the soil. For example, elevated O_3 can affect leaf residue decomposition by decreasing nonstructural carbohydrate and increasing ash-free lignin concentrations, which, in turn, can reduce N mineralization when substrate quantity is not the key factor limiting mineral N release (Booker et al., 2005). The hypothesis that N mineralization is reduced is supported by higher concentrations of total soil N under elevated O_3 than ambient O_3 treatments (Table 2). For woody species, Holmes et al. (2006) observed that elevated O_3 significantly decreased gross N mineralization under both elevated CO_2 and O_3. They concluded that these changes were caused by a modification of the CO_2 effect by O_3 on plant litter production, either by decreasing root turnover or chemical changes in the belowground litter input.

4.2. Responses of N-transforming microorganisms to elevated CO_2 and O_3

While elevated O_3 did not affect the abundance of the *amoA* gene, it increased the abundance of *nosZ* in both rhizosphere and bulk soil. The latter response was likely driven by the higher SOC observed under elevated O_3 providing a carbon source for the reaction (Table 4). Denitrifying microorganisms are dependent on organic C as their source of energy (Wallenstein et al., 2006), and thus the observed increase in the *nosZ* gene abundance, is consistent with the higher SOC observed under elevated O_3.

We hypothesized that the higher plant residue inputs to the elevated CO_2 soil would increase NH_4^+ availability and, in turn, nitrifier populations, as well as favor the heterotrophic denitrifier community. Our measurements of *amoA* and *nosZ* gene copy numbers, however, suggest that elevated CO_2 has little influence on abundances of either nitrifier and denitrifier populations (Table 4), although it is not appropriate to rule out that some group members may not be detected by the primer sets currently used for qPCR. The abundance of *amoA* gene in the soil is governed by factors that control NH_4^+ availability in the soil, such as N mineralization, microbial N immobilization, and plant NH_4^+ uptake (Forbes et al., 2009). In this study, soil NH_4^+ content under elevated CO_2 was similar to the amount found under ambient CO_2 (Fig. 1), even though the input of plant substrate into the soil was greater under CO_2 enrichment. This suggests that the NH_4^+ was not available for the nitrifying bacteria, but rather may have been taken up by plants and heterotrophic microorganisms (Bowatte et al., 2008; Hungate et al., 1999). Similarly, Horz et al. (2004) investigated the response of soil bacteria to multi-factorial global change parameters and observed that the abundance of *amoA* decreased in response to elevated CO_2. Elevated CO_2 stimulated growth of heterotrophic microorganisms and autotrophic nitrifying microorganisms were poor competitors for common resources. Thus, Horz et al. (2004) postulated that the nitrifiers' inability to effectively compete explained the decreases in *amoA* gene abundance under elevated CO_2.

The abundance of denitrifying bacteria is controlled by soil O_2, the availability of C substrates, and NO_3^- concentrations (Barnard et al., 2005). Thus, the lack of response of *nosZ* gene abundance may be expected from the lack of change in soil moisture and NO_3^- content under elevated CO_2 conditions. Elevated CO_2 failed to increase the abundance of *nosZ* genes and other genes involved in the denitrification process in the rhizosphere of Phaseolus vulgaris L. under two levels of N (Haase et al., 2008). Although elevated CO_2 stimulates C deposition through root exudates, it apparently has only a small effect on the denitrifier community.

We found neither any interactive effect between elevated CO_2 and O_3 on populations of nitrifiers and denitrifiers, nor any interactions between the FACE treatments and plant phenological stage or soil environment on either microbial population (Table 4). The abundance of the nitrifier populations is driven, in part, by the amount of NH_4^+ in the soil. Consequently, the lack of interaction between elevated CO_2 and O_3, and plant phenological stage or soil environment on the concentration of NH_4^+, is mirrored in the abundance of *amoA* genes. The abundance of denitrifiers is controlled by SOC, soil moisture and NO_3^- concentration in the soil (Wallenstein et al., 2006). No changes in the abundance of *nosZ* were expected because of the absence of any interactions between elevated CO_2 and O_3, and plant phenological stage or soil environment affecting these variables.

5. Conclusion

N transformations at the SoyFACE site were less impacted by elevated CO_2 than elevated O_3, and any differences were unaffected by the plant phenological stage or the presence of a plant rhizosphere. Although elevated CO_2 increases plant biomass production, this increase had limited effects on belowground N processes. Also, though increases of tropospheric O_3 can diminish plant-available N by decreasing plant inputs and mineralization, and by increasing denitrification, we observed an accumulation of total N. To explore further if elevated O_3 limits N availability, research should focus on changes in specific components of plant residues (e.g., cellulose, lignin), to more carefully track decomposition patterns under elevated O_3.

Acknowledgments

This research was funded by the National Science Foundation (grant # NSF-DEB 0543218). Additional support was provided by Award Number P42ES004699 from the National Institute of Environmental Health Sciences. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.

References

Ainsworth EA, Long SP. What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy. New Phytologist. 2005; 165:351–371. [PubMed: 15720649]

Alcoz MM, Hons FM, Haby VA. Nitrogen fertilisation, timing effect on wheat production, nitrogen uptake efficiency, and residual soil nitrogen. Agronomy Journal. 1993; 85:1198–1203.

Andersen CP. Sourceesink balance and carbon allocation below ground in plants exposed to ozone. New Phytologist. 2003; 157:213–228.

Barnard R, Barthes L, Le Roux X, Harmens H, Raschi A, Soussana JF, Winkler B, Leadley PW. Atmospheric CO_2 elevation has little effect on nitrifying and denitrifying enzyme activity in four European grasslands. Global Change Biology. 2004; 10:488–497.

Barnard R, Leadley PW, Lensi R, Barthes L. Plant, soil microbial and soil inorganic nitrogen responses to elevated CO_2: a study in microcosms of *Holcus lanatus*. Acta Oecologica-International Journal of Ecology. 2005; 27:171–178.

Booker FL, Miller JE, Fiscus EL, Pursley WA, Stefanski LA. Comparative responses of container-versus ground-grown soybean to elevated carbon dioxide and ozone. Crop Science. 2005; 45:883–895.

Bowatte S, Carran RA, Newton PCD, Theobald P. Does atmospheric CO_2 concentration influence soil nitrifying bacteria and their activity? Australian Journal of Soil Research. 2008; 46:617–622.

Cheng WX, Johnson DW, Fu SL. Rhizosphere effects on decomposition: controls of plant species, phenology, and fertilization. Soil Science Society of America Journal. 2003; 67:1418–1427.

de Graaff MA, van Groeningen KJ, Six J, Hungate BA, van Kessel C. Interactions between plant growth and nutrient dynamics under elevated CO_2: a meta analysis. Global Change Biology. 2006a; 12:1–15.

de Graaff MA, Six J, Blim H, van Kessel C. Prolonged elevated atmo-spheric CO_2 does not affect decomposition of plant material. Soil Biology and Biochemistry. 2006b; 38:187–190.

Dermody O, Long SP, DeLucia EH. How does elevated CO_2 or ozone affect the leaf-area index of soybean when applied independently? New Phytologist. 2006; 169:145–155. [PubMed: 16390426]

Doane TA, Horwath WR. Spectrophotometric determination of nitrate with a single reagent. Analytical Letters. 2003; 36:2713–2722.

Fiscus EL, Reid CD, Miller JE, Heagle AS. Elevated CO_2 reduced O_3 flux and O_3 - induced yield losses in soybeans: possible implications for elevated CO_2 studies. Journal of Experimental Botany. 1997; 48:307–313.

Forbes MS, Broos K, Baldock JA, Gregg AL, Wakelin SA. Environmental and edaphic drivers of bacterial communities involved in soil N-cycling. Australian Journal of Soil Research. 2009; 47:380–388.

Forster, JC. Soil nitrogen. In: Alef, K.; Nannipieri, P., editors. Methods in Applied Soil Microbiology and Biochemistry. Academic Press; San Diego, CA: 1995. p. 79-87.

Haase J, Brandl R, Scheu S, Schadler M. Above- and belowground interactions are mediated by nutrient availability. Ecology. 2008; 89:3072–3081.

Hallin S, Jones CM, Schloter M, Philippot L. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. The ISME Journal. 2009; 3:597–605. [PubMed: 19148144]

Henry S, Bru D, Stres B, Hallet S, Philippot L. Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG, nirK*, and *nosZ* genes in soils. Applied and Environmental Microbiology. 2006; 72:5181–5189. [PubMed: 16885263]

Holmes WE, Zak DR, Pregitzer KS, King JS. Soil nitrogen transformations under *Populus tremuloides, Betula papyrifera* and *Acer saccharum* following 3 years exposure to elevated CO_2 and O_3. Global Change Biology. 2003; 9:1743–1750.

Holmes WE, Zak DR, Pregitzer KS, King JS. Elevated CO_2 and O_3 alter soil nitrogen transformations beneath trembling aspen, paper birch, and sugar maple. Ecosystems. 2006; 9:1354–1363.

Horz HP, Barbrook A, Field CB, Bohannan BJM. Ammonia-oxidizing bacteria respond to multifactorial global change. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:15136–15141. [PubMed: 15469911]

Hungate BA, Chapin FS, Zhong H, Holland EA, Field CB. Stimulation of grassland nitrogen cycling under carbon dioxide enrichment. Oecologia. 1997; 109:149–153.

Hungate BA, Dijkstra P, Johnson DW, Hinkle CR, Drake BG. Elevated CO_2 increases nitrogen fixation and decreases soil nitrogen mineralization in Florida scrub oak. Global Change Biology. 1999; 5:781–789.

Intergovernamental Panel on Climate Change (IPCC). Contribution of Working Group I to the Fourth Assessment Report of the Intergovernamental Panel on Climate Change The physical Sciences Basis. C. C; 2007; 2007.

Johnson DW, Hungate BA, Dijkstra P, Hymus G, Drake B. Effects of elevated carbon dioxide on soils in a Florida scrub oak ecosystem. Journal of Environmental Quality. 2001; 30:501–507. [PubMed: 11285911]

Kanerva, T. Ph.D. Thesis. Department of Biological and Environmental Sciences; University of Helsinki, Finland,Yliopistopaino, Helsinki: 2006. Below-ground processes in meadow soil under elevated ozone and carbon dioxide.

Kanerva T, Palojarvi A, Ramo K, Ojanpera K, Esala M, Manninen S. A 3-year exposure to CO_2 and O_3 induced minor changes in soil N cycling in a meadow ecosystem. Plant and Soil. 2006; 286:61–73.

King, JY.; Milchunas, DG.; Mosier, AR.; Moore, JC.; Quirk, MH.; Morgan, JA.; Slusser, JR. Initial impacts of altered UVB radiation on plant growth and decomposition in shortgrass steppe. In: Herman, JR.; Gao, W., editors. International Soc. Optical Eng; Ultraviolet Ground- and Space-Based Measurements, Models, and Effects III. SPIE Proceedings, vol. 5156. Slusser; Bellingham, WA. 2003; p. 384-395.

Lynch JM, Whipps JM. Substrate flow in the rhizosphere. Plant and Soil. 1990; 129:1–10.

Luo YQ, Hui DF, Zhang DQ. Elevated CO_2 stimulates net accumulations of carbon and nitrogen in land ecosystems: a meta-analysis. Ecology. 2006; 87:53–63. [PubMed: 16634296]

Morales SE, Cosart T, Holben WE. Bacterial gene abundances as indicators of greenhouse gas emission in soils. The ISME Journal. 2010; 4:799–808. [PubMed: 20182521]

Morgan PB, Ainsworth EA, Long SP. How does elevated ozone impact soybean? A meta-analysis of photosynthesis, growth and yield. Plant, Cell and Environment. 2003; 26:1317–1328.

Morgan PB, Mies TA, Bollero GA, Nelson RL, Long SP. Season-long elevation of ozone concentration to projected 2050 levels under fully open-air conditions substantially decreases the growth and production of soybean. New Phytologist. 2006; 170:333–343. [PubMed: 16608458]

Mosier AR, Morgan JA, King JY, LeCain D, Milchunas DG. Soil–eatmosphere exchange of CH_4, CO_2, NO_x, and N_2O in the Colorado shortgrass steppe under elevated CO_2. Plant and Soil. 2002; 240:201–211.

Niklaus PA, Leadley PW, Stocklin J, Korner C. Nutrient relations in calcareous grassland under elevated CO_2. Oecologia. 1998; 116:67–75.

Okano Y, Hristova KR, Leutenegger CM, Jackson LE, Denison RF, Gebreyesus B, Lebauer D, Scow KM. Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. Applied and Environmental Microbiology. 2004; 70:1008–1016. [PubMed: 14766583]

Ort, DR.; Ainsworth, EA.; Aldea, M.; Allen, DJ.; Bernacchi, CJ.; Berenbaum, MR.; Bollero, GA.; Cornic, G.; Davey, PA.; Dermody, O.; Dohleman, FG.; Hamilton, JG.; Heaton, EA.; Leakey, ADB.; Mahoney, J.; Morgan, PB.; Nelson, RL.; O'Neill, B.; Rogers, A.; Zangerl, AR.; Zhu, XG.; DeLucia, EH.; Long, SP. SoyFACE: the effects and interactions of elevated [CO_2] and [O_3] on soybean. In: Nosberger, J.; Long, SP.; Stitt, GR.; Hendrey, GR.; Blum, H., editors. Managed Ecosystems and CO_2: Case Studies, Processes and Perspectives. Springer; Berlin: 2006. p. 71-85.

Pausch RC, Mulchi CL, Lee EH, Meisinger JJ. Use of ^{13}C and ^{15}N isotopes to investigate O_3 effects on C and N metabolism in soybeans. Part II. Nitrogen uptake, fixation, and partitioning. Agriculture, Ecosystems and Environment. 1996; 60:61–69.

Prather, M.; Ehhalt, D.; Dentener, F.; Derwent, R.; Dlugokencky, E.; Holland, E.; Isaksen, I.; Katima, J.; Kirchhoff, V.; Matson, P.; Midgley, PM.; Wang, M. Atmospheric chemistry and greenhouse gases. In: Houghton, JT.; Ding, Y.; Griggs, DJ.; Noguer, M.; van der Linder, PJ.; Dai, X.; Maskell, K.; Johnson, CA., editors. Climate Change 2001: The Scientific Basis; Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change; Cambridge University Press, Cambridge, UK. 2001; p. 239-287.

Rich JJ, Heichen RS, Bottomley PJ, Cromack K Jr. Myrold DD. Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. Applied and Environmental Microbiology. 2003:5974–5982. [PubMed: 14532052]

Ritchie, SW.; Hanway, JJ.; Thompson, HE.; Benson, GO. How a Soybean Plant Develops. Cooperative Extension Service; Iowa State University of Science and Technology: 1997.

Ruyters S, Mertens J, T'Seyen I, Springael D, Smolders E. Dynamics of the nitrous oxide reducing community during adaptation to Zn stress in soil. Soil Biology & Biochemistry. 2010; 42:1581–1587.

SAS Institute. Statistical Analysis System, v.8.2. SAS Institute; Cary, NC: 2002.

Singh JS, Gupta SR. Plant decomposition and soil respiration in terrestrial ecosystems. The Botanical Review. 1977; 44:449–528.

Suzuki MT, Taylor LT, DeLong EF. Quantitative analysis of small-subunit rRNA genes in mi′-nuclease assays. Applied and Environmental Microbiology. 2000; 66:4605–4614. [PubMed: 11055900]

Tarnawski, S.; Aragno, M. The influence of elevated CO_2 on diversity, activity and biogeochemical function of rhizosphere and soil bacterial communities. In: Nosberger, J.; Long, SP.; Stitt, GR.; Hendrey, GR.; Blum, H., editors. Managed Ecosystems and CO_2: Case Studies, Processes and Perspectives. Springer; Berlin: 2006. p. 393-409.

Tyree MT, Alexander JD. Plant water relations and the effects of elevated CO_2 – a review and suggestions for future research. Vegetation. 1993; 104:47–62.

Vitousek PM, Howarth RW. Nitrogen limitation on land and in the sea e how can it occur. Biogeochemistry. 1991; 13:87–115.

Wallenstein MD, Myrold DD, Firestone M, Voytek M. Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. Ecological Applications. 2006; 16:2143–2152. [PubMed: 17205893]

Zak DR, Pregitzer KS, Curtis PS, Teeri JA, Fogel R, Randlett DL. Elevated atmospheric CO_2 and feedback between carbon and nitrogen cycles. Plant and Soil. 1993; 151:105–117.

Zak DR, Pregitzer KS, King JS, Holmes WE. Elevated atmospheric CO_2, fine roots and the response of soil microorganisms: a review and hypothesis. New Phytologist. 2000a; 147:201–222.

Zak DR, Pregitzer KS, Curtis PS, Holmes WE. Atmospheric CO_2 and the composition and function of soil microbial communities. Ecological Applications. 2000b

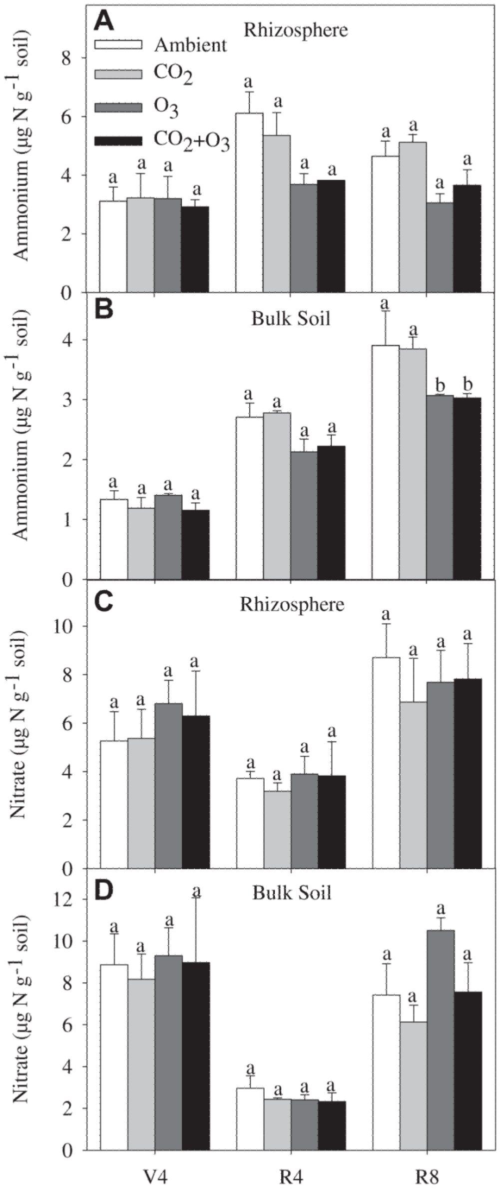

Fig. 1.

Concentrations of ammonium (NH_4^+) in the rhizosphere (a) and bulk soil (b) and concentrations of nitrate (NO_3^-) in the rhizosphere (c) and bulk soil (d) under elevated CO_2 and O_3 treatments (V4 = Fourth trifoliate leaf; R4 = Full pod; R8 = Full maturity).

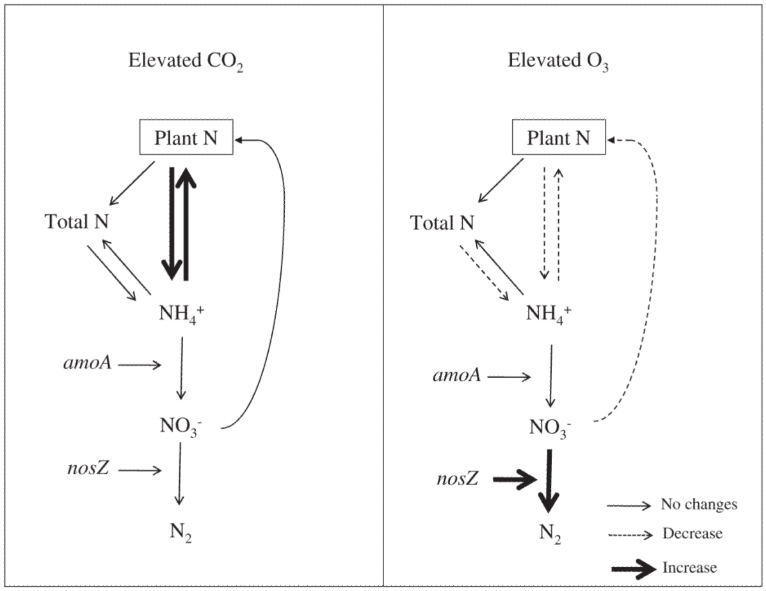

Fig. 2.

Flows of N between the plants and soil under elevated CO_2 and O_3. Even though elevated CO_2 increases plant biomass production and thus increases the plant demands of N, the mineral N released in the decomposition process is quickly taken up by the plants. In contrast, elevated O_3 decreases plant biomass production and thus the demand of N by the plant. Since the input of organic material is reduced under elevated O_3, the mineralization process is also decreased, which leads to an accumulation of total N. Lastly, possibly due to the availability of organic material under elevated O_3, a higher abundance of the denitrifier gene was observed compared to ambient O_3 plots.

Table 1

P-values for soil moisture ($d\theta$), total N (TN), soil organic carbon (SOC), ammonium (NH_4^+), nitrate (NO_3^-), total bacteria (16S rRNA), nitrifying (*amoA*), and denitrifying bacteria (*nosZ*) by analysis of variance (ANOVA).

Source[a]	**dθ**	**TN**	**SOC**	**NH_4^+**	**NO_3^-**	**16S rRNA**	***amoA***	***nosZ***
CO_2		–	–	–	–	–	–	–
O_3	–	0.01	0.01	0.03	–	0.01	–	0.06
Soil environment (SE)	0.001	–	–	<0.0001	–	–	–	0.01
Plant phenological stage (PS)	<0.0001	–	–	<0.0001	<0.0001	<0.0001	–	0.06
PS × CO_2	–	–	–	–	–	0.04	–	–
PS × SE	<0.0001	–	–	0.01	0.04	–	–	–

(–) Not significant at $P < 0.05$.

[a] $CO_2 \times O_3$, SE × CO_2, SE × O_3, SE CO_2 + Oxl, PS × O_3, PS × $CO_2 \times O_3$, PS × SE × CO_2, PS × SE × O_3, PS × SE × $CO_2 \times O_3$ interactions wrer not significant for these variables.

Table 2

Soil nitrogen (Total N) and soil organic carbon (SOC) concentration under elevated CO_2 and O_3 treatments in the rhizosphere and bulk soil during the 2008 growing season. Values in parentheses are standard errors (n = 12). Means followed by the same letter within a column are not statistically different ($P > 0.05$).

Treatment	**Total N (g N kg^{-1} soil)**		**SOC (g C kg^{-1} soil)**	
	Rhizosphere	**Bulk**	**Rhizosphere**	**Bulk**
Ambient	1.76 (0.09) B	1.83 (0.07) B	19.66 (1.14) B	20.86 (1.00) B
Elevated CO_2	1.82 (0.06) B	1.83 (0.05) B	20.45 (1.18) B	20.48 (0.84) B
Elevated O_3	2.07 (0.06) A	2.06 (0.08) A	24.25 (1.27) A	23.91 (0.99) A
Elevated CO_2 + O_3	2.01 (0.05) A	2.01 (0.06) A	22.89 (0.91) A	22.9 (0.82) A

Table 3

Total bacterial abundance (16S rRNA gene copies per gramof soil) under elevated CO_2 and O_3 treatments during the 2008 growing season at three sampling times (V4 = Fourth trifoliate leaf; R4=Full pod;R8 = Full maturity). Values are means with standard errorsinparentheses. Means withina column followed bythe same lowercase letterorwithin a row followed by the same uppercase letter are not significantly different ($P > 0.05$).

	16S rRNA (copies g^{-1} soil)		
	V4	**R4**	**R8**
Ambient	1.7×10^8 (1.8×10^7) Bb	2.2×10^8 (1.9×10^7) Aa	2.4×10^8 (3.2×10^7) Ab
CO_2	1.8×10^8 (1.8×10^7) Bb	1.9×10^8 (1.9×10^7) Ba	3.0×10^8 (3.3×10^7) Aab
O_3	2.2×10^8 (1.6×10^7) Aa	2.3×10^8 (1.1×10^7)Aa	2.8×10^8 (1.8×10^7) Aab
$CO_2 + O_3$	2.3×10^8 (1.7×10^7) Ba	2.2×10^8 (1.3×10^7) Ba	3.2×10^8 (2.6×10^7) Aa

Table 4

Abundance of nitrifier (*amoA*) and denitrifier (*nosZ*) genes under elevated CO_2 and O_3 treatments in the rhizosphere and bulk soil during the 2008 growing season at three sampling times (V4 = Fourth trifoliate leaf; R4 = Full pod; R8 = Full maturity). Values are means with standard errors in parentheses ($n = 4$).

Soil environment	**Treatment**	***amoA* (copies g^{-1} soil)**			***nosZ* (copies g^{-1} soil)**		
		V4	**R4**	**R8**	**V4**	**R4**	**R8**
Rhizosphere	Ambient	1.2 × l07 (2.3×10^6)	9.2×10^6 (3.8×10^5)	7.6×10^6 (1.3×10^6)	2.6×10^6 (6.2×10^5)	3.7×10^6 (8.3×10^5)	7.2×10^5 (1.4×10^5)
	CO_2	1.3×10^7 (1.1×10^6)	6.5×10^6 (4.3 × 10s)	9.5×10^6 (2.4×10^6)	1.9×10^6 (6.3×10^5)	1.6×10^6 (5.5×10^5)	8.9×10^5 (1.1×10^5)
	O3	1.5×10^7 (2.0×10^6)	1.1×10^7 (9.5×10^5)	8.8×10^6 (9.4×10^5)	4.2×10^6 (7.9×10^5)	1.2×10^6 (8.3×10^4)	1.3×10^6 (2.0×10^5)
	CO_2 + O3	1.3×10^7 (1.5×10^6)	7.6×10^6 (1.0×10^6)	1.2×10^7 (2.5×10^6)	2.2×10^6 (5.3×10^5)	1.4×10^6 (1.9×10^5)	1.2×10^6 (1.8×10^5)
Bulk soil	Ambient	1.2×10^7 (6.5×10^5)	8.4×10^6 (1.2×10^6)	5.1×10^6 (9.7×10^5)	1.0×10^6 (2.3×10^5)	1.2×10^6 (2.1×10^5)	7.0×10^5 (2.2×10^5)
	CO_2	8.0×10^6 (6.5×10^5)	1.4×10^7 (1.0×10^6)	1.1×10^7 (1.3×10^6)	2.9×10^5 (8.8×10^4)	1.9×10^6 (5.8×10^5)	9.1×10^5 (1.3×10^5)
	O3	1.0×10^7 (2.1×10^5)	1.0×10^7 (1.3×10^6)	6.7×10^6 (6.8×10^5)	1.8×10^6 (5.3×10^5)	2.5×10^6 (9.2×10^5)	7.5×10^5 (1.1×10^5)
	CO_2 + O3	1.1×10^7 (1.6×10^6)	7.3×10^6 (7.6×10^5)	8.0×10^6 (2.4×10^5)	2.8×10^6 (7.3×10^5)	2.3×10^6 (4.5×10^5)	7.7×10^5 (1.0×10^5)