Using Natural Isotope Variations of Nitrogen in Plants as an Early Indicator of Air Pollution Stress

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Model experiments (container experiments) were used to investigate whether natural variations of nitrogen constitute a suitable means for the early indication of harmful effects on plants and the level at which changes in nitrogen metabolism may occur. Wheat plants were exposed under near-ambient, controlled conditions (open-top chambers) to ozone for 2 or 6 weeks. The responses of the nitrogen metabolism in these plants were compared with non-exposed organisms at various biochemical levels. In the short-term experiment (2 weeks), wheat plants exhibited altered $\delta^{15}N$ values under stress conditions right at the molecular level in the individual amino acids, whereas the biochemical N-fractions (structural protein (SP), soluble protein (LP) and non-protein nitrogen (NPN)) remained nearly unchanged. In the long-term experiment (6 weeks), the impact of ozone treatment and the combined effects of ozone and carbon dioxide on the wheat plants were examined. An increase in $\delta^{15}N$ values was found as a reaction to ozone treatment in both the LP amino acids detected and in all biochemical N-fractions. Combined exposure to ozone and carbon dioxide was found to have a lesser impact at the level of the N-fractions and also at the molecular level of amino acids. The results show an example of how stable isotopes can be used as sensitive ecotoxicological indicators to provide early warning of environmental damage. **© 1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Organisms often react to various kinds of stress, including air pollutants, by changes in the protein balance.[1] Various investigations have focused on pollutant-induced changes of the plant protein metabolism by common air pollutants such as ozone, nitrogen oxides and SO_2, which are extremely phytotoxic gases.[2–6] The effects of ozone as the main component of photochemical 'summer smog' and the air pollutant with the greatest damage potential for vegetation have been described using parameters such as biomass production (shoot and root growth, dry matter partitioning, including assimilate partitioning) and carbon allocation, photosynthesis (chlorophyll level), respiration and visible symptoms of harm (chlorosis, necrosis and premature senescence) compared with non-stressed controls.[7–14]

Detecting complex effects such as those caused by the presence of a number of different pollutants and which involve antagonistic reactions (e.g. the rising level of CO_2 in the atmosphere due to the increasing combustion of fossil fuels decreasing the impact of phytotoxic gases such as ozone on plant organisms[9,14]) necessitates investigations at cellular and subcellular levels. In this context, physiological and biochemical studies are concentrated on leaf-gas exchange (or stomatal conductance) measurements, on the determination of cellular antioxidants (such as ascorbate and glutathione) and of the non-structural carbohydrate content as indicators of the altered provision of substrates for detoxification and repair processes,[7,9–12,14] as well as on measurements of free amino acids and the activities of enzymes such as peroxidase, ascorbate peroxidase, glutamine synthetase, glutamate dehydrogenase, nitrate and nitrite reductase.[15–20] The use of stable isotopes has been established as an alternative method because changes in the isotope ratios due to anthropogenic causes are often more sensitive than the macroscopic reactions of organisms.[21] The main advantage of the ^{15}N isotope technique in environmental studies in the fact that nitrogen is a basic element of all natural compartments, including living organisms.[22]

As an alternative to classical tracer studies,[20,23,24] the development of new technical equipment[25] over the past few years has led to an increase in the number of studies of the natural isotope variations of nitrogen in ecotoxicological research.[21] This method allows studies of the metabolic reaction of organisms to environmental stress without additional tracer effects.

The isotopic composition of a sample is expressed on the $\delta^{15}N$ scale, defined as

$$\delta^{15}N = \frac{(^{15}N/^{14}N)_{sample} - (^{15}N/^{14}N)_{standard}}{^{15}N/^{14}N_{standard}} \times 1000\ (‰)$$

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where ‰ (per mil) is parts per thousand and $^{15}N/^{14}N$ is the ratio of the number of ^{15}N atoms to the number of ^{14}N atoms in the sample or standard. The standard generally used is atmospheric air, defined as 0‰.[21] Interpretation of the $\delta^{15}N$ data has so far been mainly empirical and may best be performed iteratively working between the field and the laboratory.[22]

The main application of natural variation studies is in field investigations.[26–29] Investigations into complete needles and twigs of Norway spruce (*Picea abies* L.) from two different forest stands showed (in contrast to $\delta^{13}C$ values) a significant increase in the $\delta^{15}N$ values of the declining stand compared with the healthy stand. The reasons for these changes are thought to be the reduced anabolic activity and the earlier onset of catabolic decay in the needles of the declining stand.[26]

Similar results were obtained in a biomonitoring study of pine trees (*Pinus sylvestris* L.) carried out in 1992–94 in the Leipzig–Halle region, which is subject to emissions from the chemical industry, power stations and coal treatment plants. The $\delta^{15}N$ values of 1-year-old pine needles in this region were found to vary depending on their geographical location by a factor of up to one order of magnitude (−9.6 to +0.4‰): pine stands with positive or slightly negative $\delta^{15}N$ values in the needles were concentrated around conurbations and in the highly polluted industrial areas, whereas more negative $\delta^{15}N$ values were found in more rural regions.[29]

To answer the question of whether and on what level natural variations of nitrogen caused by complex mixtures of organic and inorganic pollutants provide a useful means for the early indication of harmful impact, the individual effects of selected pollutants must first be examined. In this investigation, model experiments were carried out to examine the impact of ozone on the plant metabolism of wheat, and also the combined effect of ozone and CO_2.

In earlier studies of ^{15}N isotope variations, often only complete parts of plants and soils were investigated[26–29] because of the lower natural abundance of ^{15}N *vs.* ^{13}C. Such bulk analyses (total combustion using an elemental analyzer connected to an isotope ratio mass spectrometer; EA/IRMS) merely allow the interpretation of changes in $\delta^{15}N$ values as sum parameters. Therefore, we first divided the tops (leaves and stalks) into different biochemical fractions (structural protein (SP), soluble protein (LP) and non-protein nitrogen (NPN) as described previously[20] before analyzing isotope ratio changes using EA/IRMS. These analyses are still on bulk samples, because the different biochemical fractions include not only proteins and amino acids but also other N-containing components.

One aim of the present investigation was to connect the bulk analyses with the precise determination of minuscule changes in the N-isotope ratios on a molecular level in the nanomole range by using one of the latest on-line IRMS systems. This technique allows a gas chromatograph with a combustion interface to be linked to an IRMS system especially for ^{15}N isotope analysis (GC-C-II: gas-chromatographic separation of the sample mixture with on-line combustion of the eluate).[30–32] To use this technique, traditional sample preparation techniques were examined for their suitability with regard to listed conditions and replaced with new ones if necessary. In contrast to ^{13}C,[33] investigations of amino acids in ^{15}N studies with, e.g., ion-exchange chromatography for sample clean-up, leads to isotope fractionation.[34] This could be the reason why applications of this technique have previously only been published for slightly ^{15}N-enriched samples.[35,36] To obtain more detailed information about ozone-induced changes in nitrogen metabolism, in addition to the biochemical fractions (SP, LP and NPN) we studied in the second step the changes in the natural isotope variations at the molecular level of the plants (by means of the amino acids of the soluble protein fraction).

EXPERIMENTAL

Plant cultivation and exposure conditions

Four different ozone exposure experiments with wheat (*Triticum aestivum*) were carried out in open-top chambers[37] at the Braunschweig Agriculture Research Centre during the 1993–95 growing seasons. The fourth experiment was extended by combined exposure to $CO_2 + O_3$ in addition to O_3 and CO_2 alone.

The seed material was pre-steeped and pre-germinated on moist cellulose for 1 day. The seedlings were planted in plastic containers filled with a mixture of soil and sand (2:1), covered with sand and moistened with doubly distilled water, and the containers were covered with transparent foil. After 1 week under greenhouse conditions the covers were removed and the plants were reduced to two-thirds of the initial number (by removing in particular growing anomalies to ensure a more homogeneous crop). Exposure to ozone began at this point. In Experiments 1–3, ozone was dosed to charcoal-filtered air for 10 h (10 am–8 pm) daily. Filtered air without the addition of ozone was used as a control.

In Experiment 4, non-filtered air was used as a control, the ozone level was 50% higher than the ambient air and the CO_2 level was ambient air plus 320 ppm CO_2. The mean gas concentration and the different exposure conditions of all the experiments are presented in Table 1.

In comparative tracer experiments, $K^{15}NO_3$ (20 mg nitrogen, 51.3 at.% ^{15}N, dissolved in doubly distilled water) was used as the ^{15}N source. In Experiments 1–3,[22] tracer application was performed immediately after sowing of the wheat seedlings, whereas in Experiment 4 the tracer application took place 4 weeks after sowing.

During the entire experiment, the plants' water loss was replenished daily. At the end of the experiments, the plants were harvested by cutting off the plant sections above the soil and the material was immediately frozen.

Reagents

All chemicals were of analytical grade. *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, USA).

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Table 1. Exposure conditions in the different experiments

Experiment No.	Exposure (days)	Ozone treatment: target concentration (ppb)	Ozone treatment: 8 h mean[a] (ppb)	CO_2 treatment (ppm)
1	14	90	78	—
2	20	110	88	—
3	14	90/60[b]	72	—
4	54	1.5 × ambient air	52	716

[a] 8 h d^{-1} (11 : 00–19 : 00) seasonal mean ozone concentration.
[b] Ozone was dosed to a target level of 90 ppb when ozone concentration in the ambient air exceeded 40 ppb; when the ambient air concentration was below 40 ppb, ozone was added to a target level of 60 ppb.

Separation and conversion of the biochemical N-fractions

Frozen plant material (2 g) was washed with doubly distilled water and dried on filter-paper. After coarsely cutting the leaves and adding phosphate buffer (pH 6.5, 0.1 M), the mixture was homogenized with an Ultra-Turrax (supplied by Jahnke & Kunkel, Freiburg, Germany) and allowed to stand overnight at 4 °C. The solid residue (structural protein) was separated by filtration. The filtrate was centrifuged (10 000 U min^{-1}, 0 °C, 15 min) and filtered through cotton-wool, and the clear solution was treated with 20% trichloroacetic acid (1 : 1 v/v, 4 °C, 30 min). After centrifugation, the precipitate (soluble protein) was first washed with 0.5% trichloroacetic acid followed by doubly distilled water. The supernatant contained the non-protein nitrogen. An aliquot of the soluble protein was freeze-dried for GC/IRMS analysis. The biochemical fractions were converted into ammonium sulfate using the micro-Kjeldahl technique.[38] After distillation coupled with titration to determine the nitrogen content (% N fresh mass) of the corresponding fractions, the dried $(NH_4)_2SO_4$ samples thus obtained were analyzed by linking an elemental analyzer to an IRMS system.

Sample chemistry of amino acids

Samples containing ~ 5 mg of soluble protein were hydrolyzed with 10 ml of constant-boiling 6 M HCl in reaction vials. Each vial was placed in an ultrasonic bath. Evacuation (using a membrane pump) and flushing with helium gas took place alternately.[39] This procedure was repeated five times, after which the helium-filled vial was quickly removed and a PTFE-lined screw-cap was placed on the vial and tightened.

After hydrolysis (145 °C, 4 h), the vial was immediately removed from the oven and cooled to room temperature. The cold solution was filtered through glass-fiber paper, a standard (tranexamic acid solution in 0.1 M HCl) was added and finally the mixture was evaporated to dryness. The samples were dried a second time using CH_2Cl_2 under a gentle stream of helium and slow heating in an oil-bath (40–60 °C) up to five times immediately before derivatization. For the *tert*-butyldimethylsilylation, 100 µl of pyridine, 100 µl of MTBSTFA and three drops of triethylamine were added to each dry residue and heated in an oven for 30 min at 75 °C. Aliquots of these solutions were analyzed without further treatment by GC/MS and GC/IRMS. We had previously checked that this technique does not cause any fractionation.[34] The authors referred to the fact that the samples, derivatized by the described method, are stable for only a few days.

Chlorophyll determination

Pigments were extracted from fresh material with 100% acetone. Absorption at 470, 645 and 662 nm was measured photometrically and the chlorophyll content was calculated as described by Lichtenthaler and Wellburn.[40]

Isotope analysis

GC/IRMS/MS measurements were carried out with a Hewlett-Packard model 5890 II gas chromatograph, connected via a split with (a) a combustion interface to the IRMS system (GC-C-II to MAT 252; Finnigan MAT) for the isotopic determination of nitrogen and (b) via a transfer line with a mass spectrometer (GCQ; Finnigan MAT) for qualitative analysis and quantification of the amino acids (Fig. 1). The capillary column was a 50 m × 0.32 mm i.d. × 0.5 µm Ultra 2 operating with the following temperature program: initial 100 °C, held for 1 min; increased from 100 to 120 °C at 8 °C min^{-1}, increased from 120 to 190 °C at 2 °C min^{-1}; increased from 190 to 280 °C at 12 °C min^{-1}; held at 280 °C for 25 min. The head pressure was 13 psi (90 kPa).

The combustion interface was operated under the following conditions: the oxidation reactor contained CuO–NiO–Pt and was operated at 980 °C and the reduction reactor contained Cu and was operated at 650 °C.

Electron impact mass spectra (GCQ) were recorded at an electron energy of 70 eV.

EA/IRMS measurements were performed with the combination of an elemental analyzer (Carlo Erba Model 1108) for Dumas combustion of the sample with a Delta C-IRMS system (Finnigan MAT).

Emission spectrometric ^{15}N isotope analysis. For the emission spectrometric isotope analysis, the ^{15}N-labeled plant organs and biochemical fractions were also converted into ammonium sulfate using the micro-Kjeldahl

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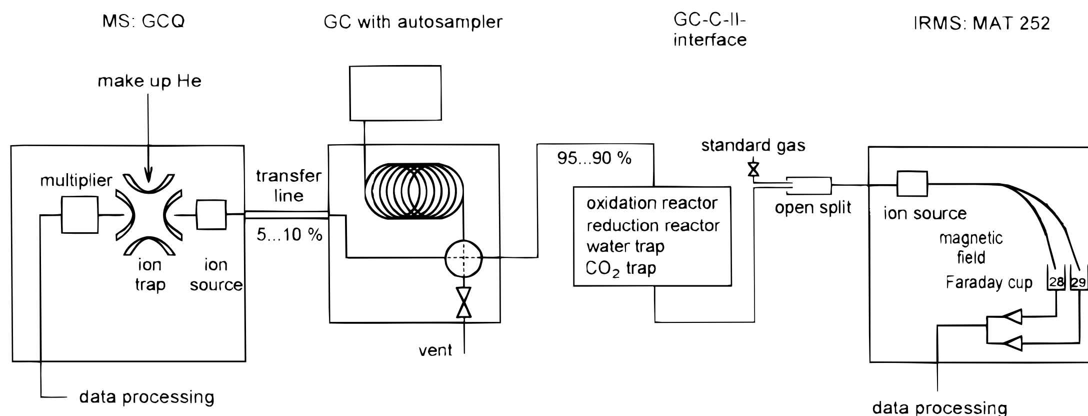

Figure 1. Set-up of the GC/IRMS/MS system.

technique. An aliquot of 10 µg of nitrogen dissolved in 25 µl of doubly distilled water was converted into N_2 according to the Rittenberg procedure for the emission spectrometric ^{15}N isotope analysis[41] with an NOI-6PC system (FAN Fischer Analysentechnik, Leipzig, Germany).

RESULTS AND DISCUSSION

Content of chlorophyll and nitrogen

The exposure experiments were carried out in order to identify damage at an early stage before macroscopic ozone caused alterations to the plants (e.g. diminished growth and visible leaf damage).[4,19,42–45] Therefore, the levels of chlorophyll (Fig. 2) and nitrogen in the biochemical N-fractions (Fig. 3) were also determined.

There was almost no effect on either chlorophyll or nitrogen content apart from a slight increase in the chlorophyll level due to elevated carbon dioxide (Experiment 4; results not presented).

Investigation of biochemical N-fractions

To obtain comparable samples of the solid SP and LP fraction and the liquid NPN fraction, all samples were converted into $(NH_4)_2SO_4$ before isotope analysis by EA/IRMS.

To check for a possible shift of $\delta^{15}N$ values during sample treatment, standards were first analyzed. A slight shift was found in the $\delta^{15}N$ values caused by the chemicals used (results not reported). This deviation remains within the overall uncertainty of the system (natural variance of plants, EA/IRMS technique: Dumas combustion and the precision of the mass

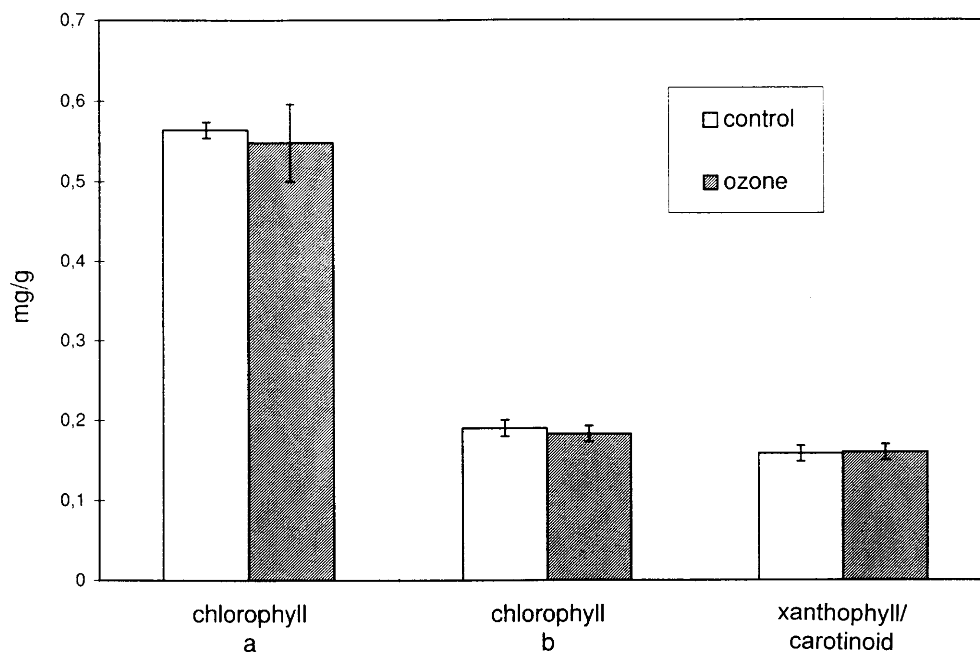

Figure 2. Pigment content of wheat in the two-leaf stage: comparison of ozone-exposed plants and control plants.

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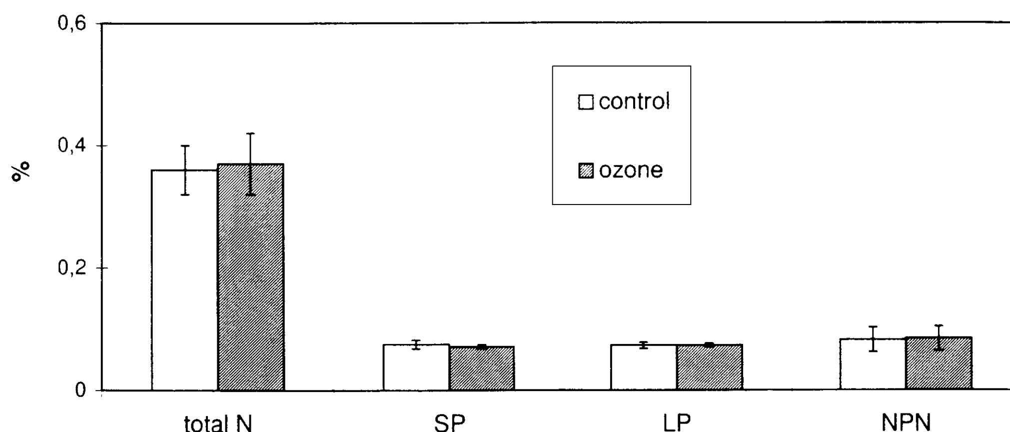

Figure 3. N content in wheat plants (total N) and in the biochemical N-fractions of wheat (SP, LP and NPN), two-leaf stage: comparison of ozone-exposed plants and control plants.

spectrometer) and, moreover, because of the symmetrical treatment of all the samples, it was not necessary to correct them.

Exposure for 2 weeks to 90 ppb of ozone (two-leaf stage, Experiments 1 and 3, Fig. 4(a)) did not alter the $\delta^{15}N$ values of the biochemical N-fractions. Exposure for 20 days with an ozone concentration of 120 ppb (Experiment 2) resulted in a slight increase in the $\delta^{15}N$ values in the N-fractions. The extension of the experiment up to the beginning of anthesis (Experiment 4, Fig. 4(b)) resulted in a significant increase in the $\delta^{15}N$ values in all fractions compared with the control treatment. We found the most significant changes of the isotope ratios in the NPN fraction, followed by the LP and the SP fractions. This shows, analogously to previous work,[46] that amino acids of the NPN exhibiting an ozone-induced change to their isotope ratio are incorporated with some delay into soluble protein and are only subsequently incorporated into structure protein.

The magnitude of the $\delta^{15}N$ values detected, especially for the NPN and LP fractions (51 and 35‰, respectively; reproducible results) of the wheat plants exposed to ozone is very rarely observed in nature (−49 to +49‰;[22,47] +46‰ for suspended particulate matter;[47] +40‰ for individual polyamines from legume nodules.[48] We generally explain this enrichment with accelerated N metabolism, which is under stress, such as an excessive ozone level, additionally strongly discriminated with a fractionation factor >1.0. The acceleration of the N metabolism was highlighted in a tracer experiment carried out simultaneously (Fig. 5): each increase in gas concentration (in the order ozone < carbon dioxide < ozone + carbon dioxide) led to an increased ^{15}N incorporation in all plant organs investigated and also in the NPN and LP fraction of these plant organs.

The literature includes some explanations of these stress-induced discrimination effects which could eventually lead to an elevation of the $\delta^{15}N$ values: (i) the variation of nitrogen isotope ratios in plants could be an indicator of nitrogen acquisition, metabolism and transport;[48] (ii) in forest research, the premature aging of the trees is postulated: the onset of the needles' catabolic activity causes accumulation in the 1‰ range;[26,49] older plants show higher $\delta^{15}N$ values than younger plants;[26,50] and (iii) legumes react to harmful gasses with the narrowing of their stomata, N fixation is changed from air more towards soil, which shows higher $\delta^{15}N$ values in comparison to air;[9] during nodule senescence, the interconversion and deamination of amino acids, the deamidation of amides and protein degradation may occur.[51]

This is not sufficient, however, to explain the effect we observed. One possible explanation could be that wheat leaves exposed to ozone (before visible injury) have increased permeability (caused by membrane alterations) to soluble substances such as carbohydrates, proteins, amino acids and inorganic salts.[52] Following general isotope effects, such a leakage would result in an increase in the relative portion of compounds with heavier nitrogen, and consequently the $\delta^{15}N$ values of the biochemical fractions should increase. On the other hand, the non-investigated, released amino acids and proteins should be isotopically lighter, resulting in more negative $\delta^{15}N$ values.

The NPN fraction represented a bulk character. Since this leakage effect concerns different N-containing substances and not only amino acids (their $\delta^{15}N$ values alone are not as high as expected; see the next section), the very high $\delta^{15}N$ values have to be accounted for by other N-containing substances, e.g. polyamines[47,53] or lipid N. Polyamines in particular show considerably higher $\delta^{15}N$ values than whole plant N, ureides and amino acids[47,53] and their level is additionally increased during ozone treatment.[42,54]

Plants exposed to elevated CO_2 levels did not show any change in the isotope ratios in comparison with the control plants (carbon dioxide does not represent a stress factor for plants[14,18,55]). The significant decrease in the $\delta^{15}N$ values of all fractions under combined exposure to CO_2 and O_3 in comparison with O_3 alone reflects the CO_2-induced increased resistance of the plant to ozone (as pointed out during other biochemical investigations).[9,14,18]

Investigations of amino acids of the soluble protein

Amino acids of the soluble protein hydrolysates from the short-term Experiment 3 (growth period up to two-leaf stage) and from the long-term Experiment 4

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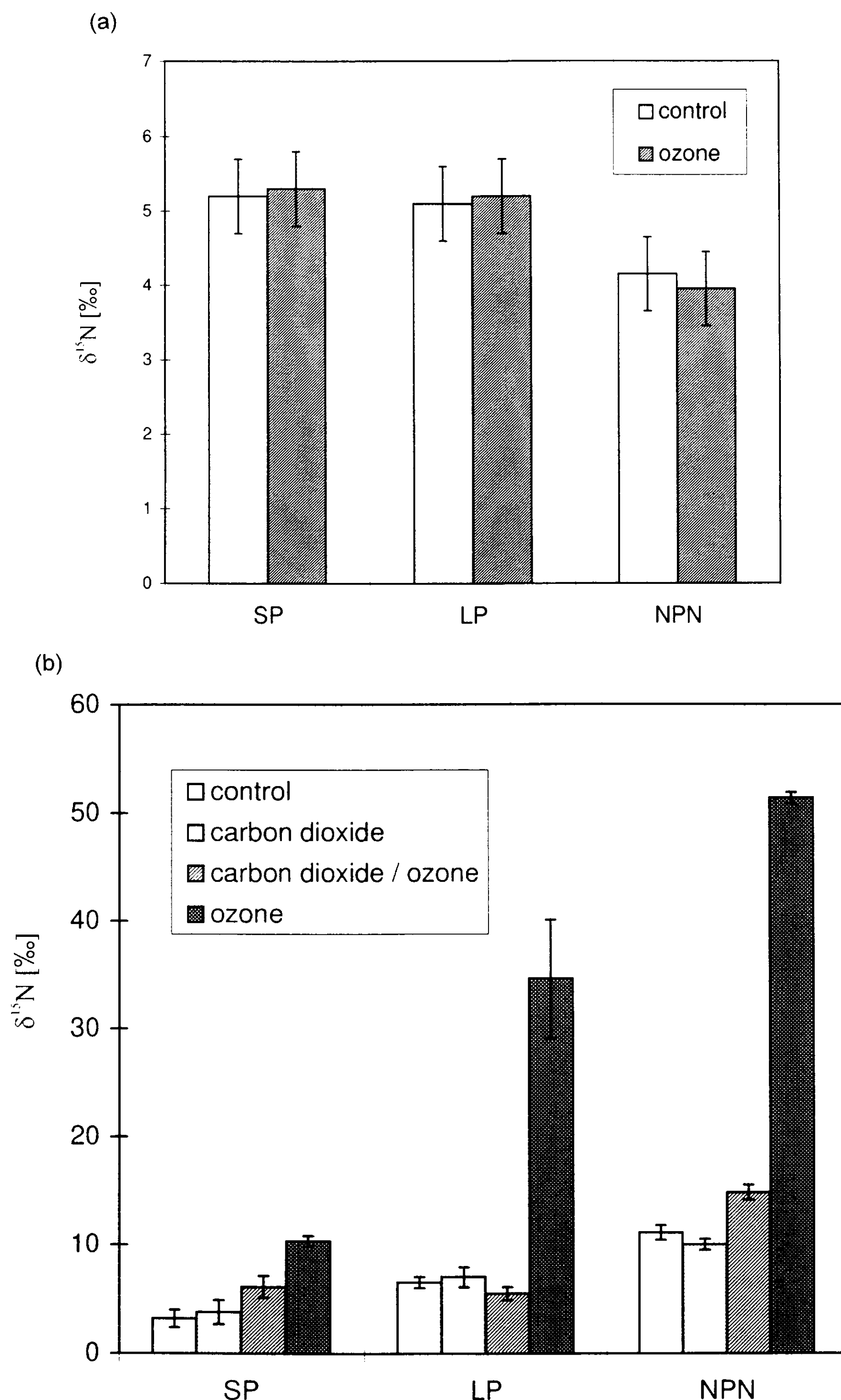

Figure 4. (a) δ^{15}N values of biochemical N-fractions (SP, LP and NPN) of wheat plants (two-leaf stage): comparison of ozone-exposed plants and control plants. (b) δ^{15}N values of biochemical N-fractions (SP, LP and NPN) of wheat plants (anthesis): comparison of plants exposed to ozone and/or carbon dioxide and control plants.

(growth period up to anthesis stage) were analyzed using GC/IRMS.

In the process of chemical treatment (hydrolysis, derivatization), not all of the proteinogenic amino acids are detectable. Glutamine and asparagine are converted into the corresponding acids by acidic hydrolysis. Therefore, the δ^{15}N values of these acids refer to the mixture of the amine and acid derivatives. The hydrolysis leads also to the degradation of tryptophan and the sulfur-containing amino acids cysteine and methionine.[39] In agreement with other workers,[56] we found that the derivatization of the amino acids arginine and histidine apparently needs stronger conditions (higher temperature, longer reaction time). Under the reaction conditions described above, the derivatization of these compounds is incomplete and may be below the detection limit. The content of phenylalanine decreased drastically with longer storage times of the samples.

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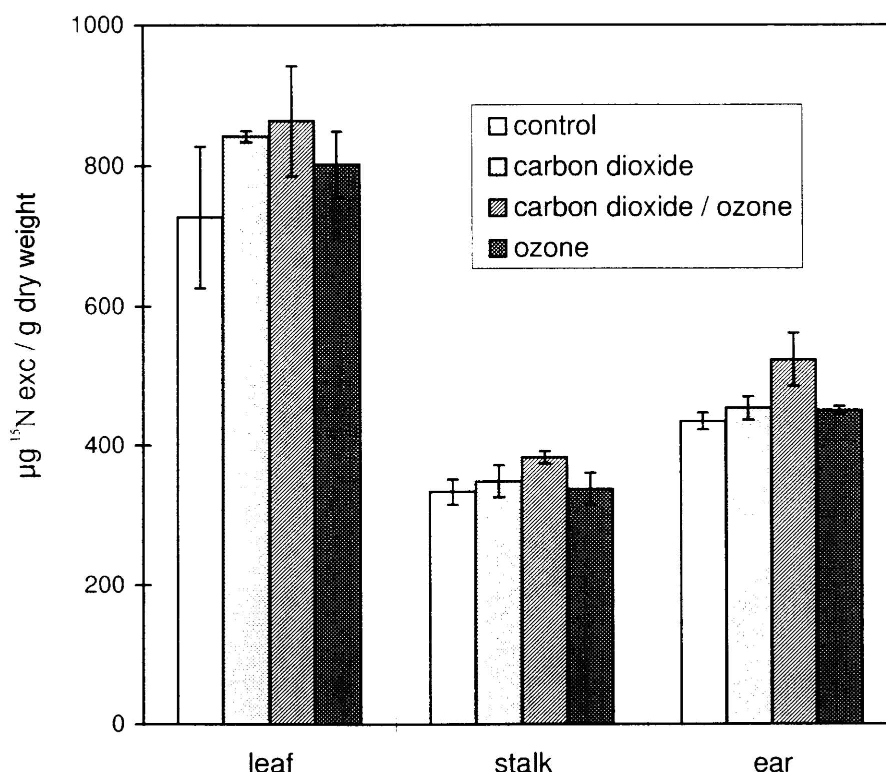

Figure 5. ^{15}N incorporation in different plant organs of wheat after $K^{15}NO_3$ labeling: comparison of plants exposed to ozone and/or carbon dioxide and control plants (tracer experiment).

Therefore, we excluded this amino acid from the discussion.

The $\delta^{15}N$ values of most amino acids of the soluble protein increased slightly during the short-term ozone exposure experiment ($\sim 1‰$ with a standard deviation of 0.5‰; Fig. 6). The noticeable increase in the $\delta^{15}N$ value of proline reflects the exceptional position of proline in metabolism.[16]

The longer ozone exposure period (Fig. 7) led to a significant increase in the $\delta^{15}N$ values up to 10‰ (with a standard deviation of 1‰) of all amino acids analyzed compared with the control (in this experiment, we could also determine arginine and glutamic acid). Alanine, serine, apartic acid, glutamic acid, leucine and glycine were above the mean of all differences between ozone-treated and control plants ($7 \pm 2‰$). This rise is observed independent of the fact that the $\delta^{15}N$ values of the individual amino acids in the soluble plant protein in the control vary. For example, glycine's low $\delta^{15}N$ control values in both the short-term and the long-term

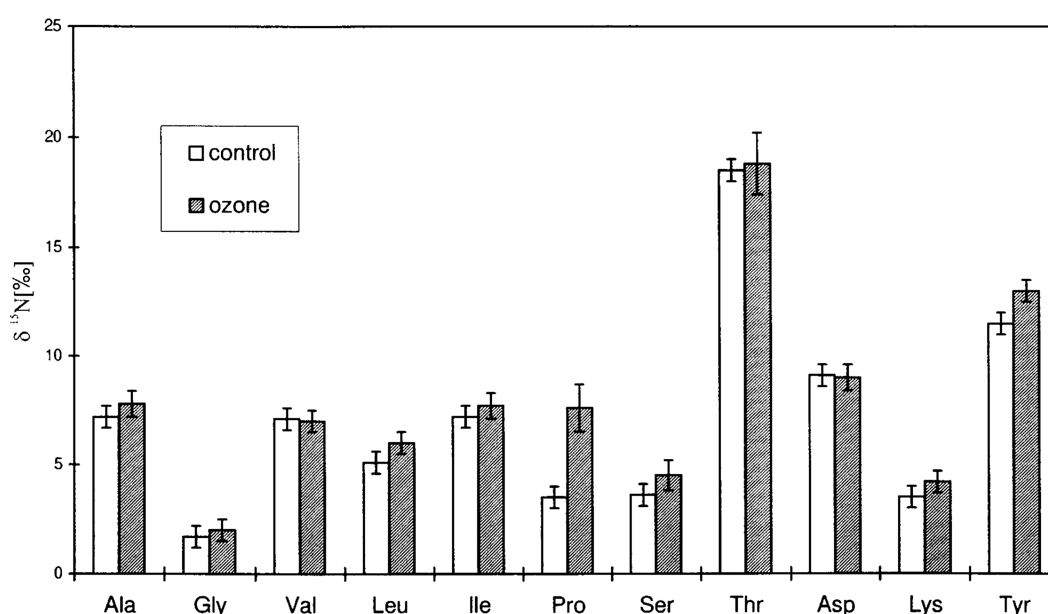

Figure 6. $\delta^{15}N$ values in amino acids of soluble protein hydrolysate of wheat plants (short-term experiment, two-leaf stage): comparison of ozone-exposed plants and control plants.

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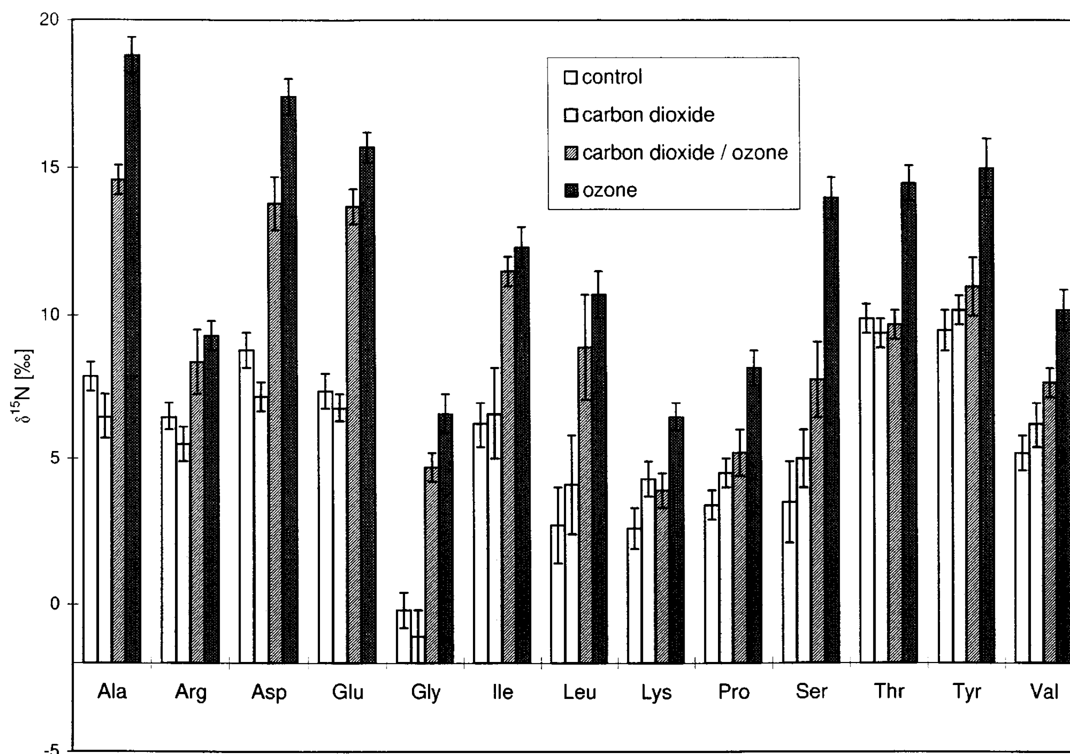

Figure 7. δ^{15}N values in amino acids of soluble protein hydrolysate (long-term experiment, anthesis): comparison of ozone-exposed plants and control plants.

experiments are especially notable. Combined exposure to $CO_2 + O_3$ resulted (as in the investigations of the biochemical N-fractions) in a decrease in the δ^{15}N values of all amino acids investigated in comparison with the merely ozone-exposed plants with an increase of up to 7‰ compared with the control (standard deviation 1‰) and a mean of all differences to the control of 4 ± 2‰.

Exposure to CO_2 alone did not affect the δ^{15}N values of the amino acids, as was shown for the biochemical N-fractions; the variations are up to ± 1.5‰ compared with the control values with a standard deviation of 1‰, while the mean of all differences from the control is 0 ± 1‰. Future investigations must establish whether information on metabolic changes caused by the impact of pollutants can be concluded from the differences among the natural isotope variations of the individual amino acids (from the various biochemical fractions).

CONCLUSIONS

The isotopic investigations described here were pilot studies to determine whether changes in natural variations of nitrogen (δ^{15}N values) can be used as an ecotoxicological indicator for the early indication of pollutant stress in plants.

The time-scaled (two-leaf stage and anthesis) exposure of wheat to ozone clearly showed that the kinetic effects in the organisms are reflected first on the (molecular) level of individual amino acids and later, but obviously more strongly, in the biochemical N-fractions (in the order NPN, LP, SP) by changes of their δ^{15}N values compared with the control material before visible symptoms of ozone stress[4,19,42–45] occur.

Exposure to elevated CO_2, which is of general interest for detoxification and repair effects,[7,9–12,14,18] did not show any significant difference in comparison with the control. However, in combination with the phytotoxic ozone a significant impact by CO_2 on the δ^{15}N values (at all levels of investigation) was observed, suggesting the decreasing (negative) impact of ozone because of declining oxidative stress connected with increasing resistance to ozone.

The results of these model experiments are qualitatively comparable to field investigations on coniferous trees (*Picea abies*[26–28] and *Pinus sylvestris*[29]). Field investigations reflect in particular time-prolonged exposure with temporally and spatially varying complex mixtures of different harmful substances. In the case of such multiple exposure, the damage effects of most of the substances are synergistic. These effects, which evidently also considerably exceed possible antagonistic effects, lead to changes of the isotope ratios and can be detected in complete plant organs.

In order to verify the validity of this isotope technique as a commonly applicable ecotoxicological indicator of harmful effects early on, the experiments need to be extended to other species with appropriate harmful substances. In this respect, a second question is the extent to which the changes in the isotope ratios caused by harmful substances are reversible and whether any regeneration effects (e.g. during environmental activities in the field) can be detected.

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