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EFFECT OF SEQUENCES OF OZONE AND NITROGEN DIOXIDE ON CHLOROPHYLL FLUORESCENCE IN RADISH

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ABSTRACT

Ozone (O_3) is the most important of the phytotoxic gaseous air pollutants. It causes substantial decreases in crop yields worldwide and bears adverse effects on vegetation in general. On the other hand, nitrogen dioxide (NO_2) is the air pollutant most likely to be associated with O_3 because it is a precursor of O_3. The objective of this study was to determine the effects of sequential exposures to nitrogen dioxide (NO_2) and ozone (O_3) on chlorophyll fluorescence in radish, *Raphanus sativus* L. Radish plants were exposed daily to O_3 or NO_2, or sequences of the two gases. The exposure profiles for both gases approximated sine waves with peak concentrations of 120 ppb (parts per billion by volume, nl l^{-1}). In the case of O_3, this is close to the reported threshold for adverse effects; while for NO_2 it is below the reported threshold. The sequences involved different combinations of exposures to NO_2 from 06:00 to 10:00h and/or 18:00 to 22:00hr and O_3 from 10:00 to 18:00hr. Relative to the control, early and early + late NO_2 resulted in stimulations of quantum yield (Y) and photochemical quenching (qP), with late NO_2 resulting in little or no change. In contrast, early, late and early + late NO_2 in combination with O_3 resulted in progressive reductions in these variables. The overall effect of O_3 treatment was to stimulate quantum yield and qP, both of which are indicative of increased photochemistry. Late NO_2 exposures caused no significant effects relative to the control. However, late NO_2 failed to result in a significant stimulation of photochemistry in the chloroplast, but caused significant residual increases in non-photochemical quenching (qN) during the middle of the day, responses which imply increased photo-protection capacity. Stimulation of quantum yield and photochemical quenching by O_3, O_3 + late NO_2, O_3 + early NO_2, early NO_2 and late + early NO_2 implies that CO_2-fixation was limited by processes at PSII. However, apart from the early NO_2 related stimulation of qN, all exposures involving O_3 led to a decrease in qN implying inability to regulate photosnynthesis resulting from changes in the thylakoid membrane. In the case of NPQ, all exposures including O_3, decreased this parameter suggesting impaired proper functioning of the xanthophyll cycle associated with the light-harvesting complex of photosystem II.

Key Words: Photochemical quenching, PSII, *Raphanus sativus*

RÉSUMÉ

L'ozone (O_3) est un gaz phytotoxique le plus important responsable de pertes substantielles des rendements des cultures de par le monde, avec des effets néfastes sur la végétation en général. D'autre part, l'oxyde d'azote (NO_2) est un polluant atmosphérique probablement associé avec O_3 du fait qu'il est son précurseur. L'objectif de cette étude était de déterminer les effets d'exposition séquentielle au dioxyde d'azote (NO_2) et à l'ozone (O_3) sur la fluorescence chlorophyllienne du radis, *Raphanus sativus* L.. Les plants de radis étaient quotidiennement exposés à O_3 ou NO_2, ou en séquences de ces deux gaz. Les profiles d'exposition pour les deux gaz a approximé les *sine waves* avec un volume de 120 ppm (parties par millions, nl l^{-1}). Dans le cas de O_3, ceci est proche des effets néfastes reportés, alors que pour NO_2 cette valeur est en deçà des valeurs reportées. Les séquences ont impliqués différentes combinaisons d'exposition au NO_2 de 06 :00 à 10 :00hr et/ou 18 :00 à 22:00 hr et O_3 de 10:00 à 18:00 hr. Concernant le témoin, l'application du NO_2 faite tôt le matin ou faite tôt et tard a entrainé des stimulations de rendement (Y) et le *quenching* photochimique (qP), l'application retardé de NO_2 ayant résulté en aucun changement ou un changement mineur. Au contraire, l'application tôt du NO_2, retardée, ainsi que l'application de NO_2 tôt et

tard dans la combinaison avec O_3 ont induit des réductions progressives dans ces deux variables. L'effet général du traitement O_3 avait induit la stimulation du rendement et qP, ces deux derniers étant indicatifs de l'accroissement photochimique. Les expositions retardées du NO_2 ont causee des effets non significatifs en rapport avec le témoin. Cependant, l'application retardee de NO_2 n'a pas pu induire une stimulation significative de la photochimie dans le chloroplaste, mais a plutôt causé des augmentations résiduelles significatives dans le *quenching* non photochimique (qN) au milieu de la journée, une réponse qui implique une capacité accrue de la photo-protection. La stimulation du rendement et du *quenching* photochimique par O_3, O_3+traitement NO_2 (retardé), O_3+NO_2 (tot), NO_2 (tot) et NO_2 (tard) + NO_2 (tot) Implique que la fixation du CO_2 était limitée par les processus à PSII. Cependant, à l'exception de la stimulation du qN par NO_2 (tot), toutes les expositions impliquant O_3 ont abouti à une diminution dans le qN impliquant l'incapacité de réguler la photosynthèse résultant des changements dans la membrane thylacoïdale. Dans le cas du NPQ, toutes les expositions incluant O_3, ont réduit ce paramètre suggérant ainsi un handicape dans le fonctionnement du cycle de xanthophylle associé au complexe de *light-harvesting* du photosystème II.

Mots Clés: *Quenching* photochemique, PSII, *Raphanus sativus*

INTRODUCTION

Ozone (O_3) is the most important of the gaseous air pollutants phytotoxic to plants. However, ozone is a secondary pollutant associated with nitrogen dioxide (NO_2) which is its precursor in polluted air. Since O_3 maxima typically occur during the early afternoon, while NO_2 maxima tend to occur in the morning and evening hours, the response of plants to O_3 exposures cannot be dissociated from daily sequences of exposure to both NO_2 and O_3 under field conditions. Although there are very few studies of the effects of such sequences, it is clear that the two gases have complicated interactive effects on vegetation (Runeckles and Palmer, 1987).

Much of the early work on the effects of O_3 and NO_2 utilised "sudden" exposures to relatively high steady-state concentrations (>200 ppb) of either pollutant. More recent work has emphasised the importance of using exposures involving concentrations that increase to and decrease from a peak level (Bicak, 1978; Dann and Pell, 1989; Bahl and Kahl, 1995; Wellburn and Wellburn, 1996), since such exposure profiles more closely resemble those occurring in ambient air. Most studies involving open-top field exposure chambers have used exposures to fluctuating O_3 profiles as described by Heagle *et al.* (1988).

Chlorophyll fluorescence has been used as an indicator of O_3-induced stress following the initial work of Schreiber *et al.* (1978). Fluorescence studies are non-intrusive, allowing time courses to be monitored, fundamentally associated with the most important process in all life and these can be viewed before symptoms appear or in situations where symptoms do not appear at all. Ozone has been reported to cause a decline in maximal fluorescence (Fm) (Schmidt *et al.*, 1990), the ratio of variable to minimal fluorescence (Fv/Fo; Grimm and Fuhrer, 1992; Godde and Buchhold, 1992), the ratio of variable to maximal fluorescence (Fv/Fm; Farage *et al.*, 1991; Salam amd Soja, 1995), non photochemical quenching (qN), and increased photochemical quenching (qP) (Godde and Buchhold, 1992). A NO_2-induced decline in Fv/Fo reported by Schmidt *et al.* (1990) was also a result of short-term "sudden" exposure to a high NO_2 concentration (5ppm for 4h). Little information is available on the possible modification of chlorophyll fluorescence parameters resulting from NO_2-O_3 sequences. The objective of this study was to determine the overall effect of sequential exposures to NO_2 and O_3 on chlorophyll fluorescence.

METHODS AND MATERIALS

Plant materials. Four replicate experiments were carried out. Radish (*Raphanus sativus* L. cv Cherry Belle) plants were grown from seed in 13 cm diameter plant pots containing about 982 cm^3 standard potting soil (85 loam; 15% peat). A slow release fertiliser,14:14:14 NPK (Osmocote; Sierra Chemical Company), was applied to the standard potting soil at planting at 1g pot^{-1}. About 15 radish seeds were sown per pot and the seedlings were thinned to 4 plants per pot 10 days after sowing. Soon after thinning, they were

transferred to a control growth chamber, where they acclimatised for 3 days before the commencement of exposure to various sequences of O_3 and NO_2 (four pots per treatment). Plants were maintained in the growth chambers under a photoperiod of 16.5 hr (light of intensity 150 µmol m^{-2} s^{-1}) and 7.5 hr (dark) and day/night temperatures of 24°C/18°C. The photoperiod started at 05:45 hr whereas the thermoperiod ran from 06:00 hr to 22:00 hr.

Air pollutant exposures. Growth chambers (Model EF7, Conviron Ltd.) were modified to allow measurement of chlorophyll fluorescence parameters in *situ*. The chambers were supplied with air filtered through activated charcoal and Air Repair (alumina coated with $KMnO_4$) (Air Repair Products Inc.) to remove ambient ozone (O_3), nitrogen dioxide (NO_2) and nitric oxide (NO). To simulate field conditions, exposures to NO_2 and O_3 both approximated sine waves, with <10 ppb minimum and 120 ppb maximum concentrations. The exposure regime was such that O_3 was applied from 10:00 to 18:00 hr and NO_2 from 06:00 to 10:00 hr and again from 18:00 to 22:00 hr. A chamber with filtered air was used as a control. The complete range of seven treatments was obtained by moving plants from chamber to chamber as required. Typical exposure regimes for the treatments were, namely, the control (C), O_3 alone (~O~), early NO_2 alone (N~~), late NO_2 alone (~~N), early NO_2 + late NO_2 (N~N), early NO_2 + O_3 (NO~), O_3 + late NO_2 (~ON) and early NO_2 + O_3 + late NO_2 (NON). These symbols are maintained in all the presented figures and are shown in Figure 1. The curves in Figure 1 were obtained with the distance weighted least squares (DWLS) smoothing function of SYGRAPH (SYSTAT Inc.) and show the typical daytime rise in ambient O_3, the tendency for NO_2 maxima to occur early and late in the day, and for NO_2 to have a morning maximum.

Fluorescence measurements. A Portable Fluorometer (Model PAM2000; H.Walz GmbH, Effeltrich, Germany), with integrated Poqet PQ-1024 computer, was used to measure fluorescence parameters. The parameters determined were derived from the measurement of Fo, Fm, Fm' and Ft, defined as in Schreiber *et al*. (1994). The fluorescence parameters were measured on the first true leaf of one randomly selected plant from four pots for each treatment. On day seven of exposure to O_3-NO_2 sequences, plants were dark-adapted using a leaf clip supplied with the fluorometer, before the measurements were made. All the measurements were made while the plants were in the growth chambers with the lights on. At the end of dark adaptation, the following measurements were taken:

(i) Fo was measured using the measuring light (0.1 ìmol m^{-2} s^{-1}),
(ii) a saturating pulse (12 000 ìmol m^{-2} s^{-1}) was applied to measure Fm, and
(iii) the saturating pulse was applied every 20 seconds for the next 5 min 20 s, to measure Ft and Fm'.

Table 1 presents the full definition of the fluorescence parameters used in the present study.

Since the last six data points were found to represent a steady state in fluorescence, the means of the last four readings of Y, qP, and qN were used for data analysis. Photochemical quenching (qP), non-photochemical quenching correlated with 'thylakoid membrane energization'(qN), non-photochemical quenching (reflecting heat dissipation of excitation energy in the antenna system) (NPQ), and fluorescence quantum yield (Y) were calculated as in Table 1. Data were collected between 07:00 and 17:00 hr. The experiment was a randomised block design with 3 blocks, blocked in time.

Statistical analyses. SYSTAT/SYGRAPH (Systat Inc.) was used for all statistical analyses. Orthogonal contrasts were used to compare treatment means. The DWLS (distance weighted least squares smoothing) function of SYGRAPH was used for curve fitting.

RESULTS AND DISCUSSION

There was no difference due to treatment in the minimal fluorescence of dark-adapted leaves (Fo), recorded at the onset of data collection at about 07:00hr over one hour after the start of the

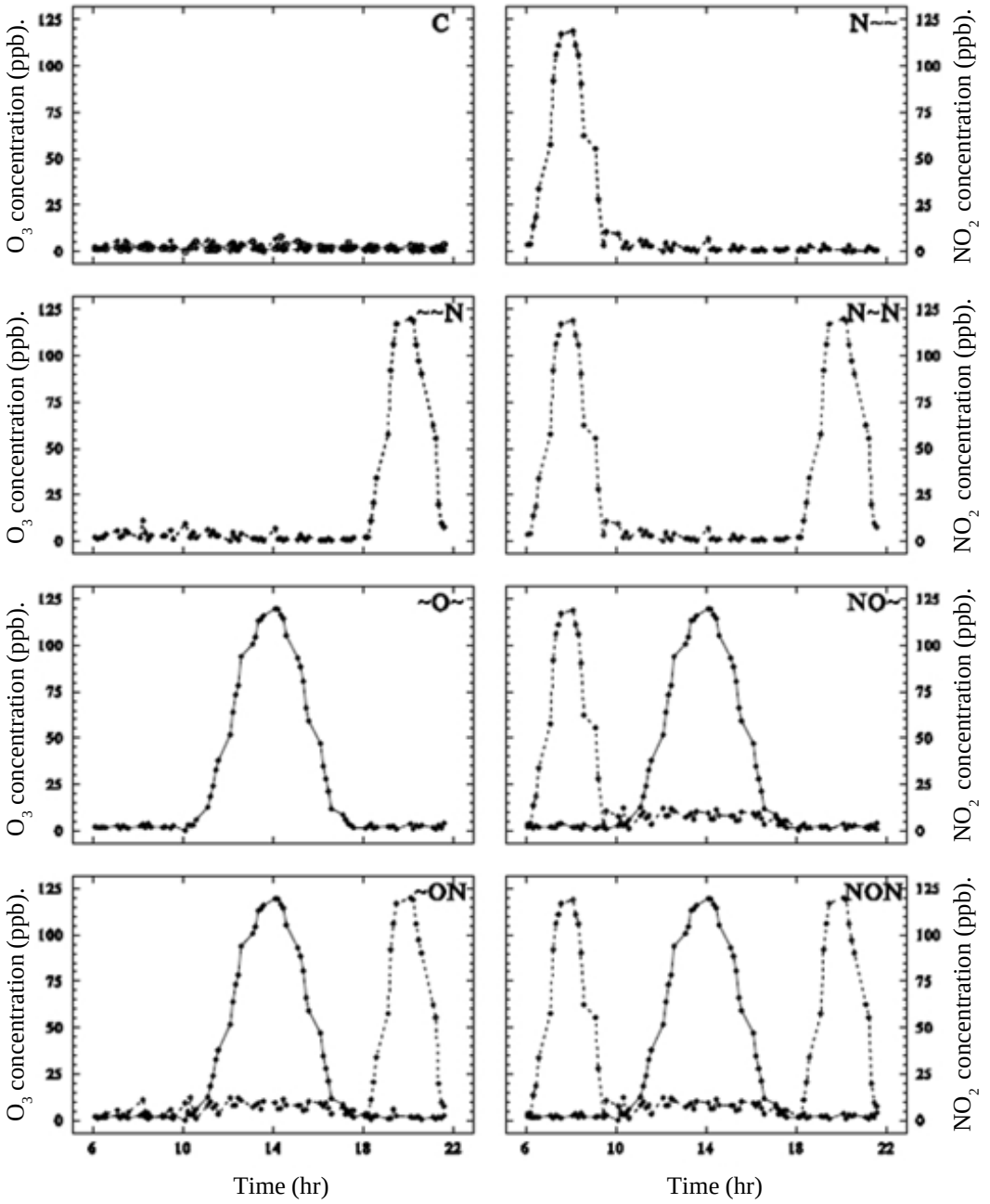

Figure 1. The exposure regime for the NO_2 and O_3 sequences. The sequences were: charcoal filtered air C, exposed to O_3 alone (~O~), early NO_2 (N~~), late NO_2 (~~N), both late and early NO_2 (N~N), early NO_2 plus O_3 (NO~), O_3 plus late NO_2 (~ON), and both late and early NO_2 plus O_3 (NON). The early NO_2 (broken line) was applied from 06:00 to 10:00 hr late NO_2 was applied from 18:00 to 22:00 hr and O_3 (solid line) was applied from 10:00 to 18:00 hr.

TABLE 1. Definition of chlorophyll nomenclature used in the present study

a. Fluorescence intensity indicators		
Fo	minimal fluorescence (dark)	fluorescence intensity with all PSII reaction centers open i.e, dark or low light adapted; qP=1 and qN=0.
Fm	maximal fluorescence (dark)	fluorescence intensity with all PSII reaction centers closed (i.e. qP=0) after dark or low light adaptation and all non photochemical quenching processes at a minimum (i.e. qN=0).
Fv	variable fluorescence (dark)	maximum variable fluorescence with all non-photochemical processes are at a minimum; i.e. Fm-Fo.
Ft	fluorescence at any time	fluorescence intensity with a light-adapted sample
Fm'	maximal fluorescence (light)	fluorescence intensity with all Photosystem II (PSII) reaction centers closed in a light-adapted state; qP=1 and qN≥0.
b. Fluorescence quenching parameters		
qP	photochemical quenching	(Fm'-Ft)/(Fm'-Fo)
qN	non-photochemical quenching	(Fm-Fm')/(Fm-Fo), the coefficient of non-photochemical quenching correlated with 'thylakoid membrane energization'.
NPQ	non-photochemical quenching	(Fm-Fm')/Fm', the coefficient of non-photochemical quenching reflecting heat-dissipation of excitation energy in the antenna system
Y	Fluorescence quantum yield	the quantum yield of photochemical energy conversion reflecting the efficiency of the overall process. Operational = (Fm'-Ft)/ Fm', and maximum = (Fm-Fo)/ Fm).

photoperiod (Fig. 2). Since the data were collected on the seventh day of exposure, this shows that exposures received during the previous six days had had no cumulative effect on Fo.

With regard to mean maximal fluorescence (Fm) (Fig. 2A), O_3 alone, early NO_2 and the combination of early + late NO_2 resulted in stimulation relative to the control. Ozone suppressed the effect of early NO_2 (cf. N~~ vs. NO~; $P<0.1$) but had no effect on late NO_2 or late + early NO_2. The maximum fluorescence quantum yield (Y) (Fig. 2B) calculated from (Fm Fo)/Fm, however, showed no distinct treatment effects, other than stimulation with early NO_2 indicating that this parameter had less variability even when Fm was variable. Values for Y were in the same range (0.75 to 0.85) as values reported in other studies (Bolher-Nordenkampf *et al.*, 1989).

In light-adapted leaves, mean Ft and mean Fm' showed greater variability among treatments (Fig. 2C). Mean Ft was lower than the control in all treatments except ~~N and NON. As is also shown in Figure 2C, in all treatments with O_3, Fm' was significantly increased relative to the control. Mean Fm' was also increased by early NO_2 (cf.. N~~ vs. C; Fig. 2C).

Operating quantum yield (Y; derived from Fm' and Ft) and photochemical quenching (qP) showed that Fm' was the overriding factor in determining yield values (Fig. 2A vs. Fig. 2C). Early NO_2 increased yield, while the level in late NO_2 did not differ from the control. The combination of early + late NO_2 led to an intermediate stimulation. Higher quantum yields also resulted from exposure to O_3 alone or in combination with early or late NO_2 (cf. C vs. ~O~, NO~, ~ON; Fig. 3), although the increase with early NO_2 was not additive (cf. ~O~ and N~~ vs. NO~). Late NO_2 decreased the O_3-induced stimulation (cf. ~O~ and ~~N vs. ~ON), and with the early + late NO_2 combination, the O_3--induced stimulation was completely suppressed (cf. ~O~ and N~N vs. NON). The time course of Y and qP recorded on a typical exposure day from 6:00 to

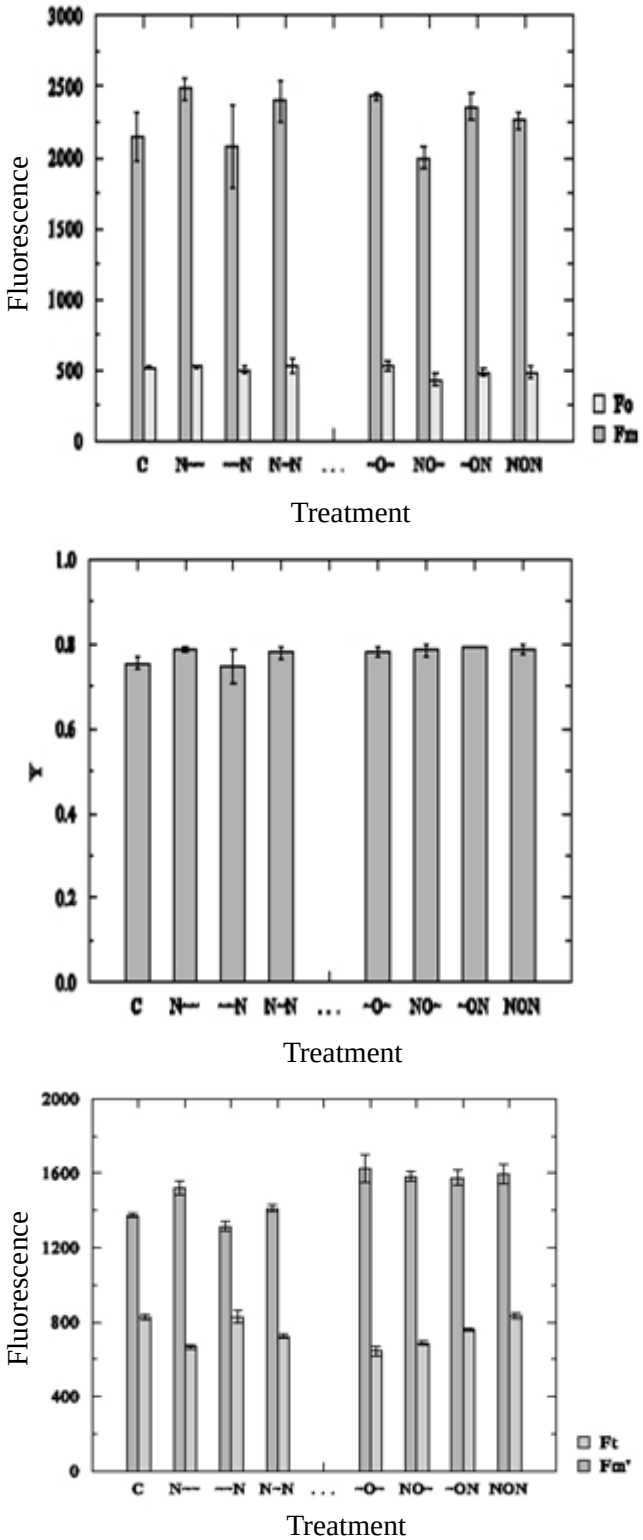

Figure 2. Fluorescence parameters depicted by each treatment after exposure for seven days to ozone and/or nitrogen dioxide alone or in sequence. A=Fm, Fo after dark adaptation, B=maximum yield after dark adaptation and C= Fm' and Ft in light.

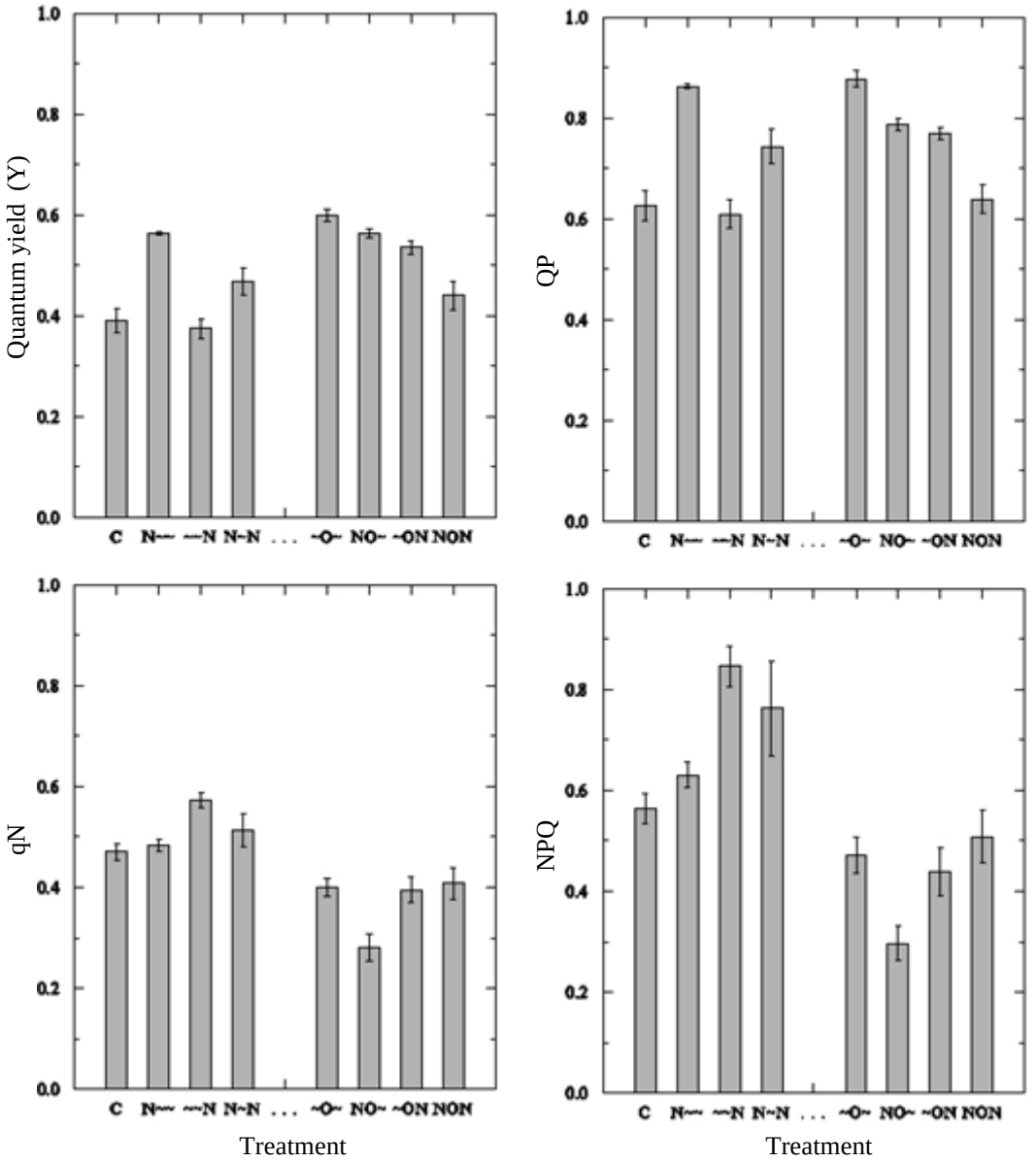

Figure 3. Effects of treatment on mean quantum yield (Y), qP, qN, and NPQ. Bars are standard errors (n=16).

18:00 hr confirmed the above observations (Figs. 4 and 5). A complete summary of the response of the fluorescence parameters is given in Table 2.

With regard to non-photochemical quenching (qN), Figure 3 shows that regardless of sequence, early NO_2 had no effect on the overall level, relative to the control, but late NO_2 alone increased qN. Exposure to O_3 alone and in all combinations with NO_2 reduced qN (cf. C vs. ~O~, NO~, ~ON, and NON; Fig. 3). A greater overall reduction occurred with early NO_2 and O_3 (NO~). Non-photochemical quenching determined as the NPQ parameter showed similar responses to treatment as qN (Fig. 3). Similarly, the time course of qN and NPQ recorded on a typical exposure day from 6:00 to 18:00 hr essentially confirmed the above observations (Figs. 6 and 7).

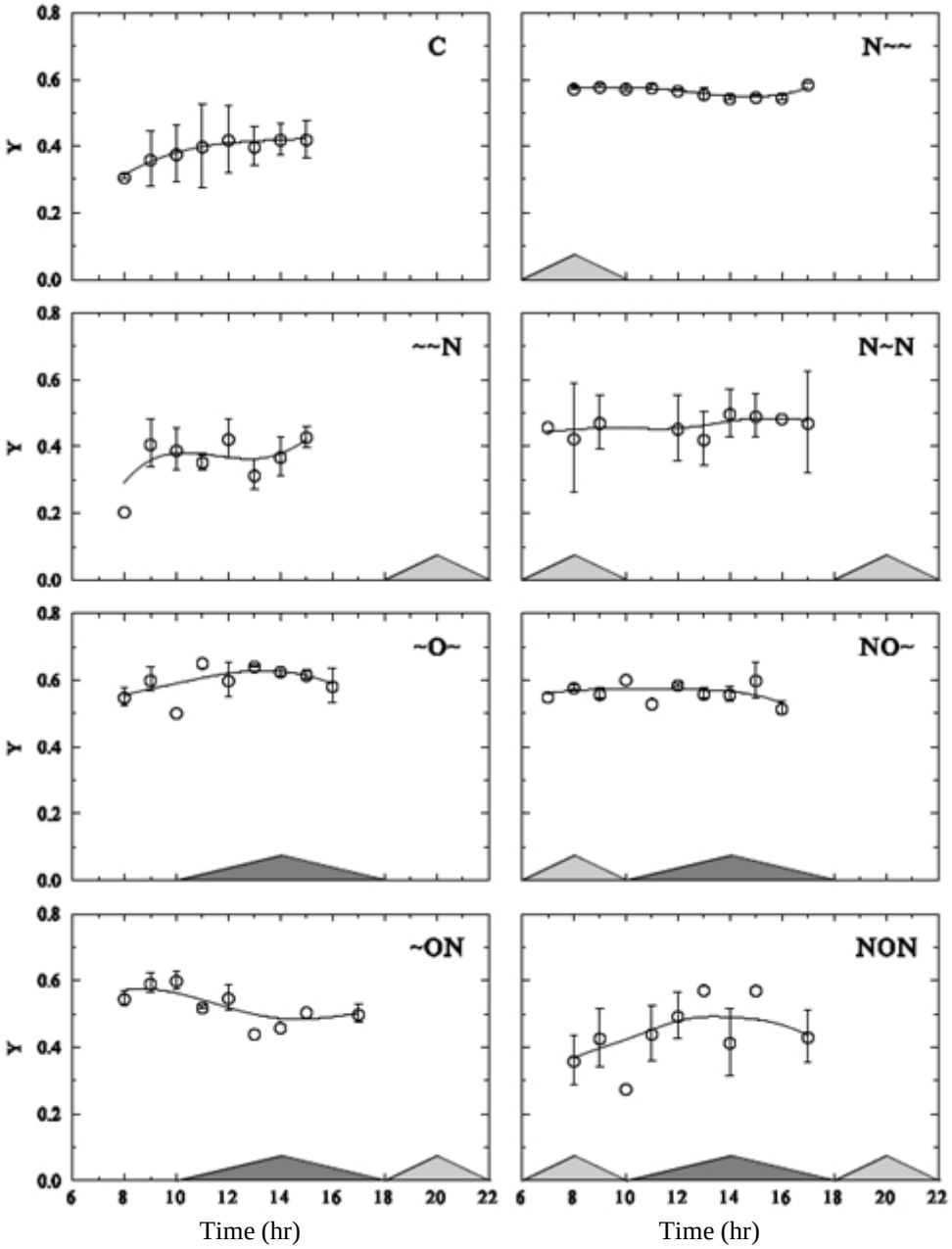

Figure 4. The time course of fluorescence quantum yield (Y) by treatment. Bars are standard errors (n=3); for data points without error bars, n=1. The shaded triangles indicate the period of exposure to NO_2 (light shading) and O_3 (dark shading).

DISCUSSION

The effects of NO_2 alone, on fluorescence parameters, showed that late NO_2 exposure caused no significant effects on Y and qP relative to the control. The nitrogen dioxide should have resulted in the formation of nitrate and nitrite on dissolution in the water film around cells on entering the leaves. Since the plants had been maintained in light up to and during this exposure the levels of nitrate and nitrite reductases should have been close to maximal, up to the end of the

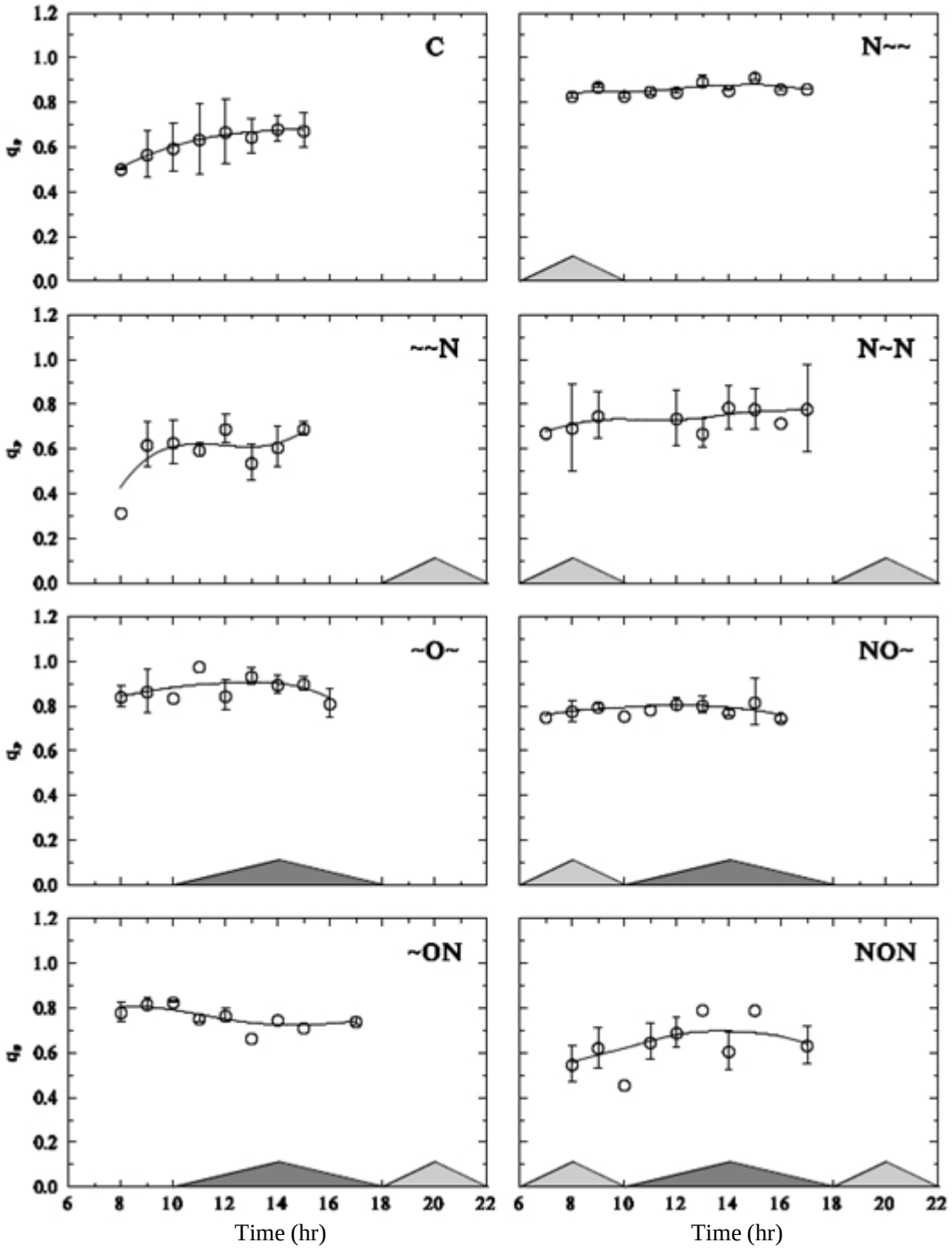

Figure 5. The Time course of photochemical fluorescence quenching (qP) by treatment.Bars are standard errors (n=3); for data points without error bars, n=1.

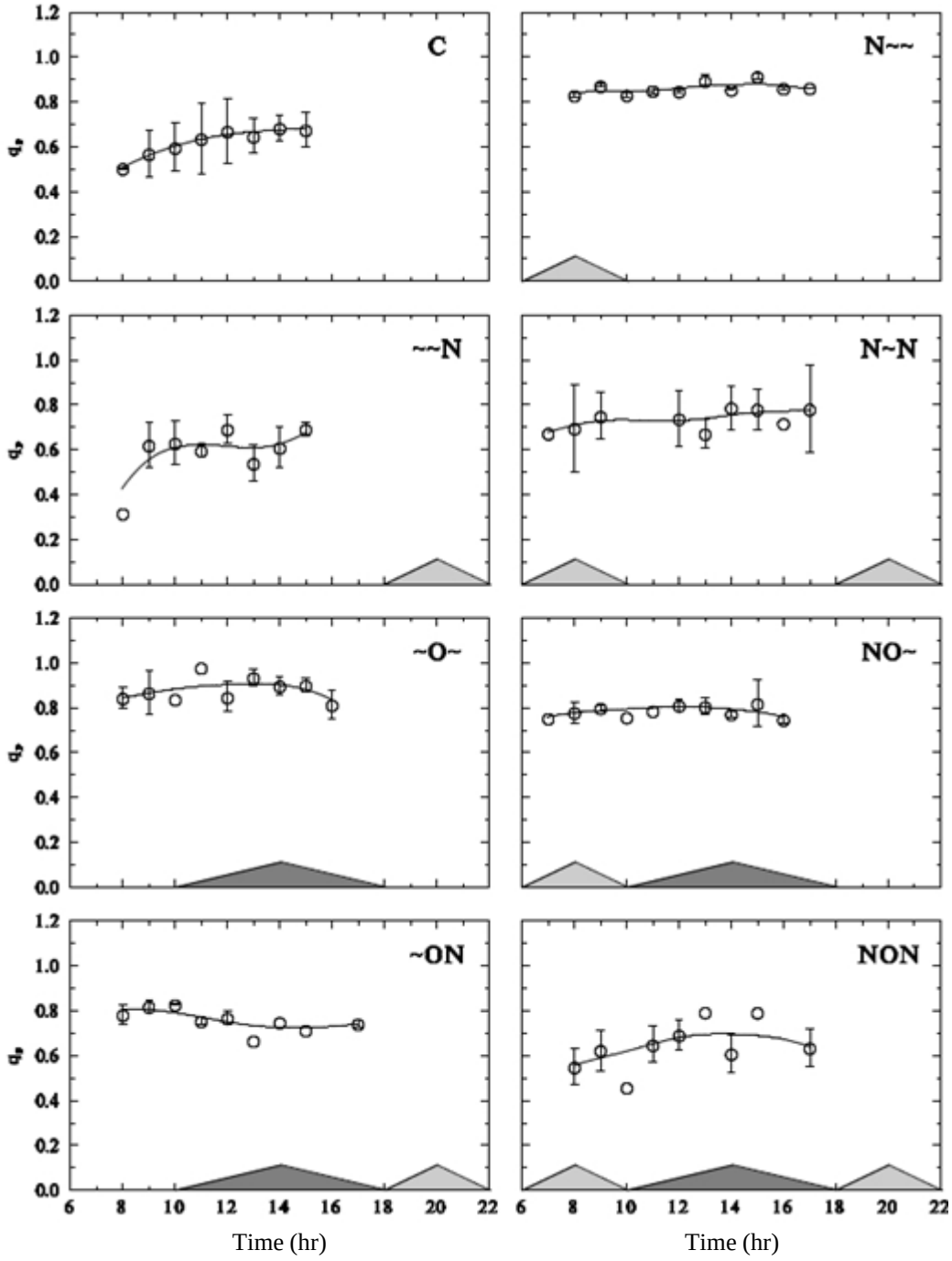

Figure 6. The time course of non-photochemical fluorescence quenching (qN) by treatment. Bars are standard errors (n=3); for data points without error bars, n=1. The shaded triangles indicate the period of exposure to NO_2 (light shading) and O_3 (dark shading).

photoperiod. However, although late NO_2 failed to result in a significant stimulation of photochemistry in the chloroplast, it caused significant residual increases in non-photochemical quenching observed during the middle of the day. These responses imply decreased efficiency of the photochemical processes that might be accounted for by an

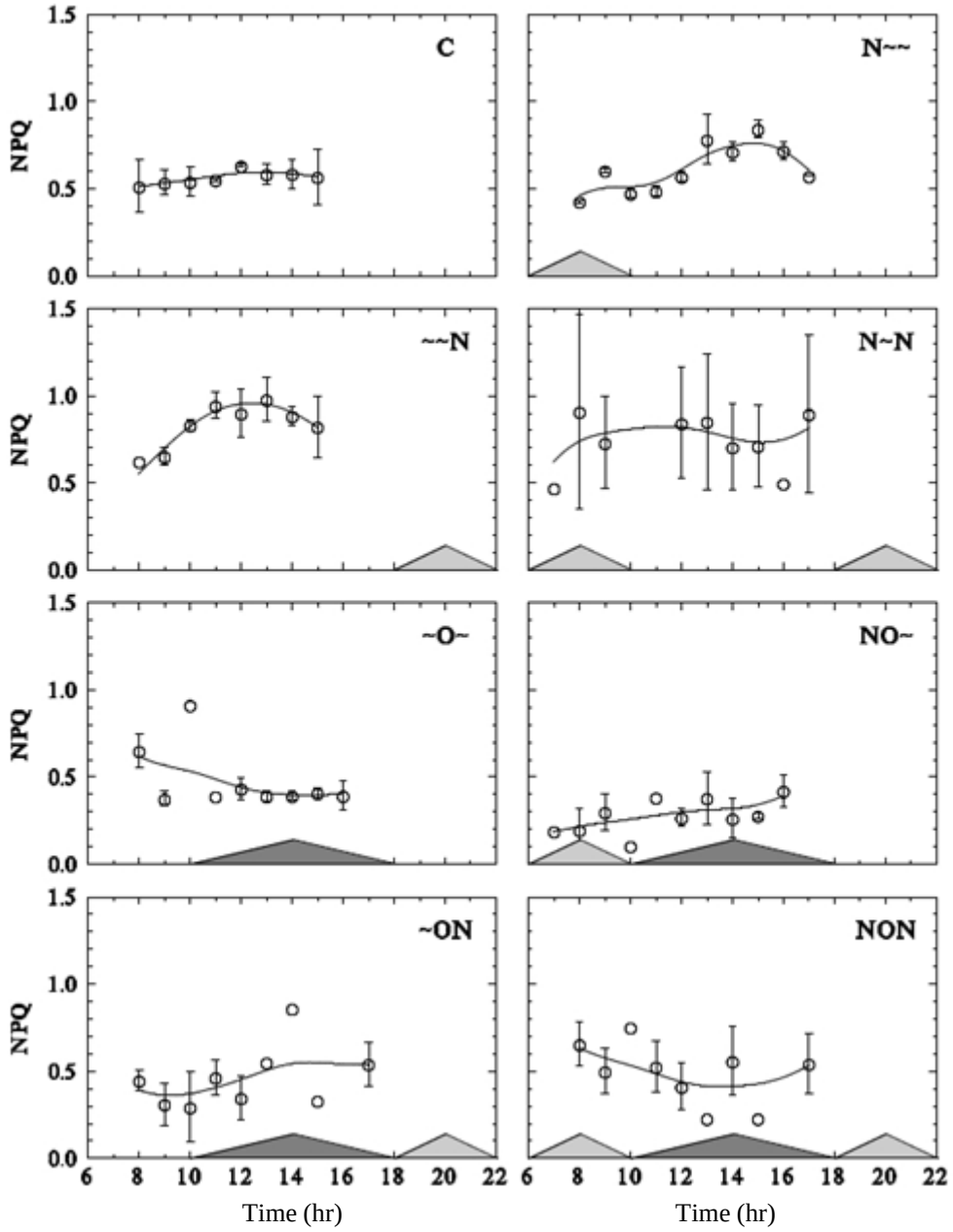

Figure 7. The time course of non-photochemical fluorescence quenching (NPQ) by treatment. Error bars are standard errors (n=3); for data points without error bars, n=1. The shaded triangles indicate the period of exposure to NO_2 (light shading) and O_3 (dark shading).

accumulation of nitrite in the leaves since exposure was at the tail end of the photoperiod. Such increases may have resulted from perturbation of the pH status of the thylakoid membrane caused by the accumulation of nitric and nitrous acid. In spinach leaves, Schmidt *et al*. (1990) reported that effects of exposures to massive (5 000 ppb) concentrations of NO_2 lasting up to 24 hr on chlorophyll fluorescence could only be observed if the exposures occurred in

darkness. They attributed the lack of responses in light to the effective removal of NO_2^- by nitrite reductase.

The overall effect of the O_3 treatment in the present study was to stimulate Y and qP, both of which are indicative of increased photochemistry (Genty *et al.*, 1989; Godde and Buchhold, 1992). Since photochemical quantum yield and the quantum yield of CO_2-fixation have been found to be linearly related (Genty *et al.*, 1989), the stimulation of photochemical quantum yield would be expected to result in increased photosynthesis and growth. However, data presented elsewhere indicated that there was no dry matter accumulation associated with O_3 exposure despite stimulation of Y and qP implying that the stimulation could have been a result of an need for repair or maintenance respiration (Amthor and Cumming, 1988; Amthor, 1988) or both. Based on the kinetics of fluorescence rise in dark-adapted bean leaves, Schreiber *et al.* (1978) attributed adverse effects of O_3 on decreased water-splitting in photosystem II. However, these effects were found using square-wave exposures to higher concentrations (250-500 ppb). Although no comparable measurements of fast kinetics were made in the present study, preventing an analysis such as that conducted by Schreiber *et al.* (1978), the lack of O_3 effects on dark-adapted quantum yield (Fig. 2B) or Fo (Fig. 2A) observed before the start of the O_3 exposure suggest that the lower O_3 levels used in the present study, did not lead to any residual effects on water-splitting activity.

Finally, the stimulation of quantum yield and photochemical quenching by O_3, O_3 + late NO_2, O_3 + early NO_2, early NO_2 and late + early NO_2 implies that CO_2-fixation could not have been limited by processes at PSII. However, apart from the early NO_2 related stimulation of qN all exposures involving O_3 led to a decrease in qN implying an inability to regulate photosynthesis resulting from changes in the thylakoid membrane. In the case of NPQ, all exposures including O_3 decreased this parameter suggesting impairment of the proper functioning of the xanthophyll cycle associated with the light-harvesting complex of photosystem II (Lavaud *et al.*, 2012).

CONCLUSION

The exposures to early NO_2 lead to effects on photochemistry that suggest increased CO_2-assimilation while exposure to O_3 suggested increased maintenance or repair or both. In addition ozone effects on non-photochemical quenching suggested effects on the functioning of the xanthophyll cycle and perturbation of structure or processes related to the thylakoid membrane. Late NO_2, because it happened at the end of the photoperiod, indicated a deleterious accumulation of nitrite. This study shows the complexity of plant responses associated with low fluctuating exposure levels to a combination of pollutants. Such exposures, however, mimic more closely the exposure regimes and profiles that plants are exposed to in real life. These results point at the need for studies that mimic ambient exposure and profiles in order to ensure findings indicative of real life situations.

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